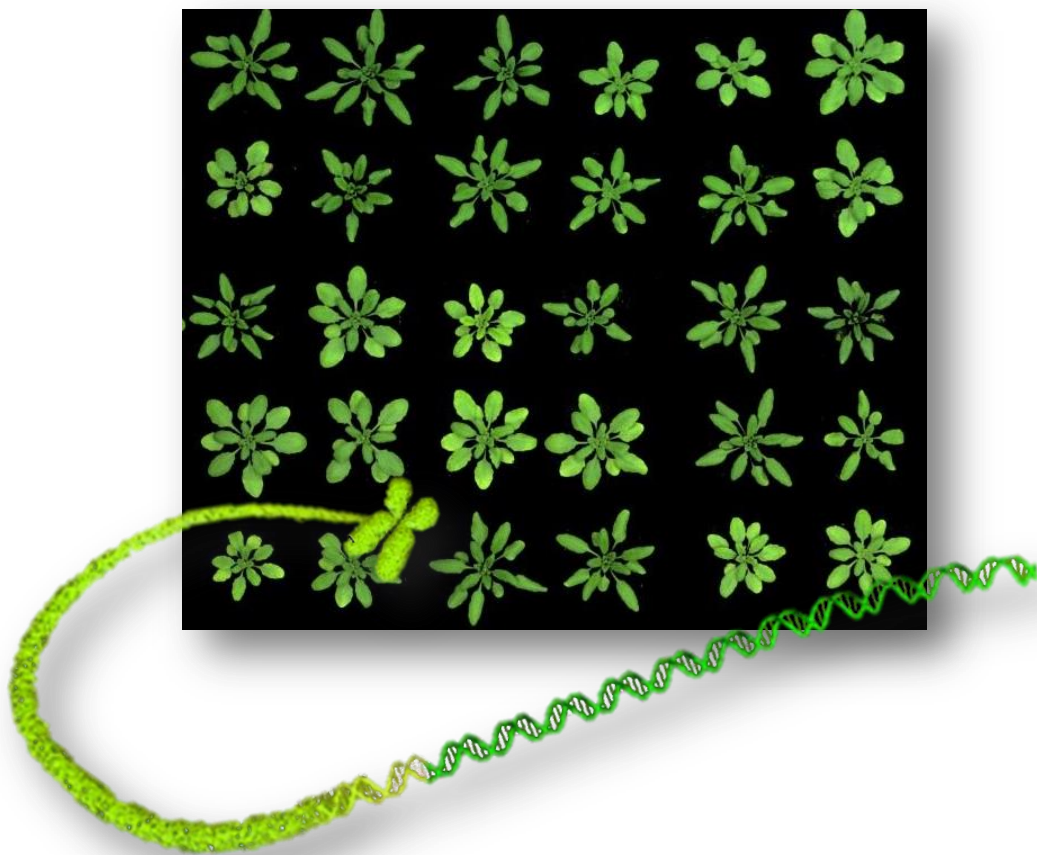# Functional analysis of unknown gene *AT4G18395* involved in heat stress tolerance in *Arabidopsis thaliana*

Irma Hoendervangers

Department of Plant Physiology
Wageningen University and Research Centre

Functional analysis of unknown gene *AT4G18395* involved in heat stress tolerance in *Arabidopsis thaliana*

Irma Hoendervangers

930329346050

Supervisor:
Emilie Fradin

Msc Thesis Plant Physiology
PPH-80424

December 2016

Department of Plant Physiology
Wageningen University and Research Centre

Acknowledgements

I want to offer my special thanks to Emilie Fradin, for being such a good and kind supervisor. You always had time when I needed something and thought along with what I wanted to do during this thesis. I appreciate your positive encouragements. It made me feel comfortable and confident during this thesis.

I'd like to thank Laura Groenenberg for the good times when we worked together. And thank you that I was allowed to use some of your data in this thesis.

I'd like to thank Juriaan and other lab assistents for their time and help in the lab. Thank you that you always had time for my questions. I thank Leo for his assistance with the chlorophyll measurements.

I thank God for bringing these wonderful opportunities and people on my path. I thank Him for providing the support, courage and strength I needed.

