Master's thesis

Functional characterization of two transmembrane proteins associated with desiccation tolerance in *Xerophyta viscosa* and *Arabidopsis thaliana*

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Introduction

Most part of land areas in Americas, Australia, Africa, southern Europe and southeast Asia will be characterized by severe drought conditions in the next 30–90 years, mainly due to decreased precipitation and increased evaporation (Dai, 2013). Climate model predictions suggest that this overall drying will intensify during the 22nd century (Sherwood & Fu, 2014), with devastating effects on agriculture. Most of the crops are indeed unable to tolerate extreme water loss and the consequences on crop production and productivity are inevitable (Bansal, Lenka, & Mondal, 2014). For this reason, it is of vital importance to improve desiccation tolerance (DT) in vegetative tissues of crops to withstand severe and prolonged drought conditions. Indeed, while drought tolerance can be considered as the tolerance of moderate dehydration, down to a moisture content below which there is no bulk cytoplasmic water present, desiccation tolerance generally refers to the tolerance of further dehydration, when the hydration shell of molecules is gradually lost (Hoekstra, Golovina, & Buitink, 2001).

To date, a group of 135 angiosperm species (Costa, Cooper, Hilhorst, & Farrant, 2017) have been found to be able to survive after the removal of almost all cellular water without irreversible damage (Leprince & Buitink, 2010). These desiccation tolerant species, namely resurrection plants, are thought to reactivate a seed-associated mechanism, which allow them to tolerate dehydration to very low water contents (orthodox seeds) (Costa, Cooper, Hilhorst, & Farrant, 2017) (Maia J. , Dekkers, Dolle, Ligterink, & Hilhorst, 2014). In particular, resurrection plants employ two different physiological strategies: retention of chlorophyll and thylakoid membrane integrity during dehydration and then quickly resume full photosynthetic function when rehydrating (homoiochlorophyllous) or regulated degradation of the photosynthetic machinery and re-synthesis upon rehydration (poikilochlorophyllous) (Costa, Cooper, Hilhorst, & Farrant, 2017) utilizing transcripts stably stored in desiccated tissues (Costa, et al., 2017).

FIGURE 1. *X. VISCOSA* FULLY DEHYDRATED PLANT (<5% RWC) VS REHYDRATED PLANT (COSTA ET AL. 2017)

Among these, *Xerophyta viscosa* (Figure 1) has been proposed as model plant to unravel the acquired desiccation tolerance in vegetative tissues, being a monocotyledonous species phylogenetically related to staple cereal crops. A high quality whole genome sequence has recently been produced and transcriptome changes during dehydration/rehydration have been assessed (Costa, et al., 2017), allowing the analysis of key genes related to desiccation tolerance.

ABI3 (Abscisic Acid Insentitive3) is a transcription factor originally discovered as a seed-specific, controlling seed development and maturation, including the acquisition of DT, by affecting the expression of downstream targets, including other transcription factors (TF), hormonal pathways and the expression of Seed Storage Protein (SSP) and Late Embryogenesis Abundant (LEA) proteins (Maia J. , Dekkers, Dolle, Ligterink, & Hilhorst, 2014). ABI3 may act as an upstream regulator of stress signalling, since its transcriptional targets in *A. thaliana* control tolerance to various abiotic stresses, including cold, salinity, and drought (Finkelstein, Gampala, & Rock, 2002). Interestingly, ABI3 plays a major role in water stress responses in vegetative tissues of desiccation tolerant plants (Costa, Cooper, Hilhorst, & Farrant, 2017), since its transcriptional targets increase in expression upon the induction of desiccation tolerance in *X. viscosa* (Costa, et al., 2016) (Costa, et al., 2017) . Bartels et al. (1992) also

reported that ABA is associated together with light in the regulation of a desiccation-related gene from *Craterostigma plantagineum*.

The above mentioned LEA proteins have been extensively studied as being ubiquitous proteins involved in protection from water-related stresses (Leprince & Buitink, 2010), such as dehydration, cold, osmotic and salt stress (Tunnacliffe & Wise, 2007). As an evidence, in *A. thaliana* LEA proteins accumulate during the later stages of embryo development, when desiccation tolerance is acquired (Candat, et al., 2014), but also in vegetative tissues under water deficit (Olvera-Carrillo, Campos, Reyes, Garciarrubio, & Covarrubias, 2010). In fully hydrated state they lack in secondary structure, but they fold into a α-helix when water decreases (Tunnacliffe & Wise, 2007) (Candat, et al., 2014), probably carrying out protective functions as water replacements molecules, ion sequesters, chaperons and or heat shields (Farrant, et al., 2015). Likewise, the accumulation of LEA transcripts is induced during dehydration in vegetative tissues of desiccation tolerant species (Costa, et al., 2016) (Costa, Cooper, Hilhorst, & Farrant, 2017).

Other than LEA genes, *X. viscosa* transcriptome analysis showed differential expression and accumulation of many interesting genes involved with activation of desiccation responses and deactivation and control of senescence. Some of the transcripts directly or indirectly involved in desiccation tolerance mechanism are going to be characterized during this thesis project, namely a protein from Abscisic acid-induced Wheat Plasma Membrane 19-like family (*AWPM19-like*) and the Arabidopsis Receptor Kinases (*ARK*) 1 and 2.

Abscisic acid-induced Wheat Plasma Membrane 19-like protein (*AWPM19-like*)

The *AWPM19-like* protein sequence is highly conserved in monocotyledonous and dicotyledonous plants (Randoford, Bryce, & Morris, 2002). The AWPM-19-like is a 855 bp gene on chromosome 1 of Arabidopsis, encoding a 19 kDa hydrophobic polypeptide with four putative membrane spanning domains (Figure 2) (Koike, Takezawa, Arakawa, & Yoshida, 1997).

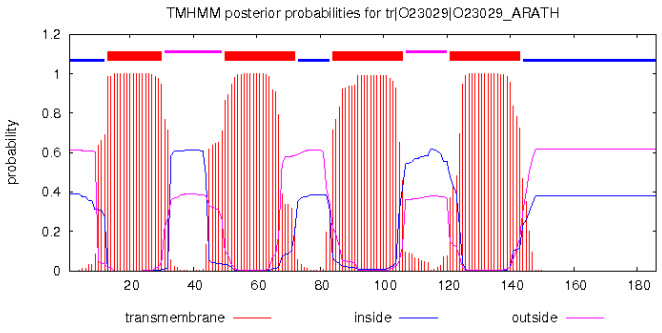

FIGURE 2. PREDICTION OF TRANSMEMBRANE HELICES IN AWPM-19 PROTEIN (TMHMM SERVER V. 2.0)

The BLASTp search showed that *A. thaliana* genome has three different genes that encode proteins with some similarity to AtPM19, suggesting that it might belong to a protein family (Alsaif, 2013).

The AWPM-19-like gene is predominantly expressed in mature seeds (Figure 4), hence its putative functions may be are related to seed traits such as germination and dormancy. In fact, AWPM-19 transcripts accumulate during seed maturation and decrease after imbibition (Bing Bai, Wageningen University and Research, unpublished data). Also, it has recently been seen that seeds from *awpm-19* mutant lines lose their capacity to germinate after ageing treatments, suggesting that they show high

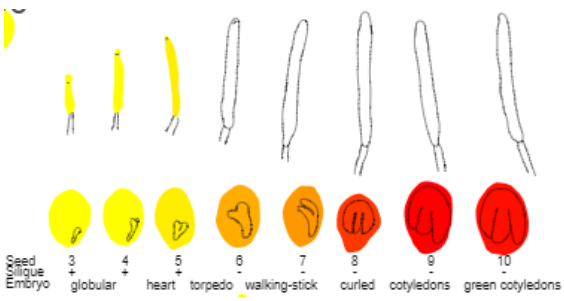

FIGURE 4. AWPM-19 GENE EXPRESSION MAP OF ARABIDOPSIS DEVELOPMENT FROM ARABIDOPSIS EFP BROWSER

dormancy and low longevity (Bing Bai, Wageningen University and Research, unpublished data). It can be hypothesized that the gene contributes to seed viability after dry storage.

This gene was also found in the co-expression network of *X. viscosa* plants under desiccation, indicating that it is involved in water stress response, likely mediated by the abscisic acid (ABA) signalling pathway in plants and seeds.

In winter wheat *(Triticum aestivum*) accumulation of the AWPM-19 polypeptide has been associated with increased freezing tolerance of ABA-treated cells (Koike, Takezawa, Arakawa, & Yoshida, 1997). In addition, it has been proposed to act as positive regulator of seed dormancy by using near isogenic lines segregating for the QTL region in which AWPM-19 gene belong (Barrero, et al., 2015). Also in barley (*Hordeum vulgare L.*), PM19 was found to be highly expressed in dormant embryos (Randoford, Bryce, & Morris, 2002).

The role of this gene in abiotic stress tolerance in *A. thaliana*, specifically low water availability, has been verified with the development of T-DNA mutant insertion lines, whose seedlings showed sensitivity to high levels of salts in the medium (Alsaif, 2013). Also, enhanced germination of seeds of overexpression lines on water, mannitol, NaCl and low concentrations ABA suggests that AWPM-19 protein might be involved in the efflux of Na^+ and water uptake during germination (Alsaif, 2013). Another indication of the involvement with water stress tolerance is the induction of AWPM-19 transcripts in leaves under drought stress (*Hruz et al. 2008).*

The analysis of *A. thaliana* co-expression network reveals a connection between *AWPM19-like* gene and LEA genes (figure 5). Also, the analysis of genes differentially expressed in *X. viscosa* under desiccation revealed a co-expression of *AWPM19-like* orthologue and LEA proteins, suggesting that it could be an activator of DT- responsive genes, and most likely this gene acts indirectly in the activation of LEA gene expression. In particular, some of the LEA proteins predicted from the network in Figure 5 belong to LEA_1 and LEA_4 families, which has been found to be involved in the adaptive response of higher plants to water deficit (Olvera-Carrillo, Campos, Reyes, Garciarrubio, & Covarrubias, 2010).

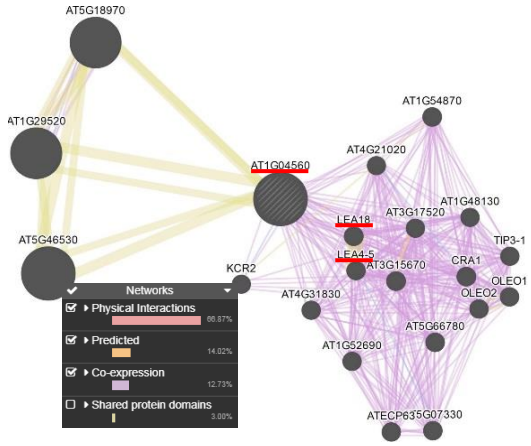

FIGURE 5. GENE MANIA CO-EXPRESSION NETWORK OF ATPM19

Arabidopsis Receptor Kinase (ARK) genes

Xerophyta viscosa transcriptome analysis showed that some genes are differentially expressed in the proximal part of the leaves and the distal part which encounters irreversible senescence after dehydration (Radermacher, A. & Farrant, J. – University of Cape Town, unpublished data). Particularly, some genes that shares sequence homology with Arabidopsis Receptor Kinases (ARK) 1, 2 and 3, which belong to the superfamily of plant receptor-like kinases (RLKs) from Arabidopsis.

Receptor-like kinases (RLK) localised in the in the plasma membrane have been shown to play important roles in sensing environmental stresses by binding specific ligands and activating signalling pathways via phosphorylation of serine/threonine kinase domains (Osakabe, Yamaguchi-Shinozaki, Shinozaki, & Tran, 2013). Functional studies in Arabidopsis revealed that many RLKs start accumulating after drying, confirming their role in drought tolerance (Osakabe, et al., 2010). Some receptor-like cytosolic kinase genes have been found to interact and regulate ABA-responsive genes like LEA genes, oleosin, ABI4 and ABI5, hence controlling ABA and osmotic stress signal transduction (Tanaka, et al., 2012). Also the transcriptome analysis of the resurrection plant *Myrothamnus flabellifolia* showed that genes encoding proteins from RLK-type families were differentially transcribed during dehydration and rehydration (Ma, et al., 2015).

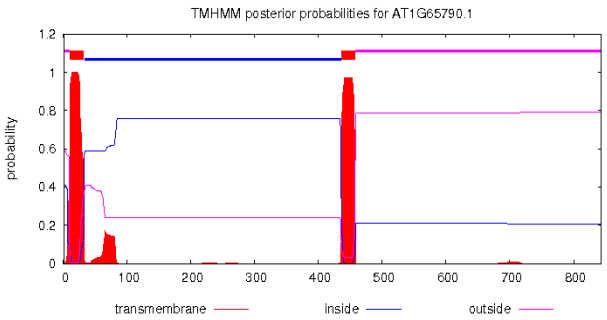

FIGURE 5 - PREDICTION OF TRANSMEMBRANE HELICES IN *ARK1* PROTEIN (TMHMM SERVER V. 2.0)

ARK1 and *ARK2* are two tandemly repeated genes encoding transmembrane proteins that share structure similarity with the S-locus receptor kinase (SRK) gene of *Brassica oleracea* (Tobias, Howlett, & Nasrallah, 1992). ARK genes structure and expression have been characterized by Tobias, et al. (1992 and 1996) and Dwyer, et al. (1994). All the *ARK* genes produce two alternative transcripts and thus alternative protein products (Figure 6): a plasma membrane-associate protein consisting of an S-domain linked to the kinase domain by a transmembrane domain (figure 5), but also a truncated protein consisting entirely of the *S*-domain and secreted into the extracellular matrix. The extracellular *S* glycoprotein domain is involved in signal transduction by binding different specific ligands and the receptor serine/threonine kinase domain is involved in auto-phosphorylation and phosphorylation of cytoplasmic substrates (Dwyer, et al., 1994).

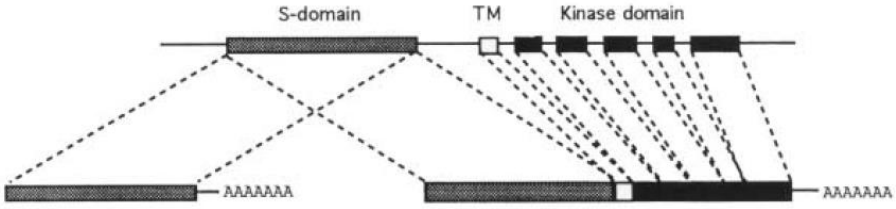

FIGURE 6 - *ARK1* ALTERNATIVE TRANSCRIPTS (TOBIAS & NASRALLAH, 1996). THE TOP LINE SHOWS THE STRUCTURE OF *ARK1* GENE. THE SECOND LINE SHOWS THE TWO ALTERNATIVE MRNAS THAT CAN BE PRODUCED.

The BLASTp search indicated 85% protein identity between *ARK1* (AT1G65790.1) and *ARK2* (AT1G65800.1) and 75% identity between *ARK1* and ARK3 (At4g21380.1), a third homolog from the S gene family of receptor kinases. This is common to most of the Arabidopsis RLKs, whose gene family expansion is mainly due to intrachromosomal duplications of the tandem regions (Shiu & Bleecker, 2001). Remarkably, the kinase domain is consistently more conserved thank *S* domain among Arabidopsis ARKs, suggesting that they may be activated by different ligands, but they phosphorylate the same or related cytoplasmic substrates (Dwyer, et al., 1994). Also in Xerophyta RLKs gene family is highly conserved, suggesting a general role in plant cell signal transduction. The alignment of ARK1 sequences in Xerophyta genome revealed 75% identity with the S-domain and 69% with the kinase domain, with the same predicted gene structure.

ARK genes are expressed predominantly in vegetative tissues like cotyledons, leaves, and sepals, specifically during the development of those structures (Dwyer, et al., 1994) and in senescent leaves (figure 7).

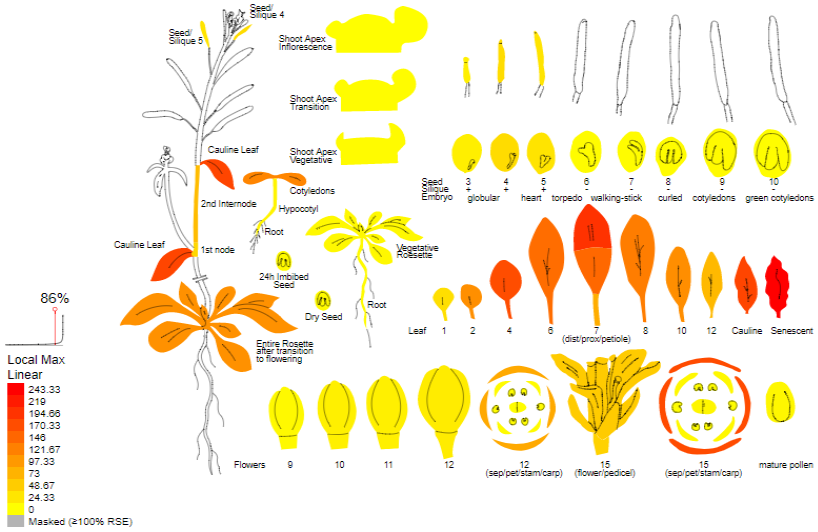

FIGURE 7 – *ARK1* GENE EXPRESSION MAP OF *ARABIDOPSIS* DEVELOPMENT FROM ARABIDOPSIS EFP BROWSER

Transgenic Arabidopsis plants overexpressing the *ARK1* transcripts showed smaller rosette leaves and reduced root system, as well as smaller inflorescence and fewer flowers, resulting in infertility (Tobias & Nasrallah, 1996). These growth abnormalities are probably due to aberrant pattern of protein phosphorylation by the kinase domain (Tobias & Nasrallah, 1996), and demonstrates the role of this gene in specific aspects of development of the plant body (Dwyer, et al., 1994).

From the protein-protein interaction network of both *ARK1* and *ARK2* it is possible to observe predicted interaction with several Plant U-Box (PUB) proteins, all containing a U-box and an ARM (Armadillo repeat) domain and having a E3 ubiquitin ligase activity (Figure 8). From *in vitro* phosphorylation assays Samuel et al., 2008, showed a strong interaction between S-domain 1 (SD1) receptor kinase and PUB-ARM proteins, revealing that the latter act as substrate. In particular, the presence of *ARK1* induces a relocalization of PUB9 from the nucleus to the plasma membrane, while *ARK2* mobilises it to the cytosol. The phosphorylation of PUB proteins by the active kinase domain of ARKs may act as signal to sort these proteins to the appropriate subcellular compartment for substrate interactions (Samuel, et al., 2008).

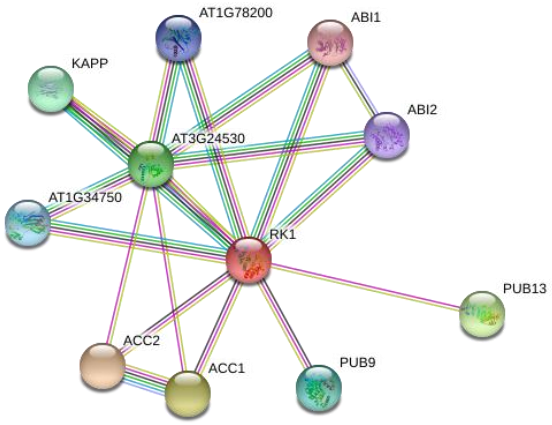

FIGURE 8 – *ARK1* (RED NODE) PROTEIN-PROTEIN INTERACTION PREDICTION FROM STRING

Interestingly, the same redistribution pattern was seen in cells treated with ABA, indicating a possible negative regulatory role of ARK and PUB9 proteins in ABA sensitivity, at or upstream the ABI3 gene. Indeed, mutant seeds of PUB9 and *ARK1* displayed hypersensitivity to 1 µM ABA and lower germination rates (Samuel, et al., 2008).

It might be relevant to highlight that PUB9 is present in both network of ARK genes and AWPM-19, being involved in physical interaction with SD1 receptor kinase and co-expressed with AWPM (figure 9).

Remarkably, from transcriptome analysis of *Xerophyta viscosa* under desiccation, ARK genes have been found to be expressed in the tip of senescent leaves (Radermacher, A., Farrant, J. – University of Cape Town, unpublished data), so it is hypothesized an additional role of these genes in controlling desiccation tolerance. It is known that in *X. viscosa* transcripts associated with the delay of senescence and anti-apoptosis accumulate during drying, giving evidence that in this species vegetative DT is uncoupled from common drought-induced senescence (Costa, et al., 2017). However, even if most part of the leaves recover after dehydration, the tip of older leaves show the typical drought-induced senescence. The reason and the nature of this physiological response in *X. viscosa* might be an efficient metabolic strategy for survival by reducing transpiration losses and allocating nutrients towards the rest of the plant. In general, as a response to abiotic external factors, if the functional capacity of a leaf is deleteriously affected by abiotic factors, targeted leaf senescence can be initiated (Law, et al., 2018). Notably, similar mechanisms are activated by dehydration in *X. viscosa* and darkness-induced senescence of individual leaves in Arabidopsis:

(1) rapid decline in photosynthetic capacity, hence cessation of carbon gain from photosynthesis; (2) accumulation of protective molecules; (3) altered sugar and amino acid metabolic pathway; (4) reallocation of nitrogen- and sulphur-rich nutrient from the senescing leaf to the rest of the plant, hence (5) altered source/sink relationship to fuel the export process (Law, et al., 2018).

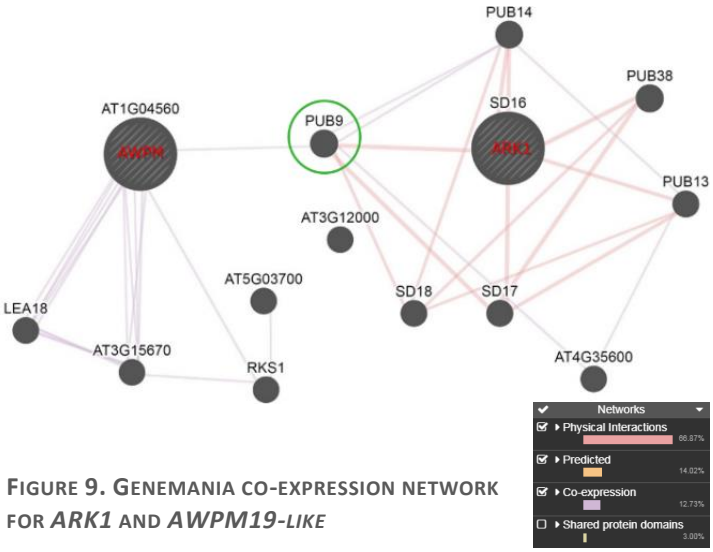

FIGURE 9. GENEMANIA CO-EXPRESSION NETWORK FOR *ARK1* AND *AWPM19-LIKE*

Research questions

Considering the above-mentioned findings, this thesis project is set up to test the following hypothesis:

AWPM-19 hypothesis (H_0)	**Mutant phenotype if H_0 is true**
H_0-1. AWPM is involved in sensing the water stress and signalling	Mutants will have lower germination rates under stresses (salt/osmotic/hormone/heat) and seedlings will show reduced growth
H_0-2. AWPM controls LEAs expression under stress	Mutants will have altered LEA protein expression

ARKs hypothesis (H_0)	**Mutant phenotype if H_0 is true**
H_0-1. ARK is either a: positive regulator of senescence → or negative regulator of senescence → and/or local regulator of senescence →	 Mutants will senescence later Mutants will senesce earlier In the mutants senescence might appear in different zones than root tip
H_0-2. ARK is a negative regulator of ABA response during seed germination	Mutant seeds will show lower germination rates with ABA
H_0-3. ARK is a regulator of plant development	Mutants will show growth abnormalities

Thesis objectives

The final aim of this thesis project is to investigate whether AWPM-19 and ARK genes may play a role in desiccation tolerance. In order to answer these questions, I will learn how to perform new techniques for phenotyping and genotyping Arabidopsis plants. The differences between mutants, overexpressors and wild-type plants will be evaluated as being an indication of the putative role of these genes.

I will conduct germination experiments to test the capacity of seeds and seedling to tolerate relatively high concentrations of compounds that can simulate water stress. Hence, I will learn how to apply stress treatments by mimicking environmental conditions, like drought and dark-induced senescence. I will make use of several bioinformatics tool, useful to elaborate and statistically analyse the results of the assays.

Laboratory techniques of molecular biology like nucleic acids purification, PCR (polymerase chain reaction) and RT-qPCR will be performed to assess the level of expression of the AWPM-19 and ARK genes or other connected genes, like LEAs, which can in turn be responsible for desiccation tolerance.

To conclude, this thesis project will allow me to gain a deeper insight of important scientific topics and techniques that will be beneficial for my future career in the plant biotechnology research field.

Overview of the project

AWPM-19 characterization

1- Phenotyping:
    a. Seed germination, dormancy, stress tolerance (osmotic, salt and heat) will be assessed.
    b. Seedlings will be analysed for their sensitivity to osmotic and salt stress.

   For the lines showing a clear phenotype compared to the wild type:

2- Genotyping both overexpression and T-DNA mutant lines by qPCR to quantify gene expression.
3- Analyse LEA genes' expression by qPCR in both overexpression and T-DNA mutant lines, to assess if AWPM gene is somehow related to LEA proteins.

ARK1 and *ARK2* characterization

1- Genotyping T-DNA mutant lines by PCR to select for plants carrying the mutation in homozygous state.
2- Phenotyping:
    a. Seed germination, dormancy, stress tolerance (osmotic, salt and heat) will be assessed.
    b. Seedlings will be analysed for their sensitivity to osmotic and salt stress
    c. Leaves will be used to analyse dark-induced senescence in adult plants, chlorophyll content and water loss.
    d. Growth and flowering will be monitored.
    e. Drought stress will be applied and survival and recovery rates monitored.

   If the mutant lines show a clear phenotype compared to the wild type:

3- Genotyping T-DNA insertion lines to quantify gene expression and confirm the knock-down or knock-down.

Materials and methods

Plant material and growth conditions

Most part of the experiments have been performed using the model plant *Arabidopsis thaliana*, Columbia ecotype (Col-0) as genetic background and as internal control for all the lines.

For the AWPM19-like gene (At1g04560), three T-DNA insertion lines were ordered (Table 1) from Salk Institute Genomic Analysis Laboratory (Table 1).

TABLE 1. INFORMATION ABOUT AWPM-19 T-DNA INSERTION LINES USED IN THE STUDY

My Code	Line code	Gene	T-DNA line	Insertion position	NASC number
WP1	AWPM-1	At1g04560	SALK_075435C	Exon	N679838
WP2	AWPM-2	At1g04560	SALK_062287	1000-Promotor	N562287
WP4	AWPM-4	At1g04560	SALK_091630	1000-Promotor	N591630

These lines have been genotyped and plants homozygous for the mutation were selected during previous experiments by M. A. Silva Artur, 2017.

Five independent overexpression lines were obtained by M. A. Silva Artur, 2017, by cloning the *Arabidopsis thaliana* AWPM19-like gene in Col-0 ecotype background, using a construct with the CaMV 35S promoter and Red Fluorescent Protein (DsRed) as selection marker. The T3 plants were grown in three biological replicates on Rockwool hydroponic system in climate room under a 22 °C and a 16/8 h light/dark regime.

For *ARK1*, two T-DNA insertion lines were ordered (one SALK insertion line from NASC Stock Center and one line from the Syngenta Arabidopsis Insertion Library (SAIL) collection). For *ARK2*, three SALK T-DNA insertion lines were ordered (Table 2). As shown in Figure 10, both SALK_024564.47.70.x and SAIL_401_F06 have a T-DNA insertion in the exon 1 of *ARK1*, probably in the S-domain. SALK_014441.43.80.x and SALK_082773.48.50.x insertions are in exon 5 and 6, respectively, in the kinase domain.

TABLE 2. INFORMATION ABOUT ARK T-DNA INSERTION LINES USED IN THE STUDY

My code	Gene	T-DNA line	Insertion	Position	NASC number
ARK1.1	AT1G65790.1	SALK_024564.47.70.x	Exon1	24469317-24469611	N524564
ARK1.2	AT1G65790.1	SAIL_401_F06	Exon1	24469224-24469748	N818550
ARK2.1	AT1G65800.1	SALK_012850.16.65.x	Exon2	24476099-24476273	N512850
ARK2.2	AT1G65800.1	SALK_014441.43.80.x	Exon5	24474880-24475177	N514441
ARK2.3	AT1G65800.1	SALK_082773.48.50.x	Exon6	24475827-24476268	N582773

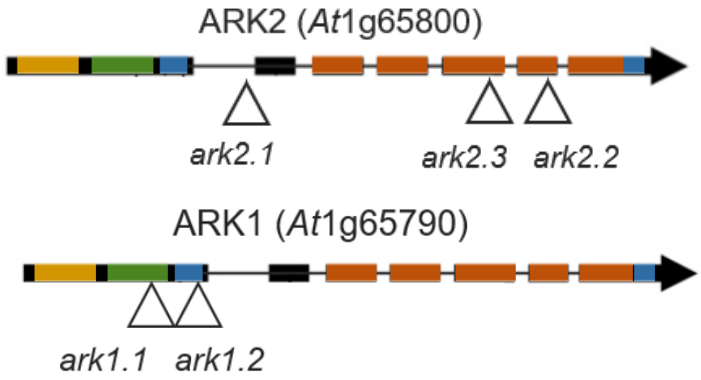

FIGURE 10 - SCHEME OF THE ARABIDOPSIS *ARK1* AND *ARK2* GENES DISPLAYING THE T-DNA INSERTION POSITIONS. DIFFERENT COLORS CORRESPONDS TO DIFFERENT PROTEIN DOMAINS. THE FIRST EXON CONTAINS THE ENTIRE S-RELATED REGION CONTAINING D-MANNOSE BINDING LECTIN (YELLOW), S-LOCUS GLYCOPROTEIN DOMAIN (GREEN), PAN-LIKE DOMAIN (LIGHT BLUE). THIS EXON IS IMMEDIATELY FOLLOWED BY AN IN-FRAME TAG STOP CODON WITHIN THE FIRST INTRON (TOBIAS, HOWLETT, & NASRALLAH, 1992). THE SECOND EXON ENCODES A 22-AMINO MEMBRANE-SPANNING REGION (BLACK). EXONS 3 TO 7 ENCODE A PUTATIVE RECEPTOR SERINE/THREONINE KINASE CATALYTIC DOMAIN (ORANGE), AND A SMALL DOMAIN OF UNKNOWN FUNCTION (DARK BLUE).

Ten plants from each ARK T-DNA line were grown on horticultural rockwool in a controlled-environment (16h/8h light/dark cycle, 21°C during the day, 18°C during the night).

For the subsequent experiments, seeds from the selected homozygous plants were harvested, stratified at 4°C for two days, sown in three replicates of at least 4 plants and grown at the same environmental conditions, apart for the dark-induced senescence, chlorophyll and drought experiments (see paragraphs below).

ARK genotyping

Every individual plant from the segregating *ark* T-DNA lines was genotyped. In order to determine the genotype of each single plant and select the individuals carrying the mutation in homozygous state, genomic DNA was extracted from the leaves. Plant material was collected by detaching the tips of rosette leaves from 19 days old plants.

The protocol used to extract the DNA was from Cheung et al., 1993, adjusted by M. A. Silva Artur. 130 µl of extraction buffer and one stainless steel bullet were added to each leaf sample in 1.5 ml tubes. The leaves were then grinded for 50 seconds at 30 Hz and incubated at 60°C for 45 minutes. The lysate was then centrifuged for 5 minutes at 17000g to remove the cell debris. 75 µl iso-propanol and 30 µl 10M ammonium acetate were added to 75 µl of supernatant to let the DNA precipitate. Total DNA was pelleted by centrifugation at 24,000g in the microcentrifuge for 10 min. The pellet was washed with 250 µl 70% ethanol and centrifuged at 24,000g in the for 5 min. Finally, it was dried and dissolved in 50 µl Milli-Q water.

The DNA samples form *ark* mutants were analyzed for the presence or absence of the T-DNA insertion by running a Polymerase Chain Reaction (PCR), to amplify the genomic fragments by using specific primers (Table 3), FIREPol® DNA Polymerase and deoxynucleotide triphosphates (dNTP). The amount of reagents used for each PCR reaction is depicted in Table 4. For each sample, two PCR reaction were carried: forward primer + reverse primer both specific for the ARK genes were used to identify the

wild-type bands; reverse primer + primer specific for the T-DNA insertion (BP SAIL or SALK, depending on the line) were used in order to detect the insertion.

All the selected plants were subjected to a second extraction of the DNA and PCR reactions were repeated to confirm the genotypes carrying the mutation in homozygous state.

TABLE 3. PCR PRIMERS DETAILS

		Primer sequence 5'-3'	Len	Product size	TM	GC	Insertion	BP+RP product size
SALK_024564.47.70.x	LP	ATTACATGACTTGGTCCGCAG	21	1161	60.01	47.62	chr1 24469611	557-857
	RP	ACGTATGGGTTGCAAACAGAG	21		60.04	47.62		
SAIL_401_F06	LP	TGTACTTTGTTGACCAAAGCC	21	972	57.80	42.86	chr1 24469748	441-741
	RP	ATTGGGATGGGATCAGAAAAC	21		60.01	42.86		
SALK_012850.16.65.x	LP	CGGCTTTGAGAGATGATTCTG	21	1209	59.96	47.62	chr1 24474880	586-886
	RP	CTTTACCGCGATTTCTTTTCC	21		60.08	42.86		
SALK_014441.43.80.x	LP	GGAAAAGAAATCGCGGTAAAG	21	1190	60.08	42.86	chr1 24476099	536-836
	RP	TATTACCGAGCGTCAATGACC	21		59.97	47.62		
SALK_082773.48.50.x	LP	CGAATTGGTAAAAGCAAGCAG	21	1191	59.90	42.86	chr1 24475827	561-861
	RP	TCGGGAATTCTGATGACAAAG	21		60.06	42.86		

TABLE 4. PCR REAGENTS

Reagent	**amount (µl)**
Milli-Q	10.05
Buffer B	1.5
MgCl2	1.5
dNTPs	0.4
primer F and R mix	0.4
FirePol	0.15
DNA template	1
Total	15

The products of the PCR amplification reaction were visualized after adding 3.5 µl of loading buffer and running a 1% agarose gel + Ethidium Bromide electrophoresis. The identification of the bands' length was done by loading in the gel 3 µl of SmartLadder marker.

Germination and dormancy assays

Germination under different abiotic stress conditions was assessed by sowing the seeds on blue germination papers in trays. Each paper was imbibed with 50 ml of solution, depending on the treatment (demineralised water, sodium chloride, mannitol, or ABA). The samples were sown in 3 replicates and each tray contained six samples of 50-100 seeds. For almost all the stress treatments, seeds were stratified in cold room at 4°C for 48 hours to ensure a more uniform germination. Seedlings were grown in a germinator incubator (growth cabinet) at 22 °C for 5 days, during which pictures were made twice a day the 1st, 2nd and 5th day, and three times a day the 3rd and 4th day. For the heat stress treatment, the seeds were incubated at 35°C for 24 hours and then transferred to the growth cabinet at 22 °C for further 4 days.

To assess germination rates, the pictures were analyzed by using the Germination system (GERMINATOR©), a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination (Joosen, Kodde, Willems, Ligterink, & van der Plas, 2010). In particular, we focused the analysis on maximum percentage of germination (Gmax) and time to reach 50% germination (t50).

Dormancy was evaluated by calculating the percentage of non-germinated seeds one week after sowing (after-ripened seeds).