Abstract

Heat stress is a serious concern for agriculture, and will be even more so in the future due to global warming. Heat stress causes yield loss by reducing plant growth, development and reproduction. This makes research on heat stress response and breeding for heat tolerance of great importance. Bac-Molenaar and co-workers found several QTL's related to heat tolerance for the developmental stages of reproduction and silique and seed development. One of the candidate genes found in that QTL analysis was *AT4G18395*, of which the function is yet unknown. This thesis attempts to get a better understanding of the function of *AT4G18395*, by phenotypic characterization of rosette shape and chlorophyll content, germination, root architecture and ion content by means of T-DNA knock-out lines and overexpression lines. Though the gene is generally only expressed in pollen tissue, overexpression on whole plant level led to a change in phenotype in various tissue. Rosettes of *AT4G18395* overexpression mutants look different from wild type plants, which could be best described with a higher solidity and leaves which are wider and have a smaller petiole. Furthermore, leaf water content is higher in plants where *AT4G18395* is overexpressed. *AT4G18395* overexpression mutants have a delayed germination. The *AT4G18395* overexpression mutants seem to be more heat sensitive than wild type plants in terms of germination and rosette growth. Leaf nitrate and sulphate content are increased in *AT4G18395* overexpression lines. Deviating phenotypes were also seen for leaf chlorophyll content and root architecture, but more extensive experiments are needed in order to confirm involvement of these two. These findings show that AT4G18395 plays a role during heat stress and, when overexpressed in the whole plant, it has a very diverse impact on different tissues and processes.

Keywords: *Arabidopsis thaliana*, heat stress, overexpression mutant, rosette shape, germination assay

Table of Contents

Introduction

Heat stress causes serious yield losses for global crop production. When a crop suffers from heat stress, it shows reduced growth, delayed plant development and reduced reproduction (Hasanuzzaman et al., 2013). Heat stress is therefore a problem in agriculture. The increasing frequency of heat waves due to climate change already causes large yield losses in worldwide crop production (Bita and Gerats, 2013). In addition, predictions are that global air temperature will rise further with 1.8 to 4.0 °C by 2100 due to climate change (IPCC, 2007). With a global temperature increase of 3.0-4.0°C yield losses of 15-35% could be expected in Africa and Asia and 25-35% in the Middle-East (Bita and Gerats, 2013). The growing consciousness on the impact that elevated temperatures have on food production in the past decades led to an increase in research to better understand plants response and adaptation to heat stress. Consequently, interest for heat-tolerance breeding has increased.

The impact of heat stress on plants is not only dependent on the temperature increase, but also on the duration of this increase (Wahid et al., 2007). First of all, elevated temperatures cause denaturation or instability of various proteins, RNA molecules, membranes and other cell structures (Figure 1, Bita and Gerats, 2013, Mittler et al., 2012). This results in malfunctioning of cellular processes and reactions, which can cause the production of reactive oxygen species (ROS) (Mittler et al., 2012, Wahid et al., 2007). Furthermore, heat stress can cause water loss and problems in the water balance of the plant (Figure 1, Wahid et al., 2007). Heat stress has a notable effect on plant development during all its life cycle, though the reproductive stage is extremely sensitive to heat and even a temperature rise of a few degrees can already arrest flowering or seed production in plants (Hasanuzzaman et al., 2013, Wahid et al., 2007). Heat can lead to sterility by failure of both male and female organs, reduced viability of pollen or ovule and disturbed fertilization among others (Hasanuzzaman et al., 2013). Germination and emergence can be slowed down or even totally inhibited when heat stress occurs during this developmental stage (Hasanuzzaman et al., 2013, Wahid et al., 2007). Next to reproduction, photosynthesis is tremendously impacted by heat stress, severely disrupting the functioning of different components and chemical processes inside the chloroplast (Hasanuzzaman et al., 2013). Heat stress has most impact on photosystem II and can even lead to inhibition of photosystem II (Wang et al., 2009). Furthermore, the structure of thylakoids is affected by heat, resulting in alteration of the Calvin cycle, photorespiration and instability of enzymes involved in carbon metabolism among others (Wang et al., 2009), and heat can lead to inactivation of Rubisco (Wise et al., 2004). In addition, the damage to photosystem II, and in lower amounts of photosystem I, results in a major production of ROS (Hasanuzzaman et al., 2013).

Plants have a natural protection system to survive heat damage (Figure 1), which is extensively studied to understand the underlying different mechanisms and to find ways to breed for enhanced thermotolerance. There are two strategies that plants use to coop with heat stress, namely acclimation or cell death (Larkindale et al., 2005, Mittler et al., 2012). The plant is able to acclimatize by protecting or repairing proteins, stabilizing membranes and regaining homeostasis by adapting metabolic processes. Another strategy is to stimulate programmed cell death, for example shedding leaves or aborting fruits, in order to survive. Plants sense an elevation in temperature at very early stage in the plasma membrane. Changes in plasma membrane fluidity lead to the opening of a calcium channel and Ca^{2+} goes into the cytoplasm (Bita and Gerats, 2013, Mittler et al., 2012). As response, Mitogen-Activated Protein Kinase (MAPK) and Calcium-Dependant Protein Kinase (CDPK) are induced by the change of Ca^{2+} in the cytoplasm and these cascades lead to the production of antioxidants to fight oxidative stress and osmolytes to adjust the water balance (Bita and Gerats, 2013, Wahid et al., 2007). One of the major responses to heat stress is the production of heat shock proteins (HSPs), which act as chaperones that protect heat-sensitive proteins against denaturation and support their stabilization and functioning (Hasanuzzaman et al., 2013, Wahid et al., 2007). Different groups of HSPs are activated at specific developmental stages, like germination or