Seedling stress experiments

Arabidopsis seeds were sterilized using the vapor-phase method with NaOCl and HCl described by Steve Clough and Andrew Bent, University of Illinois at Urbana-Champaign. After 4 hours sterilization in 1.5 ml microcentrifuge tubes, the seeds were sown on petri dishes (120mm) containing plant growth medium [0.5X Murashige and Skoog medium, 0.1% MES, 0.5% sucrose, 1% agarose, pH 5.7] in three replicates (four seedling each) per line. To ensure a more uniform germination the plates were placed in cold room at 4 ˚C. After two days cold stratification, the seeds were transferred in a climate room and kept on vertical trays at 21 ˚C under an 16h/8h light/dark cycle. After 4 days, the seedlings were transplanted to new petri dishes containing media with different concentrations of NaCl, Mannitol or ABA and grown vertically for further 6 days at the same conditions. The petri dishes were scanned 10 days after germination (Figure 11), and primary root length was measured with SmartRoot 2.0 in ImageJ, according to (Julkowska, et al., 2014). The effects of the salt and osmotic stress and ABA on seedling growth were analysed by comparing the root lengths of mutants with the wild-type seedlings. A Student T-test was carried out to test for statistical differences among treatment means (P-value<0.05).

FIGURE 11. EXAMPLE OF SCAN OF PETRI DISH SHOWING 10-DAYS-OLD SEEDLING GROWN IN 0.5X MS CONTROL MEDIUM

All the experiments were performed in sterile conditions under a fume hood cabinet.

RT-qPCR for quantification of gene expression

RNA extraction

For *awpm19* T-DNA insertion lines and AWPM overexpression lines, seeds and imbibed seeds were used for RNA extraction.

For *ark* T-DNA insertion lines, seedlings, leaves and senescing leaves from adult plants (see 'Dark-induced senescence' paragraph) were used.

RNA was isolated from the plant material with the Hot-borate extraction method (Maia J. , Dekkers, Provart, Ligterink, & Hilhorst, 2011). The RNA samples were then loaded on the nanodrop-1000 (Thermo-Scientific) to measure the amount (ng/µl) and OD 260/280 and OD 260/230 to assess quality.

The quality was also determined by adding 150 ng of RNA from each sample to a gel electrophoresis. Two bands corresponding to ribosomal RNA bands should be visible.

Absence of genomic DNA in the RNA samples was verified with a PCR reaction using primers for genomic DNA fragments.

cDNA synthesis and RT-qPCR

Clean RNA samples were used for cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad) which includes the enzyme reverse transcriptase and the iScript reaction mix. 600 ng of RNA template for each sample were used to be converted to cDNA. The complete reaction mix was incubated in a thermal cycler using the protocol supplied. After the reaction, 185 µl DEPC-treated water was added to dilute the samples to have 5 ng/µl.

The primers used for the AWPM and ARK genes were designed and tested in the cDNA samples with a PCR reaction. The specific primers as well as the primers used for housekeeping genes are summarised in Table 5.

TABLE 5. QPCR PRIMERS' DETAILS

Name		Sequence 5'-3'
AWPM	fwd	GGAATGATAGTTTAGCCGCTG
	rev	CCTCTCCATCCTCCTATGTTT
ARK1 S-domain	fwd	AAGTGTGCGGGAATTTCG
	rev	TCAGCCTCGTCTTCCTCA
ARK2 Kinase domain	fwd	ATTGGTAAAAGCAAGCAGGAG
	rev	CAATACCAAAACCACCTTGTCC
ARK2 S-domain	fwd	GTGGATTTTTGTGGCAGAGT
	rev	GTTGAATCCTCCGCTTTTGT
Ref1 (At2g28390) SAND	fwd	AACTCTATGCAGCATTTGATCCACT
	rev	TGATTGCATATCTTTATCGCCATC
Ref2 (At4g12590) UNKNOWN	fwd	GAGATGAAAATGCCATTGATGAC
	rev	GCACCCAGACTCTTTGATG
Ref3 (At4g34270) TP41-like	fwd	GTGAAAACTGTTGGAGAGAAGCAA
	rev	TCAACTGGATACCCTTTCGCA
Ref4 (At5g46630)	fwd	TCGATTGCTTGGTTTGGAAGAT
	rev	GCACTTAGCGTGGACTCTGTTTGATC
Ref (At4g05320) UBQ10	fwd	GGTTTGTGTTTTGGGGCCTTG
	rev	CGAAGCGATGATAAAGAAGAAGTTCG
LEA18 (At2g35300)	fwd	AGCTGACCTCCATCAATCCAA
	rev	GGTAATTAGCTCCGGTGGC
LEA19 (At2g36640)	fwd	ATGGGTTTCTTGTCGGGG
	rev	ACCTTCCAATCTCATTTCCTCC
LEA30 (At3g17520)	fwd	AGTCCGCGAAAGATAAGGC
	rev	CGTCGGTCAAGCTCTCTTTA
LEA46 (At5g06760)	fwd	ACGGAACGGGACAACTGA
	rev	CGTGAGTGTTATGAGCGGT
PUB9 (At3g07360)	fwd	TCTGCCTCACACGGCTTT
	rev	AGGTTGGGATGATATTGGCT

The RT-qPCR procedure was performed by mixing 2.5 µl cDNA sample with 5 µl iQ Sybr Green supermix (Bio-Rad), 0.5 µl primer mix (containing 10 µM of the forward and reverse primers) and 2 µl DEPC-treated water.

Two technical replicates per sample were pipetted in a 96-well PCR plate. By using a MyiQ iCycler (Bio-Rad) the samples were subjected to the following reactions: 3 minutes at 95°C, 50 steps of alternating between 15s at 95°C and 1 min at 60°C, and an increase from 55°C to 95°C.

Dark-induced senescence

Seeds harvested from *ark* homozygous mutants and wild-type plants were sown in three technical replicates. Plants were grown on rockwool system in a controlled environment (climate room under a 22 °C and a 16/8 h light/dark regime) and supplemented with Hyponex nutrition solution every two days. Single leaves from 7 weeks old plants were covered with aluminium foil following the experimental design of Weaver & Amasino (2001), with the exception that similar size rosette leaves from the 6th to the 12th were chosen. Individually darkened leaves (IDL) treatments were performed for 5, 3 and 0 (control) days (Figure 12, 13). All the leaves, including the controls, were detached at the same moment 5 days after the first treatment.

The same treatments were repeated on 30 days old plants, both *ark* mutants and wild-type plants.

Photographs of representative leaves were made after the detachment to visually assess the yellowing of darkened leaves from mutant lines compared to the wild-type.

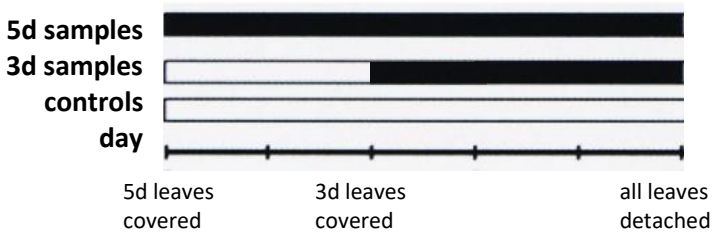

FIGURE 12. TIME COURSE OF INDIVIDUAL LEAF DARKNESS EXPERIMENT. WHITE BARS INDICATE TIME IN LIGHT AND DARK BARS TIME IN DARKNESS (ADAPTED FROM WEAVER & AMASINO, 2001)

The samples harvested from the 35-days-old plants were then frozen in liquid nitrogen and stored at -80°C before the chlorophyll extraction.

The samples harvested from the 30-days-old plants were then frozen in liquid nitrogen and stored at -80°C before the RNA extractions for the quantification of gene expression.

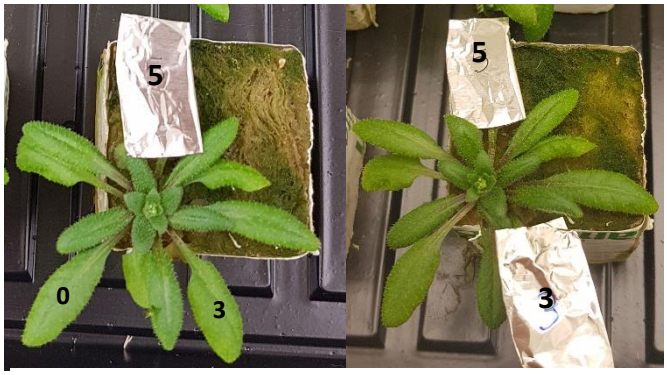

FIGURE 13. EXAMPLE OF INDIVIDUALLY DARKENED LEAVES (IDL) TREATMENTS SHOWING THE 5D, 3D AND 0 (CONTROL) LEAVES.

Chlorophyll extraction

For the quantification of the chlorophyll and carotenoids content, leaves from dark-induced senescence experiment were used, in particular leaves darkened for 5 and 0 days, hereby called senescing and control leaves. Two leaves per replicate were weighted and placed in 1.5 ml tubes. 100 µl of Dimethyl-sulphoxide (DMSO) and one stainless steel bullet were added to each tube. The leaves were then grinded for 60 seconds at 30 Hz and incubated at room temperature for 5 minutes after the addition of further 400 µl of DMSO. After centrifugation at 24000g for 5 minutes, 350 µl of supernatant were combined to a new 2 ml brown tube. The extraction was repeated with the pellet and 500 µl supernatant were mixed with the previously extracted supernatant in the brown tubes. The extracted samples were pipetted in two technical replicates in a 96-wells plate. The absorbance was measured spectrophotometrically at wavelength of 649 and 665 nm for chlorophyll a and b and 480 nm for total carotenoids. The chlorophyll a and b and carotenoids concentrations in µg/ml fresh weight were determined according to the formulas by Wellburn, 1994:

$C_a = 12.19\ A_{665} - 3.45\ A_{649}$
$C_b = 21.99\ A_{649} - 5.32\ A_{665}$
$C_{x+c} = (1000\ A_{480} - 2.14\ C_a - 70.16\ _b)/220$
Total chlorophylls = $C_a + C_b$

Water loss measurements

Three rosette leaves of similar size from 35-days-old Arabidopsis plants were detached and placed in petri dishes, previously weighted. They were kept open at toom temperature and the fresh weight was measured on an analytical balance every hour for one day.
The water loss rate (WLR) was calculated based on the formula from (Yao, et al., 2018):

$$WLR\ (\%) = \frac{(Fresh\ weight - desiccated\ weight)}{Fresh\ weight} \times 100\%$$

Drought experiment

ark mutans and wild-type plants were grown on rockwool in climate chamber and watered normally for 43 days with Hyponex solution. Then the watering was stopped for 12 days to mimic a moderate drought condition. The effects of water and nutrient deprivation were monitored visually and by the percentage of survival and recovery.

Results

AWPM-19

awpm-19 mutant lines show sensitivity to NaCl during germination

The seeds were analysed for their capacity to germinate under different concentrations of mannitol, NaCl and abscisic acid (ABA). In the first independent experiment *awpm-19* T-DNA mutant lines and AWPM overexpression lines were germinated on 200 mM mannitol and 75 mM sodium chloride, using Col-0 as reference and distillate water as control treatment. Under control and in the presence of mannitol no significant differences were observed between the wild-type seeds and the mutant lines of AWPM (Figure 14). Two out of three T-DNA lines showed significantly slower rates of germination (t50 = time to reach 50% of maximum germination) in 75 mM NaCl compared to Col-0 and most of the overexpression lines (Supplementary figure 1), indicating a sensitivity to moderate salt stress.

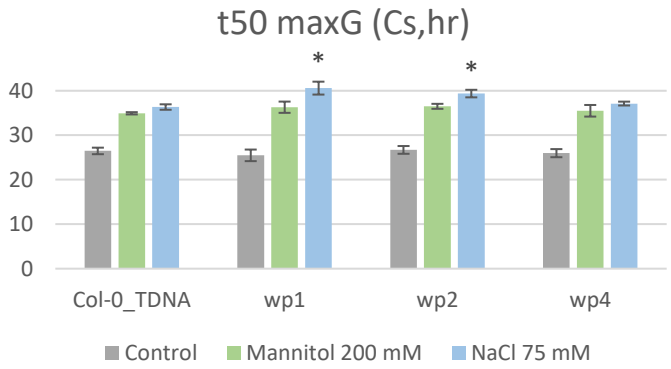

FIGURE 14. TIME TO REACH 50% GERMINATION (T50) ±SE FOR T-DNA LINES (WP1, WP2, WP4) IN 200 MM MANNITOL, 75 MM SODIUM CHLORIDE AND CONTROL TREATMENT

In the second independent experiment, higher concentrations of stress solutions were used (250 mM mannitol and 100 mM NaCl). Once more two *awpm* mutants (*wp1* and *wp2*) were affected significantly by high dose of salt, with 10 more hours needed to reach the 50% of germination (Figure 15) than Col-0 and the overexpressors. Some overexpression lines (O1 and O4) showed slightly faster germination under the same conditions compared to the wild-type.

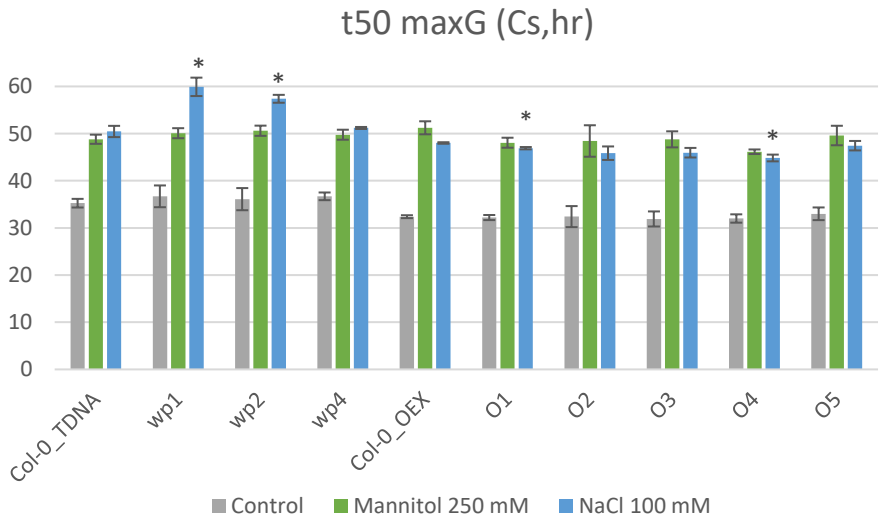

FIGURE 15. TIME TO REACH 50% GERMINATION (T50) ±SE FOR AWPM OVEREXPRESSION LINES (O1, O2, O3, O4, O5) AND T-DNA LINES (WP1, WP2, WP4) IN 250 MM MANNITOL, 100 MM SODIUM CHLORIDE AND CONTROL TREATMENT

The germination percentages for all the above-mentioned stress treatments were similar for all the lines, except for the mutant lines *wp1* and *wp2* which showed significantly lower gMAX (%) when compared to Col-0 under 100 mM of NaCl (figure 16).

These germination results suggest that AWPM gene may play a role on seed germination under salinity stress, perhaps by facilitating sodium efflux through the plasma membrane.

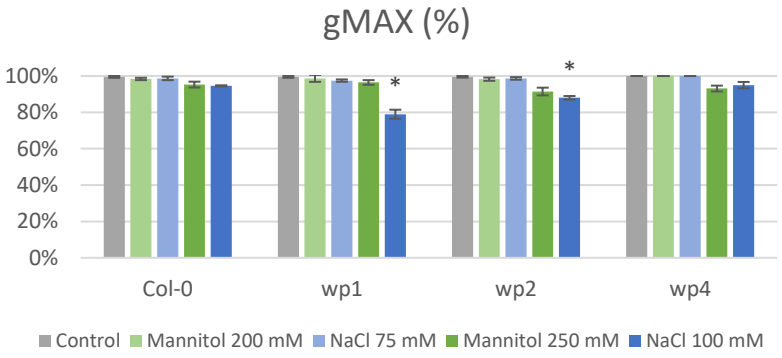

FIGURE 16. MAXIMUM PERCENTAGE OF GERMINATION (GMAX) ±SE IN AWPM T-DNA MUTANT LINES FOR ALL STRESS TREATMENTS

The osmotic stress induced by mannitol did not significantly affect the germination in any of the lines tested when compared to the wild-type.

No dormancy phenotypes were observed between any of the lines when maximum percentages of germinated seeds were counted one week after sowing (supplementary figure 2).

From the results of (Koike, Takezawa, Arakawa, & Yoshida, 1997), (Alsaif, 2013) and (Yao, et al., 2018) AWPM was found to be an ABA-responsive gene. The treatments with ABA did not induced reduction in germination rates of the insertion mutants (data not shown), compared to the wild-type.

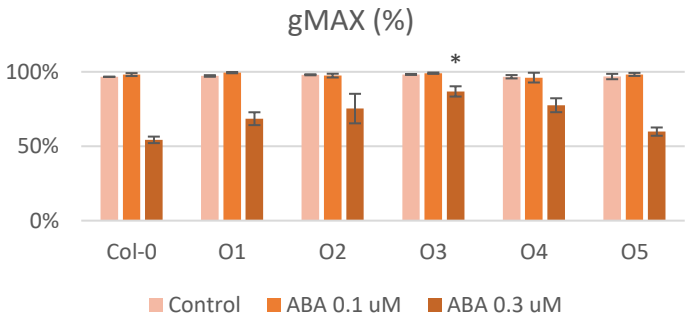

FIGURE 17. GERMINATION PERCENTAGES (GMAX) ±SE FOR AWPM OVEREXPRESSION LINES (O1, O2, O3, O4, O5) IN 0.1 UM AND 0.3 ABA

The overexpression lines tended to reach higher germination percentages (Figure 17), particularly line O3 in 0.3 µM ABA, confirming the results from Alsaif, 2013. This might indicate an induction of AWPM gene by exogenous ABA, which is in involved in several biochemical pathways of plant development and abiotic response, including seed germination and desiccation tolerance (Finkelstein, Gampala, & Rock, 2002).

Heat stress effects were assessed on seed germination as high temperature increases ABA levels and affects embryo development and seed viability (Jemaa, Saida, & Sadok, 2010). The heat shock caused a reduction in germination rates of the mutants, particularly *wp1* whose seeds needed on average 5 hours more to reach 50% of germination, compared to wild-type. (Figure 18).

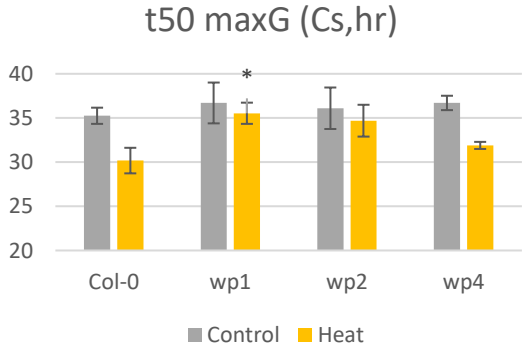

FIGURE 18. TIME TO REACH 50% GERMINATION (T50) ±SE FOR AWPM T-DNA LINES (WP1, WP2, WP4) FOR HEAT AND CONTROL TREATMENTS

Seedlings from AWPM overexpression lines are more resistant to salt but not ABA treatments

In order to investigate the effects of osmotic and salt stress on primary root length we performed plate-based experiments of seedling growth in MS media. As for the germination tests, different concentrations of NaCl, mannitol and ABA were applied to simulate abiotic stress. Contrarily to what observed from germination assays, *awpm-19* mutant seedlings were not affected by NaCl treatments more that the wild-type. On the contrary, seedlings of the overexpression line O5 (Figure 19) showed increased root length in low and moderate salt concentrations compared to wild-type. Since seedlings from line O3 appeared to be significantly different from Col-0 also in control media, I hypothesise that this phenotype may be due to differences in the initial size of the seedlings. The same hypothesis is applied to explain the increased root growth of the mutant *wp4* in mannitol. To be mentioned that *wp1* seedlings were often subjected to contamination or stopped growing after the transplant to the stress media (1/3 of the seedlings were removed from the analysis). This might be due to an intrinsic general sensitivity of this line whose mutation could affect the growth and also biotic resistance.

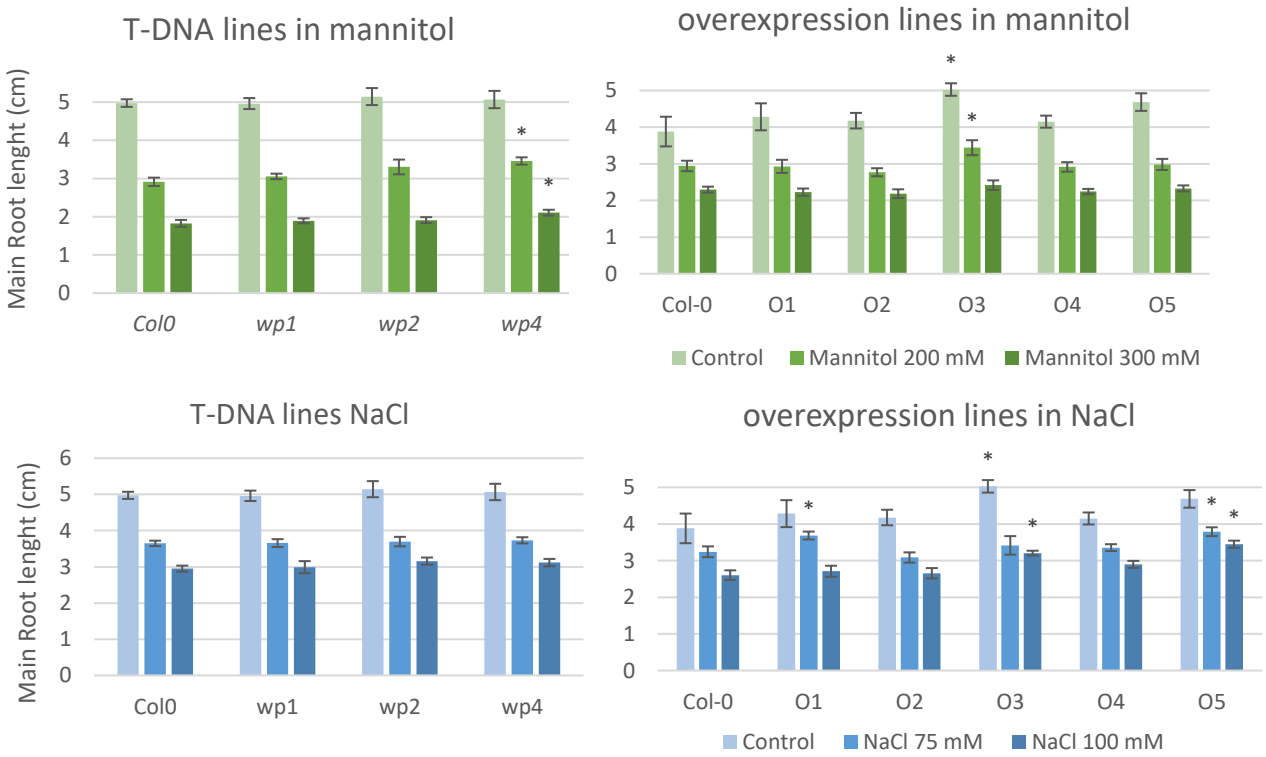

FIGURE 19. AVERAGE MAIN ROOT LENGTH ±SE FOR AWPM OVEREXPRESSION LINES (O1, O2, O3, O4, O5) AND T-DNA LINES (WP1, WP2, WP4) IN MANNITOL AND SODIUM CHLORIDE

When ABA was added to the medium, roots from mutant *wp4* line resulted to be significantly shorter than Col-0 (Figure 20). The same effects were observed for some overexpression lines (O2, O4, O5), which was not expected from the preliminary hypothesis of increased ABA-induced stress tolerance. In fact, Arabidopsis eFP Browser (http://bar.utoronto.ca) demonstrates that AWPM gene is highly expressed after 3 hours treatment with 10 µM ABA. Hence, a better performance of seedlings overexpressing AWPM was expected, in accordance to the germination test results. It may be relevant to point out that line O4 seedlings were visibly smaller compared to the other lines when transplanted to the stress media and no or few later roots were observed after 10 days in all treatments. One can hypothesise a negative-feedback regulation as a results of excessive protein levels, which may affect cell metabolism causing physiological protective degradation mechanisms.

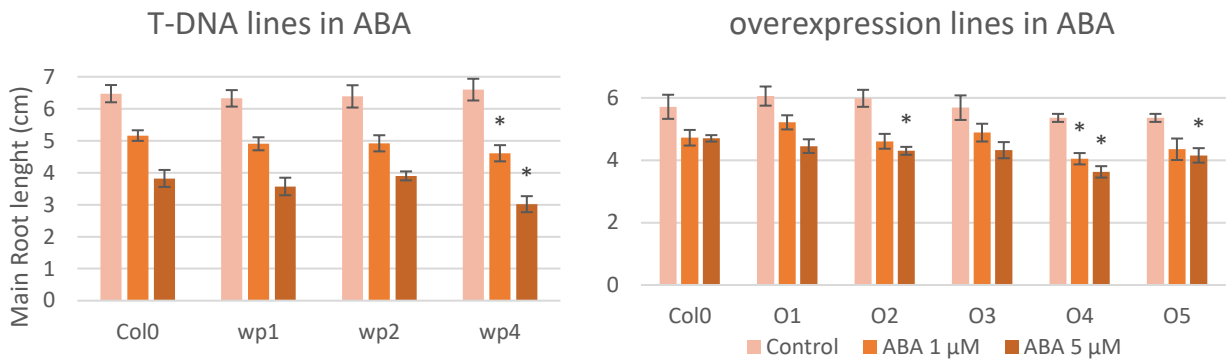

FIGURE 20. AVERAGE MAIN ROOT LENGTH ±SE FOR AWPM OVEREXPRESSION LINES (O1, O2, O3, O4, O5) AND T-DNA LINES (WP1, WP2, WP4) IN ABA

Confirmation of reduced gene expression in *awpm* T-DNA insertion lines

The RT-qPCR aimed at the confirmation of the insertion mutants and the overexpression lines by quantification of the *AWPM19-like* gene expression. The techniques and primers specific for *AWPM19-like* and the housekeeping genes used are described under 'Materials and methods'.

T-DNA lines showed the same gene expression levels both in dry seeds and imbibed seeds (Figure 21). In mutants *wp1* and *wp2* the *AWPM19-like* gene had a relative expression of 0.1-0.2 and 0.16-0.17, respectively. The *wp4* mutant showed a reduction or expression of 0.6 when compared to the wild-type, thus we considered it as a knock-down mutant.

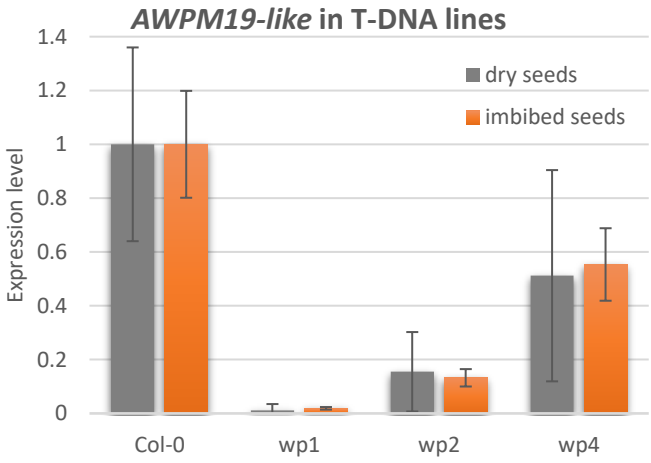

FIGURE 21. NORMALISED RELATIVE GENE EXPRESSION ±SE OF AWPM-19 GENE IN MUTANT T-DNA LINES. GREY BARS FOR EXPRESSION IN DRY SEEDS, ORANGE BARS FOR IMBIBED SEEDS (TWO TECHNICAL REPLICATES PER LINE WERE USED)

Overexpression lines showed different expression patterns in imbibed seeds and dry seeds (Figure 22, Supplementary figure 5). This is due to the low activity of the 35S promoter in dry seeds, which influence the expression of the cloned gene. Hence, the expression levels measured in imbibed seeds are more reliable in this case. Line O1 shows a little down-regulation, maybe due to silencing mechanisms as a result of the overexpression. Slightly higher transcript levels are observed in lines O2, O4 and O5, in which *AWPM19-like* appears to be 0.2 times more expressed than in wild-type seeds. Overall, the *AWPM19-like* gene expression is comparable to the wild-type in the overexpression lines used in this study, which might explain the inconsistent phenotyping results.

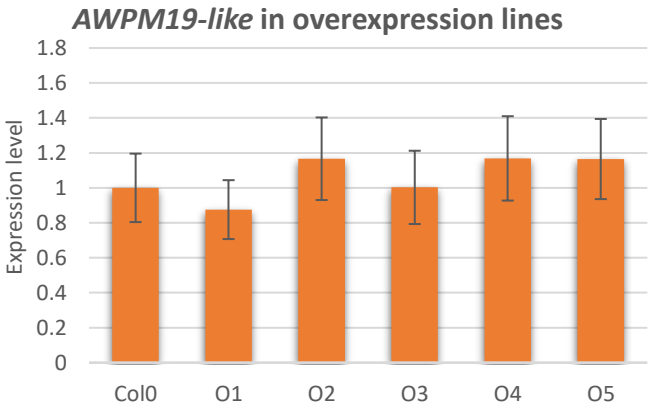

FIGURE 22. NORMALISED RELATIVE GENE EXPRESSION ±SE OF AWPM-19 GENE IN OVEREXPRESSION LINES IMBIBED SEEDS (TWO TECHNICAL REPLICATES PER LINE WERE USED)

Some LEA genes are co-expressed with AWPM gene

The LEA genes' expression pattern in the *AWPM19-like* lines used in this study is fundamental to have an insight of the putative relation between *AWPM19-like* and some LEA genes. In particular, we will focus on the expression of genes from LEA4 group, which were found to be differentially expressed during embryogenesis and in response to water deficit treatments in Arabidopsis (Olvera-Carrillo, Campos, Reyes, Garciarrubio, & Covarrubias, 2010).

The results of the RT-qPCR showed that LEA gene 18 transcripts tend to decrease in loss-of-function mutants *wp1* and *wp2* (Figure 23, top), but no specific expression pattern is observed in the knock-down mutant *wp4* nor the overexpression lines. It is common to observe differences between knockout and knockdown mutants, mainly due to changes in the expression of other genes within the same network as compensation mechanisms in knockouts but not in knockdowns (El-Brolosy & Stainier, 2017).

On the contrary, LEA30 and LEA46 genes seem to specifically follow the expression of AWPM-19 (Figure 23): their transcript numbers increase in all *AWPM19-like* T-DNA insertion lines, while an increase is observed in almost all the overexpression lines, up to 6-fold for LEA30. This trend suggests a co-expression or induction between the *AWPM19-like* and LEA genes from group 4, indicating a complex regulation pathway among *AWPM19-like* and LEA genes.

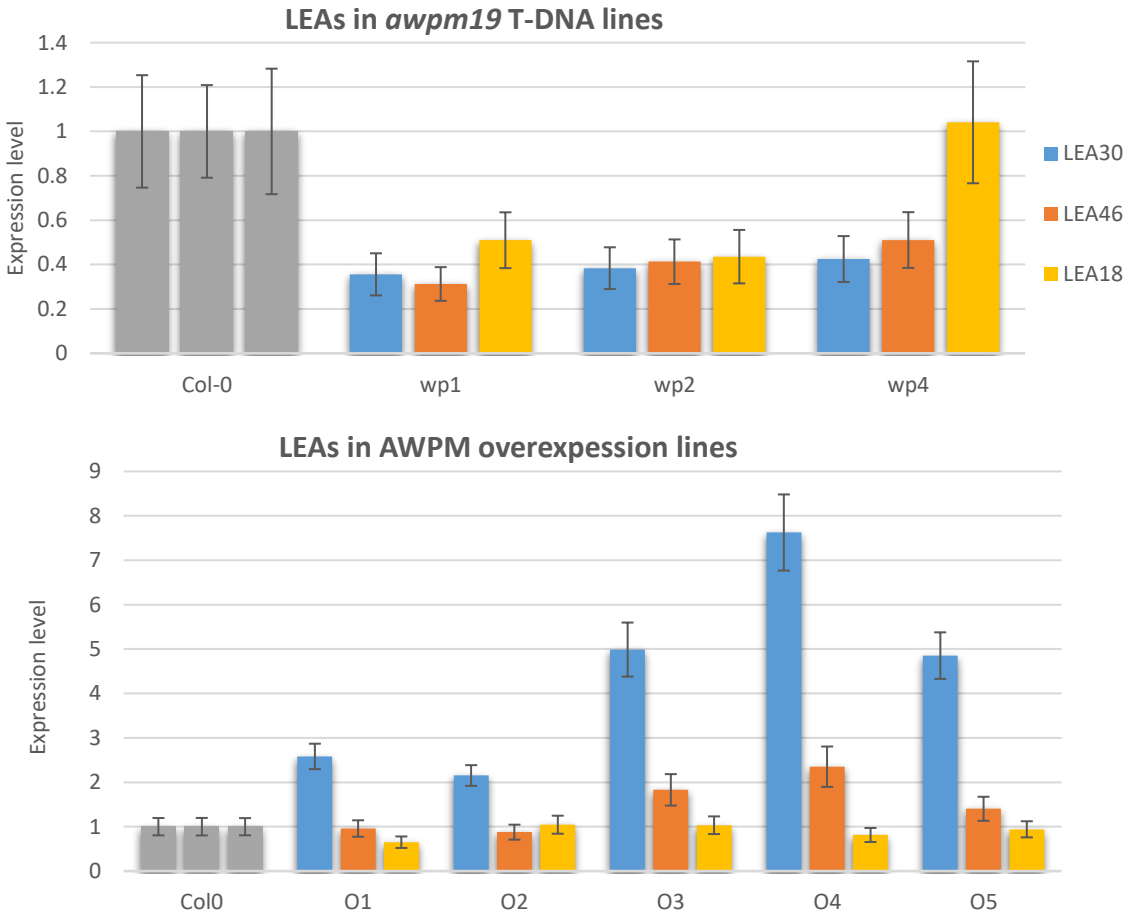

FIGURE 23. NORMALISED RELATIVE EXPRESSION OF THREE DIFFERENT LEA GENES IN AWPM T-DNA LINES (TOP) AND AWPM OVEREXPRESSION LINES (BOTTOM) IMBIBED SEEDS.

TABLE 6. SUMMARY OF LEA GENES BEHAVIOUR IN KNOCK-OUT/KNOCK-DOWN MUTANTS AND OVEREXPRESSION LINES

If **AWPM** is...	Knocked-out ×	Knocked-down ↓	Overexpressed ↑
...LEA18	↓	=	
...LEA30 and LEA46	↓	↓	↑

ARK1 and *ARK2*

Identification of T-DNA insertion mutants

Among the parental T-DNA lines ordered from the stock, plants not carrying the insertion in the gene of interest can still be present (wild-type or heterozygous). For this reason, homozygous segregants for the mutation were identified following the procedures described under 'Materials and methods'. PCRs were carried out using the genomic DNA of all the individuals from the parental stock.

For the *ARK1* mutant lines, one homozygous plant was selected in line *ark1.1* (Figure 18, top line). Six plants carrying the mutation were identified for *ARK2* mutants, respectively two plants from line *ark2.2* and four plants from line *ark2.3* (Figure 24, bottom line).

In lines *ark1.2* and *ark2.1*, all the plants resulted to be wild-type. The reason could be consequent to the mutation, which could have compromised the production of vital seeds. In particular, the insertion in line *ark1.2* is located in the PAN domain, probably affecting the putative binding site, fundamental for protein-protein interactions. In *ark2.1* the insertion is probably located at the beginning of the Serine/threonine kinase domain. Another explanation could be that the insertion in the T-DNA lines caused off-target effects in the genome that knocks out one or more genes important for viability.