flowering (Hasanuzzaman et al., 2013). Next to the calcium influx, the presence of ROS or unfolded proteins are signals that also trigger the production of antioxidants which fights oxidative stress (Mittler et al., 2012, Wahid et al., 2007).
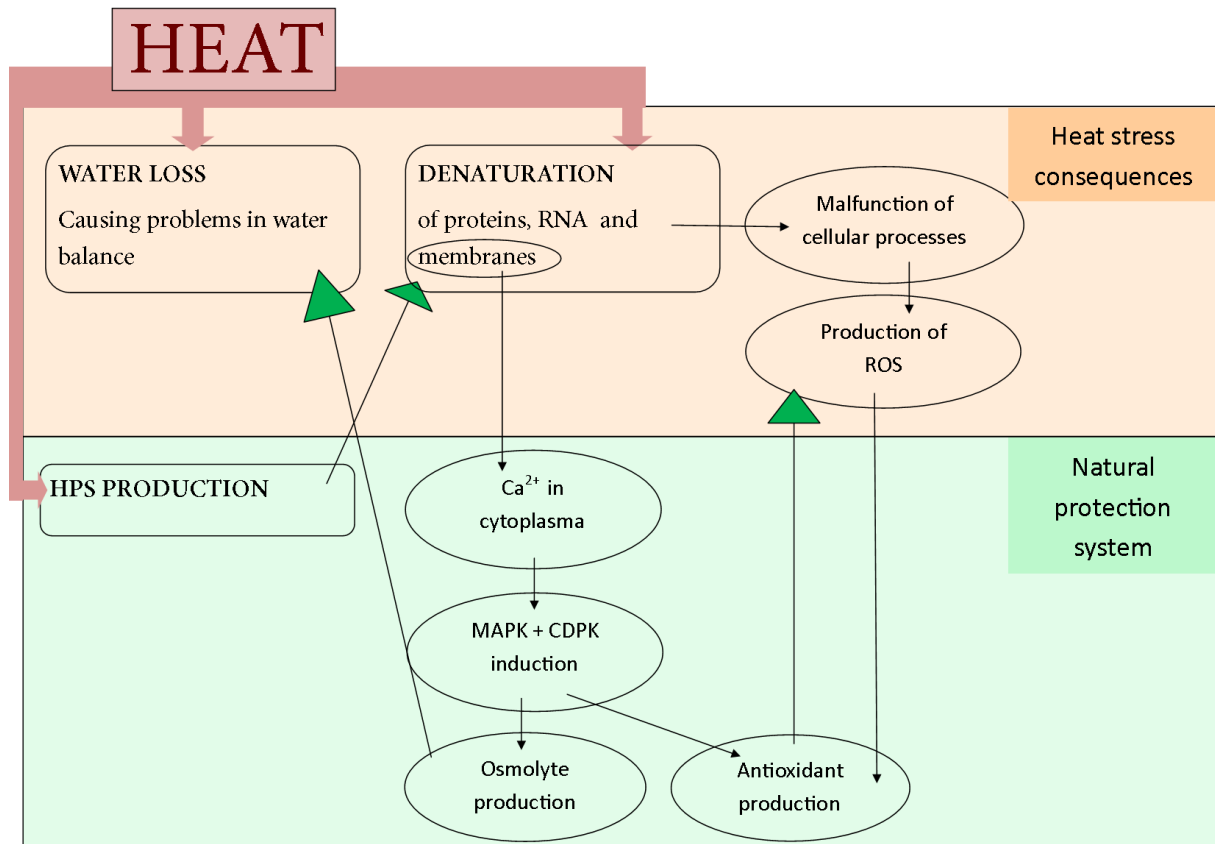

Figure 1: EFFECT OF HEAT STRESS ON A PLANT CELL. Schematic representation of the impact of heat stress on cell level. Red arrows indicate direct processes that occur during heat stress and black arrows indicate indirect reactions. The upper part describes the negative consequences of heat stress and the lower part describes the plants natural protection response. Green arrows show what problems the different heat stress responses tackle. ROS=Reactive Oxygen Species, HPS = Heat Shock Proteins, MAPK = Mitogen-activated protein kinase, CDPK = calcium-dependant protein kinase