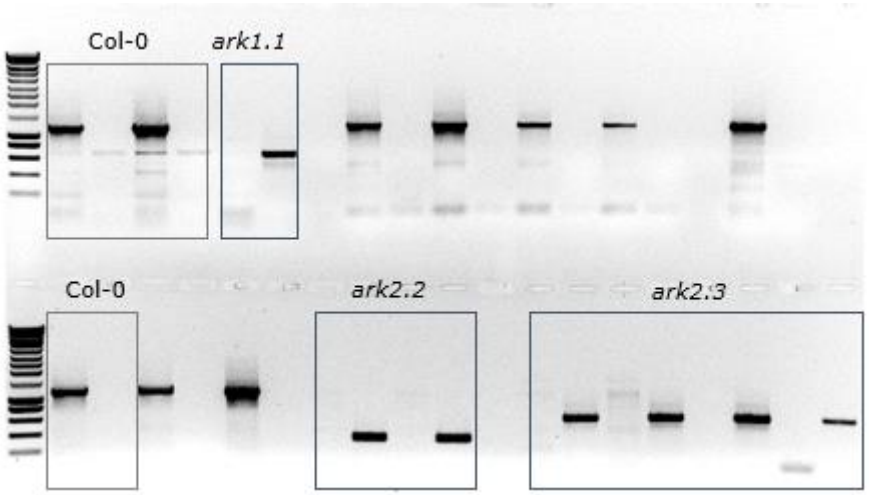

FIGURE 24. PCR PRODUCTS SHOWING THE PRESENCE OF THE INSERTION IN HOMOZYGOUS STATE FOR THE SELECTED MUTANTS

ARK2 mutants respond better to salt and hormone stress treatments

A first assessment of seed dormancy was carried out after harvesting *ark* mutant seeds by sowing them on distilled water without stratification at 4°C. Both *ARK2* mutant lines showed increased germination rates (Figure 25), but all reached 95% of germination (data not shown).

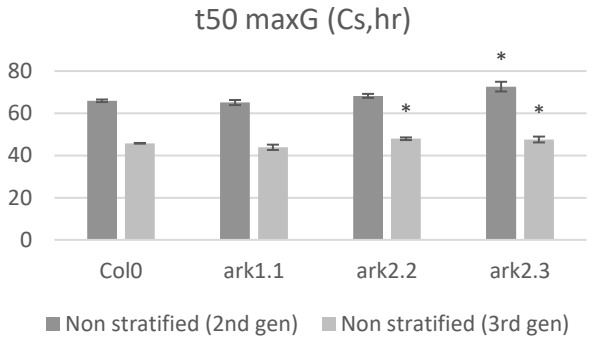

FIGURE 25. TIME TO REACH 50% GERMINATION (T50) ±SE FOR ARK T-DNA LINES FROM TWO GENERATIONS OF SEEDS

However, when tested under salinity stress, both *ARK2* mutants appeared to be less affected in their germination speed compared to Col-0 and *ark1.1* (Figure 26, right). It is still not

clear why the mutants performed better, but it can be hypothesised that the *ARK2* gene is somehow involved in the regulation pathway of the response to salt stress during germination.

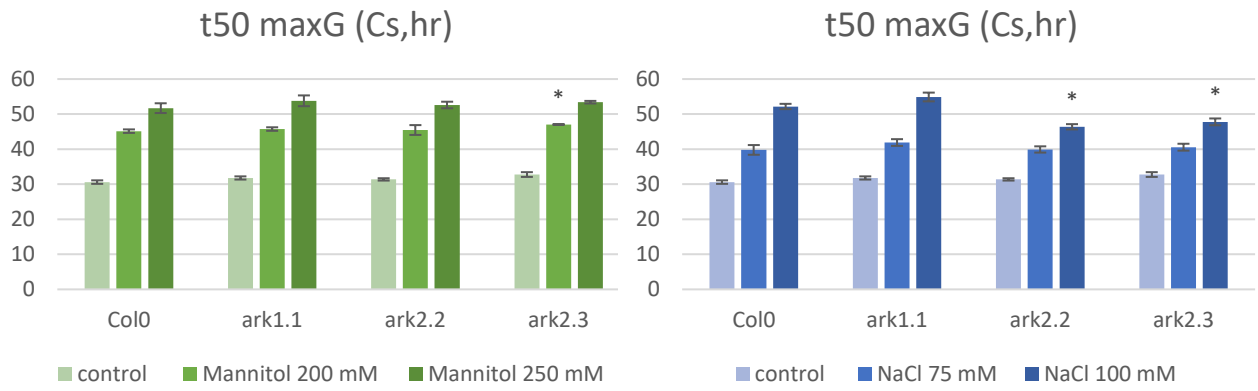

FIGURE 26. TIME TO REACH 50% GERMINATION (T50) ±SE FOR ARK T-DNA LINES IN DIFFERENT CONCENTRATIONS OF MANNITOL (LEFT) AND SODIUM CHLORIDE (RIGHT)

No significant results on germination rates were observed for the heat shock treatment, nor for ABA treatments (Figure 27). However, *ARK2* mutants reached higher germination percentages under 0.3 µM ABA compared to wild-type. Hence, also in this case it can be hypothesised that the gene functions are related to ABA-mediated response.

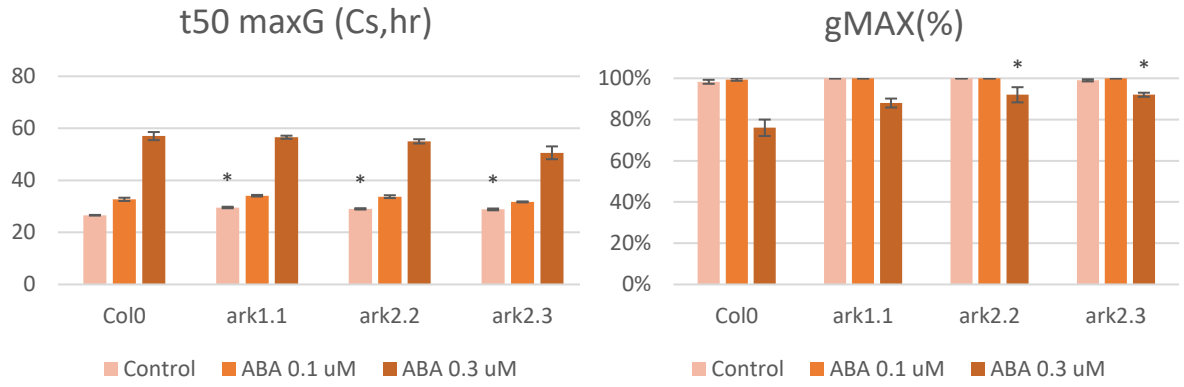

FIGURE 27. TIME TO REACH 50% GERMINATION (T50) ±SE (LEFT) AND GERMINATION PERCENTAGES (GMAX) ±SE (RIGHT) FOR ARK T-DNA LINES IN 0.1 UM AND 0.3 UM ABA

Root elongation of mutants was not affected by NaCl, mannitol and ABA treatments

The stress treatments on seedlings in MS media did not affect the primary root growth differently from the wild-type (Supplementary figures 3 and 4), suggesting that the mutation does not have effects on plant development during seedling stage.

Mutant *ARK2* lines shows some growth abnormalities during different developmental stages

The different stages of plant growth were monitored to assess abnormalities. From preliminary functional studies aberrant phenotypes were observed in transformants overexpressing *ARK1* (Tobias & Nasrallah, 1996), that showed smaller rosette leaves and roots, tendency to become chlorotic, altered stomata morphology, smaller and fewer flowers. Rosette growth appeared to be slower in *ARK1* mutant plants, as 30 days-old plants showed smaller rosette leaves and visibly smaller diameter compared to wilt-type and *ARK2* mutant plants (Figure 28). The same alteration was observed in the next generation of plants, whose rosette leaves showed also a chlorotic appearance (figure 29). Even though the variability observed among Col-0 plants did not allow the assessment of a reliable comparison, rosettes from *ARK1* T-DNA line were generally smaller, while the ones from *ARK2* mutants were notably bigger, with the first flower buds visible (Figure 29). In fact, most of the plants of T-DNA mutants from the first and second F1 generation started flowering earlier than Col-0 (Figure 29) or showed faster bolting with taller stems. However, in the next generations only few hours difference in bolting was observed between mutants and wild-type plants, which showed synchronized growth and flowering.

FIGURE 28. COMPARISON BETWEEN ROSETTE GROWTH OF 30-DAYS-OLD PLANTS FROM T-DNA LINE ARK*1.1* (MIDDLE), T-DNA LINE *ARK2.3* (RIGHT) AND COL-0 (LEFT) GROWN IN CLIMATE ROOM UNDER A 22 °C AND A 16/8 H LIGHT/DARK REGIME

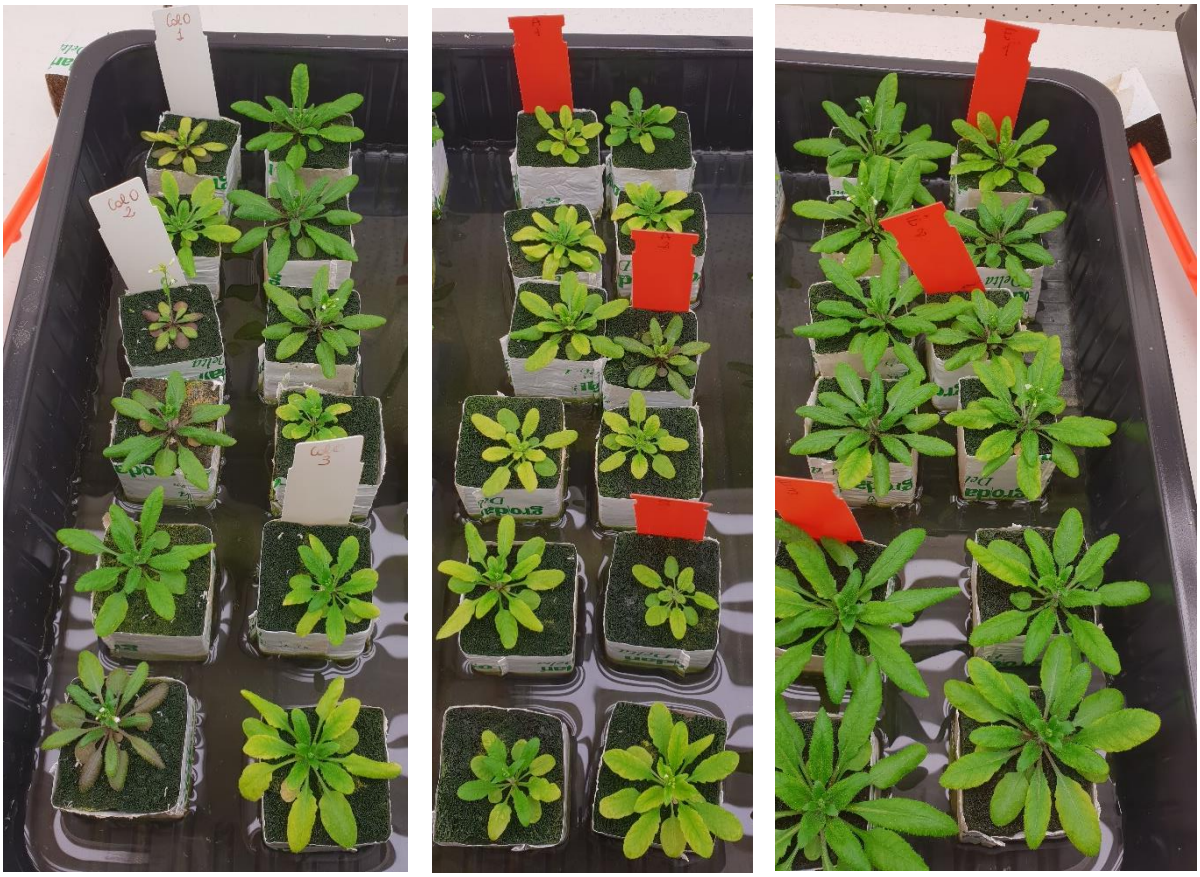

FIGURE 29. COMPARISON BETWEEN ROSETTE GROWTH OF 35-DAYS-OLD PLANTS FROM T-DNA LINE *ARK1*.1 (MIDDLE), T-DNA LINE *ARK2*.3 (RIGHT) AND COL-0 (LEFT) GROWN IN CLIMATE ROOM UNDER A 22 °C AND A 16/8 H LIGHT/DARK REGIME

FIGURE 29. FLOWERING TIME OF 30-DAYS OLD PLANTS FROM COL-0 (LEFT), ARK1.1 MUTANT (CENTRE) AND ARK2.3 MUTANT (RIGHT) PLANTS GROWN IN GREENHOUSE

Relevant abnormalities were also observed during early and late stages of senescence. When grown under greenhouse conditions, rosette leaves from *ark1.1* mutant lines showed precocious senescence 36 days after sowing (figure 30) and they completely degraded few days later. In climate rooms it was also observed that flowers were unexpectedly produced on 3-months-old *ark1.1* mutant plants (figure 31). In fact, after stage 6.9 (around 50 days after sowing), flower production should be complete (Boyes, et al., 2001). Although ARK genes expression is not detectable in reproductive tissues apart

from sepals, a role in plant development and senescence is supported by the observed phenotypes. It is also hypothesised that these receptor kinases are involved in common pathways regulating both flowering and leaf senescence (see the 'Discussion' session).

FIGURE 30. ROSETTE APPEARANCE OF 36-DAYS-OLD PLANTS OF FROM COL-0 (LEFT), ARK1.1 MUTANT (CENTRE) AND ARK2.3 MUTANT (RIGHT) GROWN IN GREENHOUSE

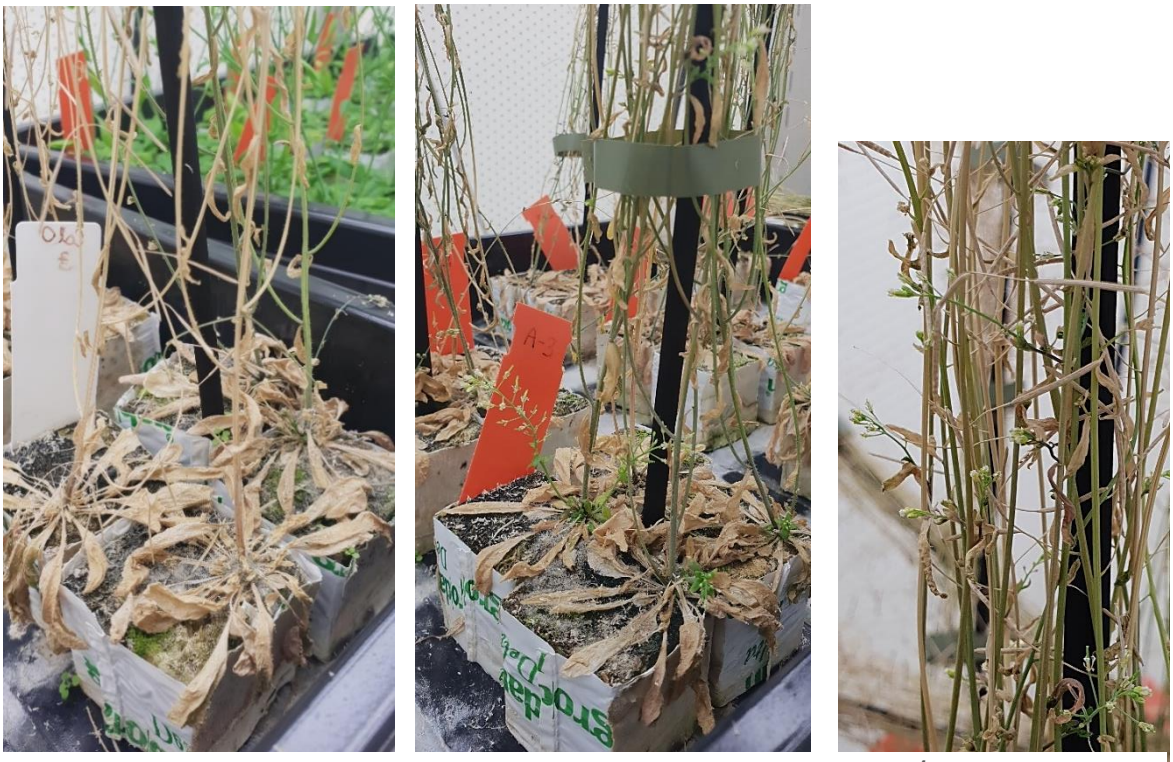

FIGURE 31. FLOWERS APPEARANCE IN 90-DAYS-OLD SENESCING PLANTS FROM *ARK1*.1 LINE (MIDDLE AND RIGHT IMAGES), COMPARED TO WILD-TYPE PLANTS (LEFT) GROWN IN CLIMATE ROOM UNDER A 22 °C AND A 16/8 H LIGHT/DARK REGIME

Chlorophyll breakdown after dark-induced senescence changes in leaves of *ark* mutant lines

Individually darkened leaves (IDL) treatments aimed at the artificial induction of senescence to test whether rosette leaves from T-DNA insertion mutants show different senescing phenotypes. From a visual analysis, no differences were noticed in the mutant leaves after 3 and 5 days in the dark, apart from a physiological yellowing starting from the tip of the leaves due to chlorophyll loss (Figure 32 and 33). Also due to variability between the biological replicates, it was not possible to assess consistent visual differences in leaf senescence with the wild-type. To quantify possible differences with Col-0

FIGURE 32. LEAVES DETACHED FROM 7-WEEKS-OLD PLANTS. FOR EACH REPLICATE, THE CONTROL (0D), 3 DAYS DARKENED (3D) AND 5 DAYS DARKENED (5D) LEAVES ARE DEPICTED FROM LEFT TO RIGHT

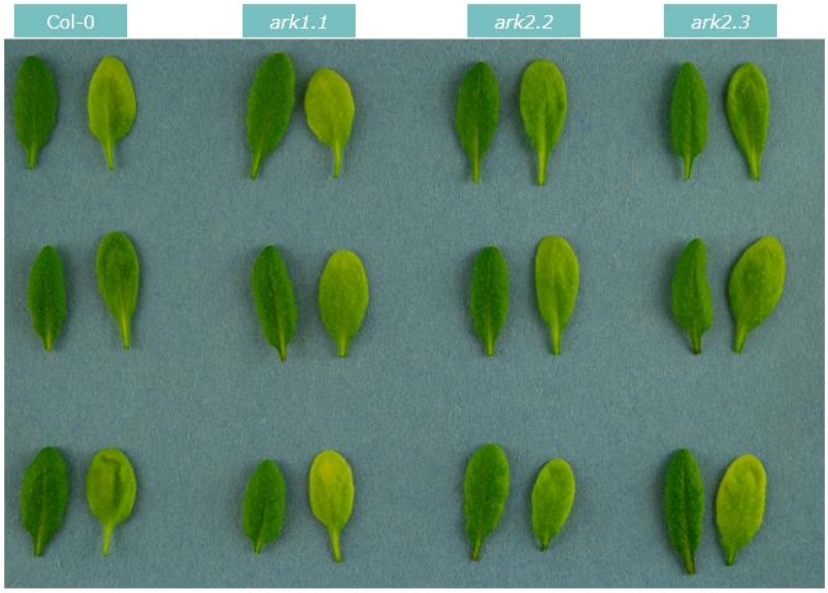

FIGURE 33. LEAVES DETACHED FROM 30-DAYS-OLD PLANTS. FOR EACH REPLICATE, THE CONTROL (0D, LEFT) AND 5 DAYS DARKENED (5D, RIGHT) LEAVES ARE DEPICTED

leaves in pigments content, chlorophylls and carotenoids were extracted from detached leaves from 7-weeks-old plants.

Individually darkened leaves for 5 days (hereby senescing) and control leaves of the same age from mutant T-DNA lines were compared with wild-type leaves for the amount of chlorophyll a, chlorophyll b, total carotenoids and total chlorophylls (figure 34). Line *ark1.1* leaves showed higher amount of chlorophylls in senescing leaves when compared to Col-0 senescing leaves, particularly chlorophyll a. On the contrary, control leaves from *ark2.2* line have a decreased total chlorophyll content than wild-type, specifically chlorophyll b. Apart from chlorophyll a, the pigments were reduced in senescing leaves compared to their internal controls.

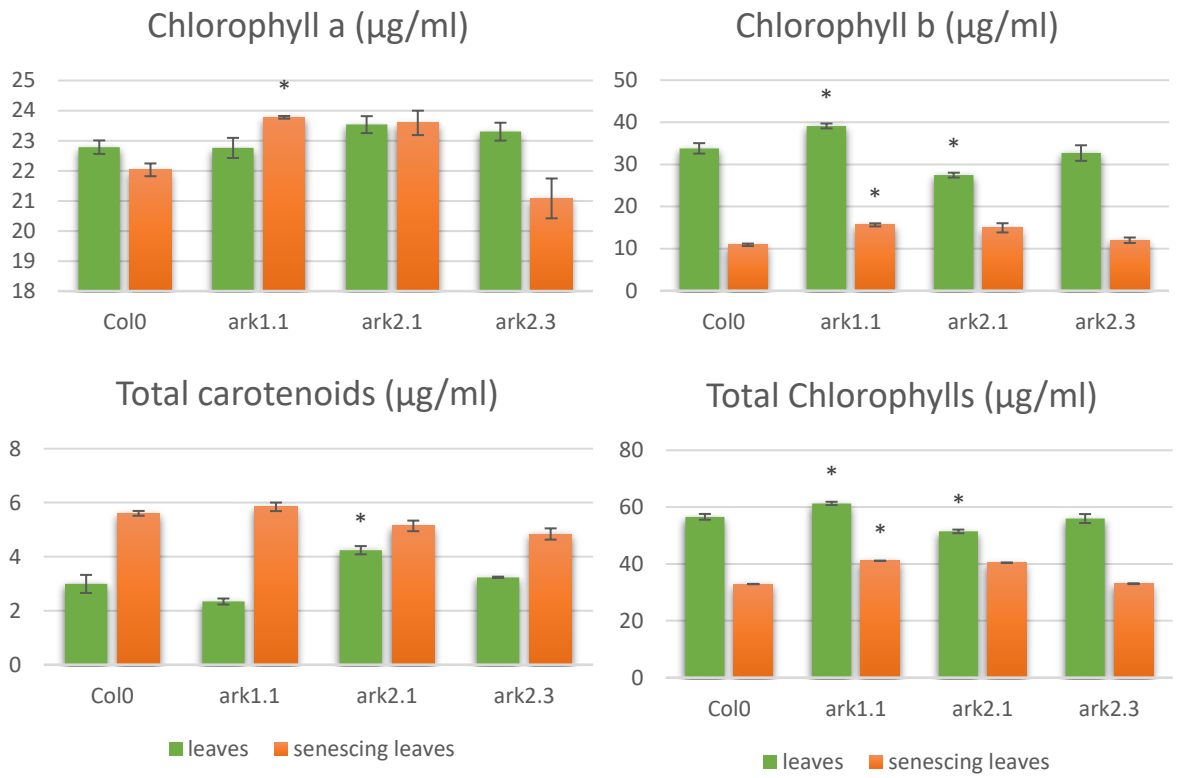

FIGURE 34. CHLOROPHYLLS AND CAROTENOIDS CONTENT (µG/ML) ± SE IN CONTROL LEAVES (GREEN BARS) AND LEAVES INDIVIDUALLY DARKENED FOR 5 DAYS (ORANGE BARS) OF THE SAME AGE FROM COL-0, *ARK1* AND *ARK2* LINES. ASTERISKS INDICATE SIGNIFICANT DIFFERENCES WITH THE WILD-TYPE

Drought stress strongly affected *ARK2* plants

Drought stress was induced on plants growing on Rockwool when most of flowers to be produced had opened. Twelve days after the dehydration was started, survival rates were measured: five wild-type plants out of twelve survived from the drought stress, seven *ark1.1* mutants, two *ark2.2* mutants and only one *ark2.3*. The plants that were able to tolerate or avoid the decreased water availability also slowly recovered to normal growth 30 days after re-watering. Based on the survival percentages observed, *ark1.1* mutant plants had improved resistance to water deficits compared to wild-type plants and both *ARK2* mutant lines, which, in contrast, were severely affected by the drought stress.

Water loss of detached leaves from *ARK2* was significantly faster than that of WT

To assess whether the decreased drought tolerance of the *ARK2* plants (or the increased tolerance of *ARK1* mutants) may be correlated with an altered response to water scarcity, water loss was monitored as being an indication of short-term avoidance of water deprivation. Indeed, the measurements of fresh weight loss of leaf tissues resulted in significantly higher water loss rates for *ARK2* mutants (Figure 35). T-DNA line *ark2.3* in particular lost on average 76% of its water content over the 24 hours after the detachment, against a 68% water loss of wild-type leaves. Eight hours after the first measurement a difference in weight loss was already visible between *ARK2* and Col-0. This altered leaf water loss could indicate an imbalance in the mechanisms regulating water uptake and water loss through the leaf surface. As a reduction in stomatal pore aperture size mediated by ABA is usually observed as short-term response (Verslues, Agarwal, Katiyar-Agarwal, Zhu, & Zhu, 2006), it is hypothesised an altered stomatal conductance in *ARK2* mutants. Although ABA did not seem to affect germination of ARK mutants, a major role of this hormone is considered for mediating water stress responses.

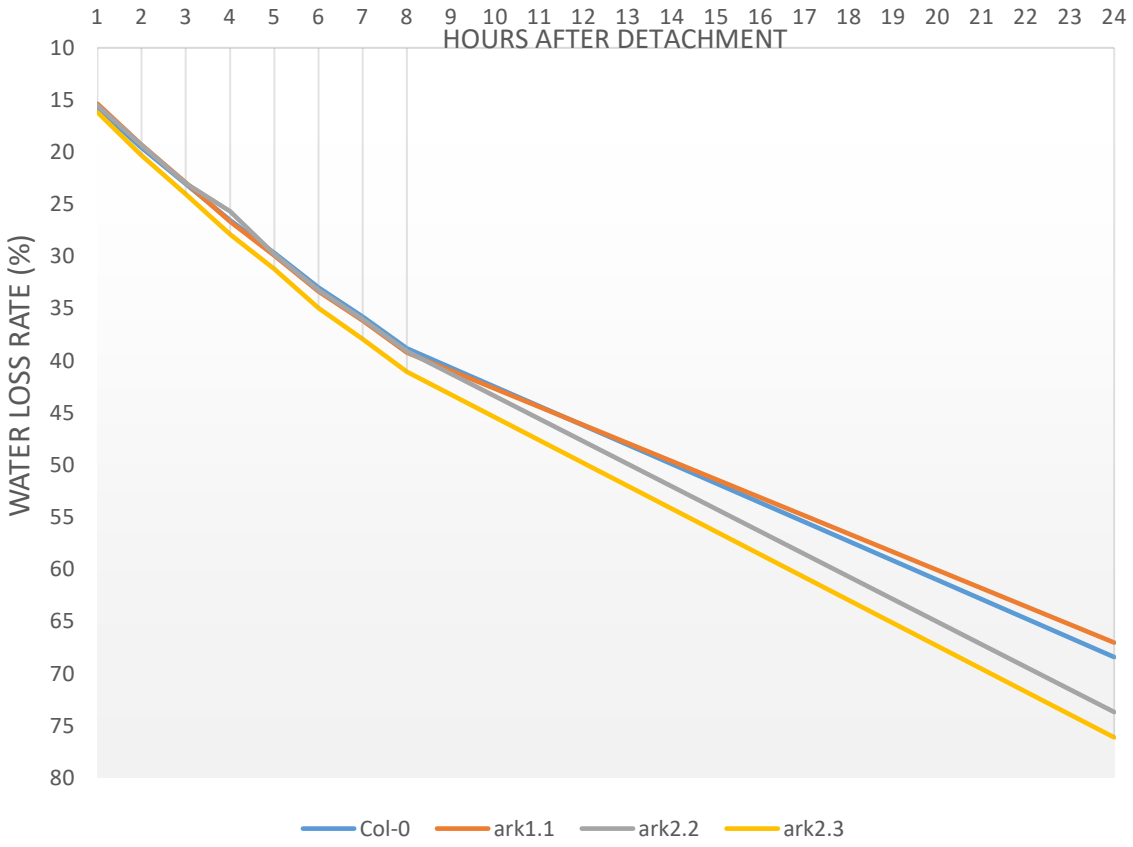

FIGURE 35. DECREASE IN LEAVES FRESH WEIGHT EXPRESSED AS WATER LOSS RATE (%) COLUMBIA AND ARK MUTANT LINES. THE WEIGHT WAS MONITORED FOR 8 HOURS AND 24 HOURS AFTER THE DETACHMENT

Confirmation of reduced ARK gene expression in *ark* T-DNA insertion lines

The quantification of the number of ARK gene transcripts in the mutant T-DNA lines aimed at the confirmation of a reduced gene expression. cDNA from leaves and individually darkened leaves of the same age (see paragraph Dark-induced senescence from materials and methods) were used for the analysis.

ARK1 specific primers were designed to amplify the region of the S-domain, in which also the T-DNA insertion is located. In *ark1.1* leaves the quantified relative expression level was close to 0 (Figure 36), thus confirming the complete knock-out of the gene in this mutant line. To be remarked that in only three out of six replicates the transcript was detected in very low levels, and in the remaining three the gene was not amplified. The same results were observed in *ark1.1* senescing leaves, of which only one replicate was amplified, showing a small number of transcripts. These results were confirmed by an electrophoretic run of the PCR products in a 1% agarose gel, that did not show any amplified fragment. The absence of amplification in most of the replicates can be considered to be due to the knock-out of the gene and not to the quality of the cDNA or of the primers. Indeed, the primers were previously tested on DNA samples. Also, a normal expression of the *ARK2* gene was observed in these *ark1.1* samples (figure 37).

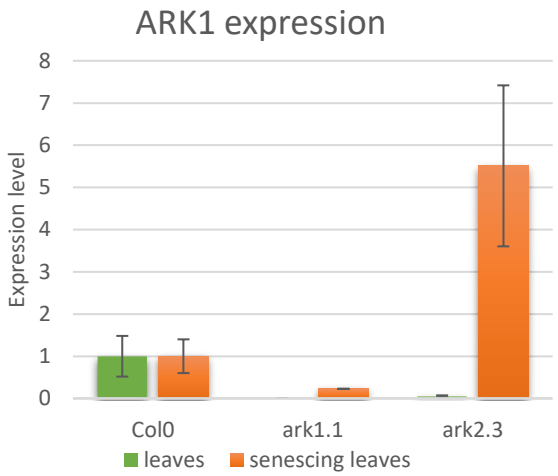

FIGURE 36. NORMALISED RELATIVE EXPRESSION ±SE OF *ARK1* GENE USING *ARK1* SPECIFIC PRIMERS IN BOTH MUTANT T-DNA LINES, CONTROL LEAVES (GREEN BARS) AND SENESCING LEAVES (ORANGE BARS), BASED ON 3 BIOLOGICAL REPLICATES X 2 TECHNICAL REPLICATES PER LINE.

The quantification of the gene expression for *ARK2* mutants was carried out only on line *ark2*.3 since the results of the phenotyping were comparable for *ark2*.2 and *ark2.3*. This might be due to the fact that the T-DNA insertion is located in the same domain. The RT-qPCR analysis showed a very low number of transcripts for *ARK2* in line *ark2.3* compared to the wild-type (Figure 37). Hence, the *ARK2* gene can be considered to be a knocked-out in this mutant line. To be noticed that in this case the primers are specific for the kinase domain, which should exclude the possibility of amplifying alternative transcripts. *ARK2* specific primers designed in the region of the S-domain were also used to analyse the expression of eventual alternative transcripts containing the S-domain only. In fact, a relative expression level of 0.5 was observed (Supplementary figure 7), thus suggesting the presence alternative transcripts coding for the S-glycoprotein domain.

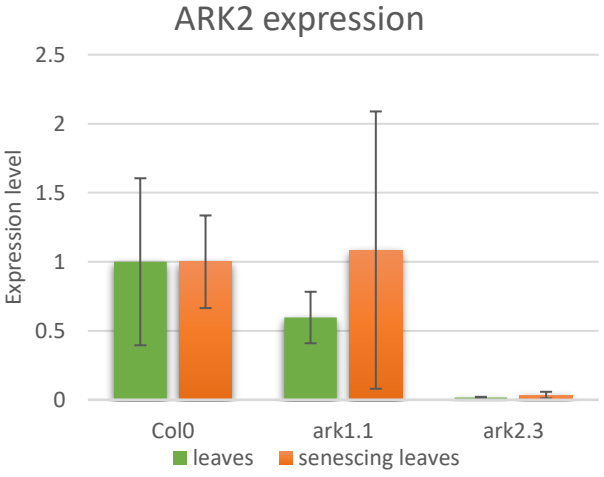

FIGURE 37. NORMALISED RELATIVE EXPRESSION ±SE OF *ARK2* GENE USING *ARK2* SPECIFIC PRIMERS IN BOTH MUTANT T-DNA LINES, CONTROL LEAVES (GREEN BARS) AND SENESCING LEAVES (ORANGE BARS), BASED ON 3 BIOLOGICAL REPLICATES X 2 TECHNICAL REPLICATES PER LINE.

Due to high nucleotide sequence similarity (86% identity), the amplification of *ARK2* transcripts

using *ARK1* specific primers and viceversa is not excluded. In fact, transcripts were detected in *ark1.1* mutant line samples using *ARK2* specific set of primers (Figure 37). In addition, while *ARK1* transcripts were not observed in *ark2.3* control leaves, an up-regulation may be induced in senescing leaves (figure x, left). *ARK2* gene (80% identity) is also considered as a possible off-target of the amplification, althought it was not used in this study.

The gene coding for the ubiquitin ligase Plant U-Box 9 (PUB9) was quantified in *ARK1* and *ARK2* mutant lines, as being found to interact with S-Domain Receptor Kinases in a conserved signalling pathway (Samuel, et al., 2008). No specific expression patterns were observed for this gene, a part from a probable decrease in transcripts number in *ARK2* mutant senescing leaves (Figure 38). The big variability observed in PUB9 expression levels among the samples does not allow an assessment of significant differences.

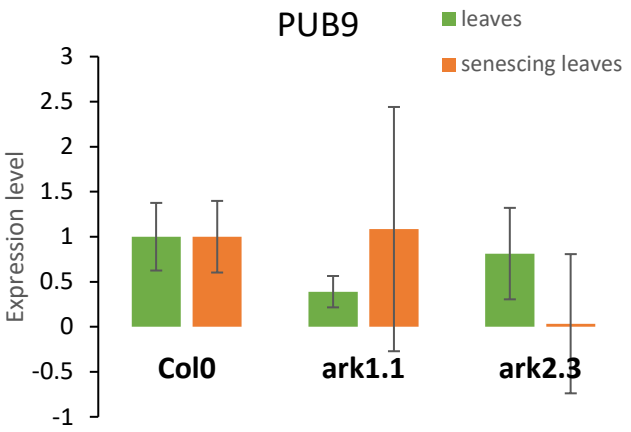

FIGURE 38. NORMALISED RELATIVE EXPRESSION ±SE OF *PUB9* GENE IN BOTH MUTANT T-DNA LINES, CONTROL LEAVES (GREEN BARS) AND SENESCING LEAVES (ORANGE BARS), BASED ON 3 BIOLOGICAL REPLICATES X 2 TECHNICAL REPLICATES PER LINE.

However, the interaction between PUB9 and ARK protein kinase is expected to be on a protein more than a transcriptional level, as shown by yeast two-hybrid interaction screens (Samuel, et al., 2008).

Discussion and conclusion

AWPM19-like

Abscisic acid-induced Wheat Plasma Membrane 19-like is part of a highly conserved protein family among land plants, which is an indication of its importance in plant physiology. It has deeply been characterized as playing crucial roles in germination and ABA-related abiotic stresses in winter wheat (Koike, Takezawa, Arakawa, & Yoshida, 1997), barley (Randoford, Bryce, & Morris, 2002), rice (Chen, et al., 2015) (Yao, et al., 2018) and Arabidopsis (Alsaif, 2013). In this study an assessment of the effects of salt, osmotic and ABA treatments on *AWPM19-like* knock-out/knock-down and overexpression lines aimed at the confirmation of previous predictive functions of this gene.

Phenotypic responses to abiotic stress that differed between transgenic and wild-type plants were used to correlate a biological role with the genotype. To allow a comprehensive overview of the phenotypic results obtained in this study, it might be relevant to first describe the genotypes of the lines used in relation to the *AWPM19-like* gene. In the three T-DNA insertion lines the gene was confirmed to be overall down-regulated, specifically mutants *wp1* and *wp2* appeared to be knock-outs, while *wp4* mutant only showed a reduction in expression levels of 0.6-fold compared to the wild-type. Concerning the five overexpression lines containing the gene under a CaMV 35S promoter, the *AWPM19-like* gene expression was comparable to the wild-type for line O1 and O3, with a little up-regulation (only 0.2-fold) in lines O2, O4 and O5. Also, the limited number of biological replicates used in the genotypic analysis by RT-qPCR did not allow to assess differences in gene expression with statistical significance. Despite the small divergences in gene expression among all the overexpression lines with the Col-0, the phenotypical analyses results are relevant in relation to abiotic stress tolerance.

Since the *AWPM19-like* gene is predominantly expressed during seed maturation and germination, and during seedlings development, we focused at these stages in this in this research project. All the significant results are summarised in Table 7. Seed germination rates and percentages under salinity stress were monitored to test the capacity of the seeds to tolerate mild and high concentrations of NaCl during germination. T-DNA insertion mutants *wp1* and *wp2* showed significantly slower germination rates and lower germination percentages, indicating a hypersensitivity to moderate salt stress. On the contrary, seeds from the overexpression lines O1 and O4 responded better to salt treatments, as showing faster germination rates than Col-0. These results collectively suggest that *AWPM19-like* plays important roles in ion stress during germination, perhaps regulating water and

TABLE 7. SUMMARY OF THE SIGNIFICANT PHENOTYPICAL RESULTS OF GERMINATION ASSAYS AND SEEDLING GROWTH EXPERIMENTS FOR AWPM T-DNA INSERTION MUTANTS AND OVERXPRESSION LINES. DEPENDING ON THE EXPERIMENT, THE ARROWS INDICATE STATISTICALLY DIFFERENCES IN PERFORMANCE OF THE TRANSFORMANTS COMPARED TO WILD-TYPE.

			T-DNA lines			overexpression lines				
			wp1	***wp2***	***wp4***	**O1**	**O2**	**O3**	**O4**	**O5**
	expression level		×	×	↓	wt	↑	wt	↑	↑
germination	NaCl	75 mM	rates ↓	rates ↓						
		100 mM	rates and % ↓	rates and % ↓		rates ↑			rates ↑	
	ABA	0.3 µm						% ↑		
	heat		rates ↓							
root growth	NaCl	75 mM				↑				↑
		100 mM						↑ but also in control		↑
	mannitol	200 mM						↑ but also in control		
		300 mM								
	ABA	1 µm			↓				↓	
		5 µm			↓		↓		↓	↓

nutrient uptake. It is hypothesised a role as ion transporter that regulates Na^+ efflux through the plasma membrane (Alsaif, 2013) or in the vacuoles during seed maturation. The root growth of seedlings from the mutant lines did not differ significantly from those of the wild-type under NaCl treatment. However, the overexpression line O5 showed higher root length in mild and high salt concentrations compared to the wild-type. A possible explanation is that the sensitivity to salinity stress changes in different developmental stage. Also, the *AWPM19-like* gene is lowly expressed only in roots of 10-days old seedlings after salt treatments (Arabidopsis eFP browser, Winter, et al., 2007).

Abscisic acid is a plant hormone known to promote seed germination and response to water, salt, hypoxic, and cold stress (Finkelstein, Gampala, & Rock, 2002), hence the addition of exogenous ABA is used to mimic abiotic stresses. The mechanisms of ABA signalling pathways have been partly elucidated in Arabidopsis, with several ABA-transporters, intracellular receptors and targets identified. A study from Chen at al. (2015) showed that the rice *AWPM19-like* homolog is induced by ABA, salt and cold stresses, leading to the hypothesis of a role in stress tolerance through an ABA-dependent pathway. Yao et al. (2018) have recently suggested a putative role as ABA influx carrier, from the evidence that the gene expression is regulated in a ABA-dependent way. The e-Northerns w. Arabidopsis Expression Browser at BAR uturonto (http://utoronto.ca/) indicate a significant increased expression in Arabidopsis seedlings after 3 hours treatment with 10 µM ABA, which may reflect similar responses as the one of the rice ortholog in ABA-dependent mechanisms. In this study germination under 0.3 µM ABA resulted in higher germination percentages in overexpression lines, particularly line O3, in accordance with the results from Alsaif, 2013. However, roots from lines O2, O4 and O5 were significantly affected by ABA treatments in growing media as showing reduced growth compared to Col-0 seedlings. A higher sensitivity was expected in seedlings of the mutant T-DNA lines, among which only *wp4* showed significantly shorter roots than Col-0. These observations suggest that AWPM might be involved with seed germination and seedling growth under ABA treatment. To further investigate if *AWPM*19-*like* works upstream of the ABA signalling pathway, it is recommended to examine the expression levels of the AWPM gene after ABA treatments. The expression of other genes known to be related to ABA response can be quantified in these lines to assess whether *AWPM19-like* regulates important downstream targets in the ABA signalling pathway. The yeast cells transformation with vectors carrying rice *AWPM19-like* used by Yao et al. (2018) in rice can be replicated in Arabidopsis in order to prove AWPM protein role as ABA transporter in the plasma membrane.

We assessed the effects of heat stress on seed germination since high temperature increases ABA levels and affects embryo development and seed viability (Jemaa, Saida, & Sadok, 2010). The heat shock treatment caused a reduction in seed germination rates of the mutants, particularly in seeds of *wp1* which needed on average 5 hours more to reach 50% of germination compared to wild-type. The observed general sensitivity of the T-DNA mutants to abiotic stresses reflects the importance of the *AWPM19-like* gene in the cell adaptative responses to water-related stresses. *AWPM19-like* might contribute to maintain cell homeostasis to avoid stress by regulating ion influx/efflux, accumulating compatible osmolytes or regulating LEAs or of Heat Shock Proteins (HSPs). Although the expression of this gene seems to be restricted to mature seeds and seedlings under stress treatments, it might be interesting to examine the AWPM overexpression and mutant lines under water deficit stress such as drought and desiccation. Further analyses of water loss rates and stomatal conductance measurements in leaves of *AWPM19-like* overexpression or mutant lines could be used for further functional investigation of short-term avoidance of water deprivation.

Late Embryogenesis Abundant proteins (LEA) have been extensively characterized as playing fundamental protection roles during different developmental stages and in response to water stress (Tunnacliffe & Wise, 2007). Several genes coding for LEA proteins have been found in the co-

expression network of the Arabidopsis *AWPM19-like* gene, leading to the hypothesis of a possible relationship between these genes on a transcriptional level. LEA genes 18, 30 and 46 were chosen as candidate for this analysis since they show similar expression patterns to *AWPM19-like* during embryogenesis. The LEA30 gene seems to play a role in acquisition of seed desiccation tolerance and longevity during embryogenesis (Mariana S. Artur, persona communication), while LEA18 and LEA 46 transcripts have been shown to accumulate after hyperosmotic, severe drought, and ABA treatments during germination and seedling stage (Olvera-Carrillo, Campos, Reyes, Garciarrubio, & Covarrubias, 2010). Transcripts of the LEA18 gene were downregulated in the loss-of-function mutants *wp1* and *wp2*, however, the expression of this gene did not differ in the *wp4* line nor in the overexpression lines when compared to the wild-type. Often, the lack of differences in gene expression in knockout and knockdown mutants are due to compensation between genes within the same co-expression network, which can perform similar functions (El-Brolosy & Stainier, 2017). On the other hand, LEA30 and LEA46 genes seem to follow the expression of *AWPM19-like*: their transcript accumulation decreases in all *awpm19* T-DNA insertion lines, while an increase is observed in almost all the overexpression lines, up to 6-fold increase in LEA30. This observation leads us to hypothesize that there may be co-expression between the *AWPM19-like* and LEA genes from group 4. If similar patterns of expression are confirmed by further experiments, it could be postulated the involvement of AWPM19-like in regulating LEA genes' expression.

TABLE 8. SUMMARY OF LEA GENES EXPRESSION PATTERN IN AWPM KNOCK-OUT MUTANTS, KNOCK-DOWN MUTANT AND OVEREXPRESSION LINES

If **AWPM** is...	Knocked-out ×	Knocked-down ↓	Overexpressed ↑
...**LEA18**	↓	=	
...**LEA30** and **LEA46**	↓	↓	↑

The phenotyping results obtained in this study suggest that the *AWPM-19 like* gene contributes to maintain the cell homeostasis in Arabidopsis, since different T-DNA insertion mutants were generally more sensitive to induced salt, ABA and heat stress during germination. As a further confirmation, a replication of the experiments using the overexpression lines of AWPM would be needed.

The high similarity between the AWPM gene in Arabidopsis with orthologues in *Xerophyta viscosa* (79%) implies its evolutionary conservation and suggests its importance in desiccation tolerance. The analysis of genes differentially expressed in *X. viscosa* under dehydration revealed a co-expression between AWPM19 and LEA genes, suggesting that AWPM19 orthologue in Xerophyta could also be an activator of desiccation tolerance responses in leaves. In particular, transcripts of LEA_1 and LEA_4 members, whose expression was found to be affected in the *AWPM*19-*like* mutants of Arabidopsis in this study, accumulate during drying and rehydration in Xerophyta (Costa, et al., 2017). The mechanism of activation and functioning of this gene is still unclear, but it is likely that in both species, in response to water-related stress, *AWPM19-like* facilitates ABA influx through the plasma membrane and indirectly induces transcriptional activation of genes associated with protective roles and induction of DT such as LEA proteins, heat shock proteins, or ABA-related transcription factor and ABA biosynthetic genes, in a positive feedback mechanism. In conclusion, it is postulated a role as an indirect positive regulator of desiccation tolerance, acting at or upstream the ABA signalling pathway, in Arabidopsis seeds and Xerophyta vegetative tissue under dehydration.

Recommendations for future studies

It must be remarked that Arabidopsis *AWPM19-like* belongs to a gene family composed by three different members that encode proteins with sequence similarities (at least 40% identity with the

AWPM protein). Hence, it is plausible a compensation mechanism for the knocked-out genes in the insertion mutants. For this reason, it is also suggested a functional characterization of lines in which all the three genes are silenced.

Although this study was limited to some LEA genes, it would be interesting to analyse the expression of other related genes in down-stream pathways or interacting proteins from translational regulatory network predictions. For instance, another group 4 LEA protein (At1g32560) was found in the same functional module from the network constructed by polysome occupancy (Bing Bai, personal communication) as being co-expressed or regulated by *AWPM19-like* during germination.

ARKs

Plasma membrane receptor-like kinases (RLK) belong to a large gene family with at least 610 members that represent nearly 2.5% of *Arabidopsis* protein coding genes (Shiu & Bleecker, 2001). They have been shown to play important roles in sensing environmental stimuli by binding specific ligands through a extracellular domain and activating signalling pathways via phosphorylation of cytosolic serine/threonine kinase domains (Osakabe, Yamaguchi-Shinozaki, Shinozaki, & Tran, 2013). In Arabidopsis, several RLKs have been characterized as regulators of abiotic stress signalling, as drought stress and ABA sensitivity in root growth and stomatal closure (Osakabe, et al., 2010) since their gene expression is induced by ABA, dehydration, high salt, and low temperatures. Other kinase genes have been found to control ABA and osmotic stress signal transduction by interacting with other membrane RLKs and regulating ABA-responsive genes like LEA genes, oleosin, ABI4 and ABI5 (Tanaka, et al., 2012).

The RLKs are classified based on their putative extracellular ligand-binding domains, whose specificity may give an insight of their role in signalling. The Arabidopsis Receptor Kinase (ARK) genes characterized in this project contain a stress-related carbohydrate-binding lectin domain which has been found to be associated with a S-locus glycoprotein, Pan/Apple, and protein kinase domain (Eggermont, Verstraeten, & Van Damme, 2017) (figure 39). This chimeric domain structure is typical of the conserved S-locus receptor kinase (SRK) of *Brassica oleracea* and to the receptor kinase ZmPK1 gene of maize (Tobias, Howlett, & Nasrallah, 1992). In Arabidopsis, three genes have been found to share sequence similarity with the S-receptor kinase genes, namely *ARK1* (At1g65790.1), *ARK2* (At1g65800.1) and *ARK3* (At4g21380.1). This redundancy is common to most of the Arabidopsis RLKs, whose gene family expansion is mainly due to intrachromosomal duplications of the tandem regions (Shiu & Bleecker, 2001). ARK genes structure and expression have been characterized by Tobias, et al. (1992 and 1996) and Dwyer, et al. (1994). All the *ARK* genes produce two alternative transcripts and thus alternative protein products: a plasma membrane-associate protein consisting of an S-domain linked to the kinase domain by a transmembrane domain, but also a truncated protein consisting entirely of the *S*-domain and secreted into the extracellular matrix. Remarkably, the alignment of the three sequences shows that kinase domains are consistently more conserved than the *S* domains. In fact, *ARK1* and *ARK2* kinase domains share 93% amino acid sequence identity, while a 79% identity is observed in the S-domain. This suggests that they may be activated by different ligands, but they phosphorylate the same or related cytoplasmic substrates (Dwyer, et al., 1994). The ARK genes expression is predominantly in vegetative tissues, specifically during maturation of cotyledons, leaves, and sepals (Dwyer, et al., 1994), but not in reproductive organs (Tobias & Nasrallah, 1996). However, *ARK3* promoter is also active in roots and in leaves at much lower levels than the *ARK2* promoter (Dwyer, et al., 1994). These results, together with the growth abnormalities observed in *ARK1* transformant plants by Tobias & Nasrallah (1996), suggest a role for the ARK genes in signalling events during plant development.

FIGURE 39 - ARABIDOPSIS ARK GENES AND PROTEIN DOMAIN ARCHITECTURE. THE FIRST EXON CONSISTS OF THE ENTIRE S-RELATED REGION CONTAINING D-MANNOSE BINDING LECTIN (YELLOW), S-LOCUS GLYCOPROTEIN DOMAIN (GREEN), PAN-LIKE DOMAIN (LIGHT BLUE), FOLLOWED BY AN IN-FRAME TAG STOP CODON IN ALTERNATIVE TRANSCRIPTS (TOBIAS, ET AL., 1992). THE SECOND EXON ENCODES A MEMBRANE-SPANNING REGION (BLACK). EXONS 3 TO 7 ENCODE A PUTATIVE RECEPTOR SERINE/THREONINE KINASE CATALYTIC DOMAIN (ORANGE), AND A SMALL DOMAIN OF UNKNOWN FUNCTION (DARK BLUE).

In this preliminary study, T-DNA insertion lines for *ARK1* and *ARK2* genes were characterized for their responses to abiotic stresses during different developmental stages in order to correlate a phenotypical response of the mutants with a putative role of these genes. It might be relevant to point

out that in the *ARK1* mutant line selected, the T-DNA insertion is located in the S-domain, while in *ARK2* mutants it is located in the kinase catalytic domain of the receptor. Hence, the possibility that alternative transcripts coding for the secreted S-glycoprotein are produced is not excluded. RT-qPCR analysis for gene expression revealed the complete knock-out of *ARK1* S-domain in *ark1.1* leaves (both mature and senescing). *ARK2* kinase domain was also found confirmed to be knocked-out in *ark2.3* leaves. Due to high nucleotide sequence similarity (86% identity), the amplification of *ARK2* transcripts using *ARK1* specific primers and vice versa is not excluded. In fact, *ARK2* transcripts were detected in *ark1.1* mutant line samples. While *ARK1* transcripts were not observed in *ark2.3* control leaves, an up-regulation may be induced in senescing leaves. *ARK3* gene (80% identity) is also considered as a possible off-target of the amplification, although it was not used in this study.

All the significant phenotypical results and observations are summarised in Table 9. The mutation in *ARK1* and *ARK2* lines did not affect primary root growth in 10-days old seedling subjected to stress treatments differently from Col-0, probably because the promoter is only active in cotyledons of older seedlings (Dwyer, et al., 1994). However, when tested for their capacity to germinate under high NaCl and ABA concentration, seeds from *ARK2* mutant lines showed higher germination rates and higher germination percentages than wild-type, respectively. The hyposensitivity observed may indicate that *ARK2* receptor kinase acts as a positive regulator in the response to salt ad ABA stress during germination. The mechanism of action might be related to *S* receptor kinase interactions with E3 ubiquitin ligase from the plant U-box-ARM family (PUB). Samuel et al. (2008) suggested that the phosphorylation of PUB proteins by the active kinase domain of ARKs may act as signal to sort these proteins to the appropriate subcellular compartment for substrate interactions. While *ARK2* presence induces a cytosolic distribution of PUB9, both *ARK1* and ABA induce relocalization of PUB9 from the nucleus to the plasma membrane, where ABA interacts with various membrane transporters and ion channels (Finkelstein et al., 2002). It is proposed that in response to intracellular or extracellular ABA during germination, S receptor kinases phosphorylates PUB9 that targets ABA-responsive genes or transcription factors for ubiquitin-mediated degradation. The hypersensitivity to 1 µM ABA observed by Samuel, et al. (2008) in *ARK1* mutants during germination may suggest that these different S receptor kinases play non-redundant or even opposite roles in ABA response in this stage.

Apart from seed maturation and germination, ABA is known to regulate vegetative growth, including cell elongation and/or stomatal regulation (Finkelstein, Gampala, & Rock, 2002) in response to environmental stresses. To assess whether *ARK* mutants show altered sensitivity to water stress, leaf water loss was monitored as being an indication of ABA-dependent short-term avoidance of dehydration. The measurements of fresh weight loss of leaf tissues resulted in significantly higher water loss rates for *ARK2* mutants. T-DNA line ark*2.3* in particular lost on average 76% of its water content over the 24 hours after the detachment, against a 68% water loss of wild-type leaves. This

TABLE 9. SUMMARY OF THE SIGNIFICANT PHENOTYPICAL RESULTS OF GERMINATION ASSAYS AND SEEDLING GROWTH EXPERIMENTS FOR ARK T-DNA INSERTION MUTANTS. DEPENDING ON THE EXPERIMENT, THE ARROWS INDICATE STATISTICAL DIFFERENCES IN PERFORMANCE OR APPEARANCE OF THE MUTANTS COMPARED TO WILD-TYPE

			T-DNA lines			
			ark1.1	***ark2.2***	***ark2.3***	
germination	NaCl	100 mM		rates ↑	rates ↑	
	ABA	0.3 µm		% ↑	% ↑	
growth	rosette leaves		smaller and chlorotic	slightly bigger		compared
abnormalities	flowering		also in senescence stage			to wt
chlorophyll	Total Chl		↑ in senescent leaves	↓ in leaves		
water loss				rates ↑	rates ↑	

increased leaf water loss could indicate an imbalance in the mechanisms regulating water uptake and water loss through the leaf surface. As a reduction in stomatal pore aperture size mediated by ABA is usually observed as short-term response (Verslues, Agarwal, Katiyar-Agarwal, Zhu, & Zhu, 2006), it is hypothesised an altered stomatal conductance in *ARK2* mutants due to ABA insensitivity or altered stomatal morphology. Abnormal and variable stomatal morphology was observed in *ARK1* transformants by Tobias & Nasrallah (1996), which could explain the slight reduction in water loss rates of *ARK1* T-DNA line used in this study.

The effects of water and nutrient deprivation were also monitored on adult plants growing on Rockwool by inducing drought stress for twelve days. Based on the survival percentages observed, *ARK1* mutant plants showed improved resistance to water deficits compared to wild-type plants and both *ARK2* mutant lines, which, in contrast, were severely affected by the drought stress. This result is in accordance with the increased water loss rates of *ARK2* mutant leaves and leads to the hypothesis that ARK genes might play important role in regulating drought stress responses in vegetative tissues.

To confirm the role of ARK genes in plant development suggested by the above-mentioned functional studies in Arabidopsis, different stages of plant growth were monitored. Rosette growth appeared to be slower in mutant plants *ARK1*, as 30 days-old plants showed smaller rosette leaves and visibly smaller diameter compared to wild-type and *ARK2* mutant plants. *ARK1* rosettes from the next generation of plants also tended to become chlorotic. The same growth abnormalities were observed in plants with constitutive ectopic over-expression of *ARK1* (Tobias & Nasrallah, 1996). On the contrary, rosettes from *ARK2* mutants appeared to have enhanced growth as they were bigger and started bolting earlier. These observations support the hypothesis of Dwyer et al. (1994) that these genes may play a role in processes related to organ maturation and/or the establishment of growth pattern transitions. It is not clear whether the extracellular domain or the kinase domain is responsible for these phenotypes. However, the differences in tissue expression and in the S-domain among the ARK genes, together with the growth abnormalities observed, we suggest that they might have nonredundant functions in specific aspects of development or growth of the plant body (Dwyer, et al., 1994)**.**

Relevant abnormalities were also observed during late senescence stage, when flowers were unexpectedly produced on 3-months-old plants from *ARK1.1* mutant line. In fact, after stage 6.9 (around 50 days after sowing), flower production should be complete (Boyes, et al., 2001). It has been shown that common pathways can regulate flowering and leaf senescence, as several mutants in protein kinases and transcription factors with senescence and flowering phenotypes have been identified (Wingler, 2011). Likewise, it is proposed that *ARK1* controls the expression of genes responsible for floral initiation and senescence induction. Since it is hypothesised a role in regulating dehydration response, the mechanisms of action could involve the perception of the stress through the extracellular domain and the activation of senescence-related transcription factors or promoters via phosphorylation by the kinase domain.

Senescence in *ARK1* and *ARK2* mutant lines was first assessed by observation of leaf yellowing. Since it was not possible to assess consistent visual differences in dark-induced leaf senescence attributable to the loss of *ARK1* and *ARK2* in independent T-DNA insertion lines, further analysis on chlorophyll and carotenoids content were carried out. Although individually darkened leaves for 5 days did not always visibly appear senescent, their pigments content was reduced than in control leaves of the same age from the same plant. Notably, line *ARK1.1* showed a higher amount of chlorophyll a in senescing leaves when compared to Col-0 senescing leaves. On the contrary, control leaves from *ARK2.2* line had a decreased total chlorophyll content than wild-type, especially due to a decline in chlorophyll b. Again,

the line lacking the *ARK1* S-domain performed differently from the lines without the *ARK2* kinase domain, as further confirmation that the putative receptor protein kinases encoded by these genes have distinct functions or that the mutation in different domains results in different phenotypical responses.

From Arabidopsis eFP Browser (Winter, et al., 2007), increased expression level is predicted in the distal part of mature leaves and in senescing leaves. This expression pattern overlaps with the putative orthologue in *Xerophyta viscosa*, that has been found to be up-regulated in the tip of senescent leaves during dehydration (Radermacher, A. & Farrant, J. – University of Cape Town, unpublished data). Despite the divergences in transcript sequences (75% identity in the S-domain and 69% in the kinase domain) and in senescing mechanisms between *Arabidopsis thaliana* and *Xerophyta viscosa*, it is hypothesized a role of these genes as local regulators of senescence and growth. The desiccation tolerance strategy employed by Xerophyta includes the reduction of transcripts associated with the promotion of senescence, photosynthesis, energy metabolism and ABA biosynthesis (Costa, et al., 2017) in adult leaves during drying. However, the distal part of older leaves shows irreversible drought-induced senescence as in desiccation sensitive species, maybe to decrease energy consumption and redirect nutrients to younger leaves. As being up-regulated in the senescing part of the leaves and silenced in the proximal DT resistant part, *ARK* genes might be involved in growth and development also in Xerophyta, hence they have to be suppressed in order to acquire DT. It is hypothesised that, when translated, ARK receptors perceive the local osmotic stress through the extracellular domain and transduce the stress signal into downstream pathways by the kinase domain. External signals could be prompted either by dehydration or ABA, and the activation through phosphorylation of specific transcription factors, promoters or ubiquitin ligases could result in the induction of senescence, which includes growth inhibition, protein degradation and apoptosis. For instance, ABI5 and ABI3 transcription factors could be indirectly deactivated by ARK kinases, which could in turn down-regulate LEA genes expression and protective mechanisms typical of resurrection plants DT. It is then proposed a general negative regulatory role in desiccation tolerance, but a positive role for growth and development, yet considering the possible differences in function and specificity between the homologs and the domains of these receptor-like kinases.

Recommendations for future studies

From the phenotypical results obtained in this study it cannot be concluded whether *ARK* genes act as a positive or negative regulator of dark-induced senescence, but the comparison in expression levels with senescence marker genes (Balazadeh, Riaño-Pachón, & Mueller-Roeber, 2008) would give an insight on the positive or negative effects on senescence.

For future studies it might be interesting to functionally characterize T-DNA double or triple mutant lines in which two or three ARK genes with high sequence homology are silenced in the S-domain, to avoid possible compensation mechanisms and production of alternative transcripts. In this way it could be possible to attribute to this ARK family genes a common role in development, abiotic stresses, flowering and/or senescence. The development of transgenic Arabidopsis plants overexpressing ARK1/2/3 is also suggested to confirm whether ARKs could act as positive or negative regulators in those physiological traits and responses.

Morphological abnormalities in stomata resulting in increased water loss could be confirmed by electron microscopy analyses of guard cells and measures of stomatal conductance.

Specific ligands of the extracellular or secreted S-domain and specific phosphorylation targets of the kinase domain could be identified to better understand the molecular pathways of signal recognition and transduction in which ARK receptors are involved.

For a complete functional characterization of *ARK* genes in relation to senescence and desiccation tolerance, it would be useful to clone the putative *X. viscosa* ARK homolog in *A. thaliana* T-DNA insertion lines. Despite the peculiarity of the physiological strategies employed by resurrection plants under dehydration (Costa, et al., 2017), this would allow to make a comparison between the ARK orthologues in the two species and confirm their putative roles in senesce and desiccation tolerance.

To be remarked that in Xerophyta transcriptome other sequences similar to the ones of ARK1, ARK2 and ARK3 were found (Supplementary Table 1). These sequences are similar to genes coding for different receptor-like serine/threonine-protein kinase in Arabidopsis, revealing that also in Xerophyta RLKs gene family is highly conserved, suggesting a general role in plant cell signal transduction. The transcriptome analysis of Xerophyta viscosa during dehydration and rehydration could be used to assess whether these receptor kinases can also play a role in desiccation tolerance.

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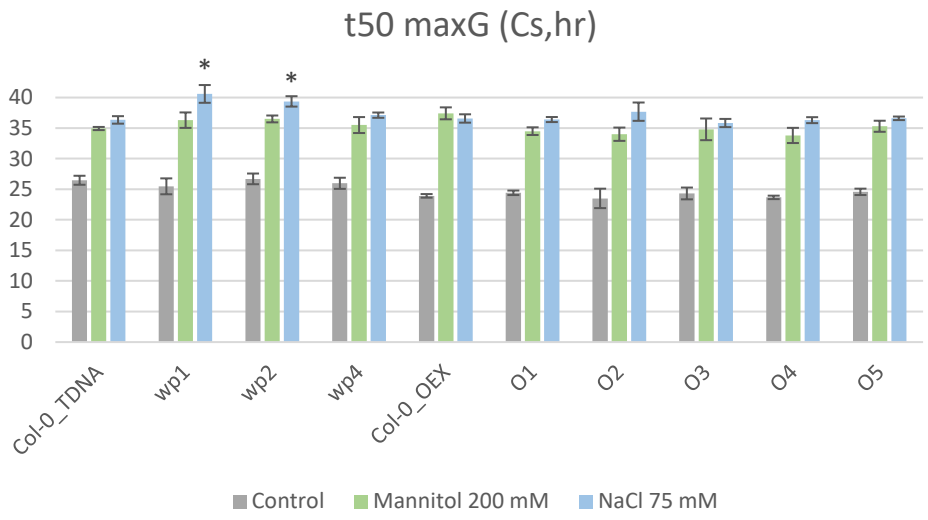
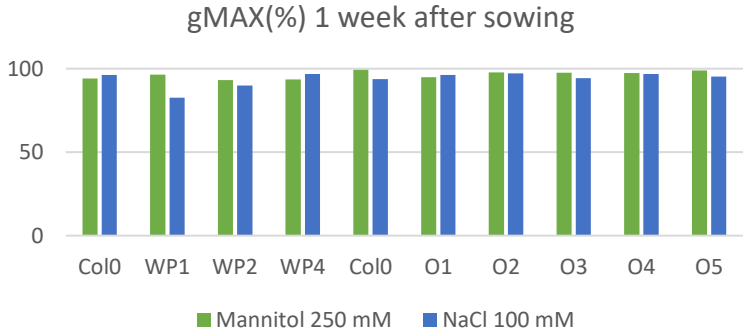
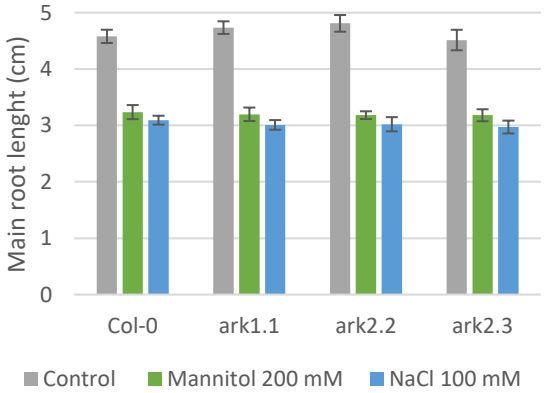
Supplementary data



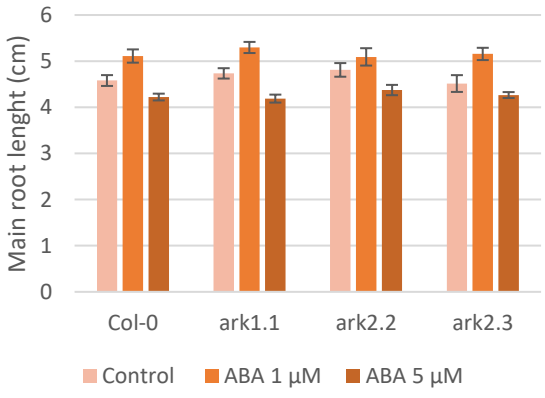
SUPPLEMENTARY FIGURE 6. TIME TO REACH 50% GERMINATION (T50) ±SE FOR T-DNA LINES AND OVEREXPRESSION LINES IN 200 MM MANNITOL, 75 MM SODIUM CHLORIDE AND CONTROL TREATMENT



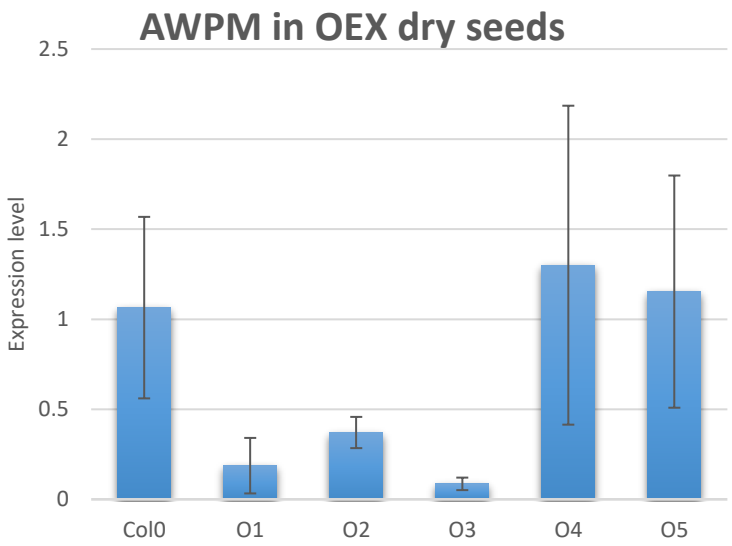
SUPPLEMENTARY FIGURE 2. MAXIMUM PERCENTAGE OF GERMINATION (GMAX) ±SE IN AWPM T-DNA MUTANT LINES AND OVEREXPRESSION LINES IN 250 MM MANNITOL AND NACL 100 MM



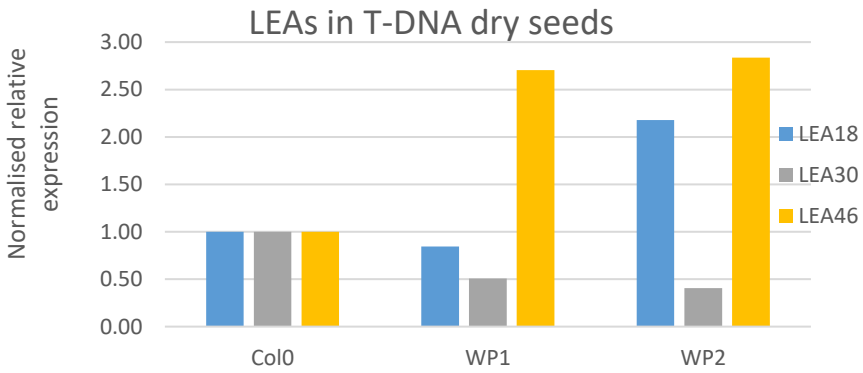
SUPPLEMENTARY FIGURE 3 AVERAGE MAIN ROOT LENGTH ±SE FOR ARK T-DNA LINES IN MANNITOL AND SODIUM CHLORIDE



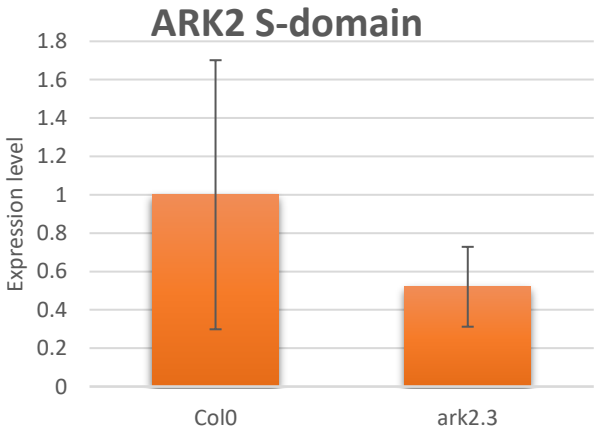
SUPPLEMENTARY FIGURE 4. AVERAGE MAIN ROOT LENGTH ±SE FOR ARK T-DNA LINES IN ABA



SUPPLEMENTARY FIGURE 5. NORMALISED RELATIVE GENE EXPRESSION ±SE OF AWPM-19 GENE IN OVEREXPRESSION LINES DRY SEEDS (TWO TECHNICAL REPLICATES X 3 BIOLOGICAL REPLICATES PER LINE WERE USED)



SUPPLEMENTARY FIGURE 6. NORMALISED RELATIVE EXPRESSION OF FOUR DIFFERENT LEA GENES IN AWPM T-DNA LINES DRY SEEDS.



SUPPLEMENTARY FIGURE 7. NORMALISED RELATIVE EXPRESSION ±SE OF *ARK2* GENE USING *ARK2* SPECIFIC PRIMERS FOR THE S-DOMAIN IN *ARK2* T-DNA LINE, CONTROL LEAVES, BASED ON THREE BIOLOGICAL REPLICATES AND 2 TECHNICAL REPLICATES PER LINE.

SUPPLEMENTARY TABLE 10. SEQUENCES PRODUCING SIGNIFICANT ALIGNMENTS TO ARK1 IN X. VISCOSA TRANSCRIPTOME FROM WWW.BIOINFORMATICS.NL/XEROPHYTA/PORTAL/BLAST/

G-type lectin S-receptor-like serine/threonine-protein kinase (B120)
G-type lectin S-receptor-like serine/threonine-protein kinase (At4g27290)
Receptor-like protein kinase THESEUS 1 (THE1)
G-type lectin S-receptor-like serine/threonine-protein kinase (SD1-1)
G-type lectin S-receptor-like serine/threonine-protein kinase (At1g11330)
Cysteine-rich receptor-like protein kinase 10 (CRK10)
Putative receptor-like protein kinase (At4g00960)
Similar to SLSG S-locus-specific glycoprotein (S13)
Putative cysteine-rich receptor-like protein kinase 35 (CRK35)
Probable LRR receptor-like serine/threonine-protein kinase (At1g53430)
Probable LRR receptor-like serine/threonine-protein kinase (At1g53440)