Breeding for improved heat tolerance can be strived thanks to this extensive understanding of the plants responses and mechanisms to heat stress. Though the natural thermotolerance of most crops is only temporary and insufficient under longer periods of heat or more extreme temperatures, knowledge on the physiological and biochemical changes under heat can be used to pursue long-lasting heat tolerance by means of genetic or epigenetic improvements (Wahid et al., 2007). Heat stress tolerance is a multigenic trait (Bita and Gerats, 2013, Larkindale et al., 2005). Different sets of genes are induced upon different responses and different developmental stages. With a good understanding of the different aspects of this multigenic trait, progress can be made with screening techniques and genetic approaches, like QTL analysis, that result in identification of genes (Bita and Gerats, 2013, Wahid et al., 2007). This will help with developing breeding tools, like markers for heat tolerance. Fortunately, genetic variation for heat stress tolerance is abundantly present within plant species as well as between species (Wahid et al., 2007). Since heat stress responses are similar in many plant species, *Arabidopsis thaliana* can serve as a good model plant for this trait (Bac-Molenaar et al., 2015).

This thesis follows on Bac-Molenaar and co-workers' work on heat tolerance during reproduction, silique development and seed development. In this research, two stages were found to be extremely sensitive to heat during the reproduction, namely 1) male and female meiosis (before anthesis) and 2) fertilization and early embryo development (Bac-Molenaar et al., 2015). Through genome-wide association mapping, four QTLs were discovered that are strongly associated with heat response (LOD-score markers of 5.5 or higher). Specific developmental stages were defined in which the QTLs are involved. Three of the QTLs are involved in heat stress response before anthesis and one QTL relates to heat stress response after anthesis. In addition, for each QTL, one or two candidate genes were found, among which genes for two QTLs in the pre-anthesis response were also confirmed to be involved in heat stress response (Bac-Molenaar et al., 2015). However, even more QTL's were found in the research of Bac-Molenaar and co-workers and this thesis focusses on the candidate gene of a QTL that is associated with four markers with a LOD-score between 4 and 5. This QTL was found for pre-anthesis response and using the trait "number of siliques with a length inferior to 5 mm", (unpublished). The candidate gene for this QTL is *AT4G18395*. Its involvement in heat stress was confirmed through knock-out and overexpression mutants. The knock-out line T28 was more susceptible to heat stress (unpublished). Publicly available T-DNA lines carried an insert in the promotor area of the gene (T28) and in the coding region of the gene (T108). Overexpression lines were made by the insertion of a construct containing the constitutive 35S promotor fused to *AT4G18395*. Plants were selected for three generations to ensure single copy and homozygosity of the insertion.

The gene has a size of only 360 base pairs and encodes a protein of 119 amino acids with four cysteines. Cysteine is characterized by thiol groups, which gives a protein higher reactivity, in terms of, for example, oxidation, metal-binding reactions or substitution reactions (Fahey et al., 1977). Thiol groups are often seen in catalytic or regulatory processes and help in binding coenzymes or activators (Fahey et al., 1977). The gene has no introns and no signal peptides. The lack of signal peptides indicates the protein is probably not transported to another cell compartment and thus remains in the cytoplasm or nucleus. The gene is expressed mostly in pollen, as shown in Table 1 where all tissues with expression levels of 100 or more relate to pollen tissue (Arabidopsis eFP Browser). Heat stress causes reduced viability and germination of pollen (Firon et al., 2006). Pollen grain production is disrupted through failing separation from pollen mother cells under heat stress in Arabidopsis. In addition, heat stress causes difficulties with the release of pollen from the pollen sacs (Kim et al., 2001). Firon and co-workers show that in tomato the maximum starch content in pollen grains is lower under heat stress and also the sucrose content in mature pollen is reduced upon heat stress (Firon et al., 2006). Heat stress can also lead to abortion of pollen, through suppression of S-adenosylmethionine decarbosxylase activity, which leads to reduced spermidine and spermine content in pollen (Song et al., 2002). Lastly, heat shock proteins (HSP) and heat stress transcription factors (HSF) play a role in the heat stress tolerance response of pollen. Higher contents of HSP and HSF were found in pollen in tolerant tomato lines, compared to heat sensitive lines (Frank et al., 2009; Bita et al., 2011).

Table 1: *AT4G18395 EXPRESSION LEVEL.* Expression signal (GCOS (GeneChip Operating System) normalized) above 100 of *AT4G18395* for different types of tissues (from Arabidopsis eFP Browser).

Tissue	Expression Level
Mature Pollen Grain	4900.8
Tricellular Pollen	3744.25
Dry pollen	1696.97
Pollen, germinated in vitro for 30 minutes	1346.56
Pollen, germinated in vitro for 4 hours	1154.02
Pollen tubes harvested after growth through pistil explants	381.57
Bicellular Pollen	137.35

Though this knowledge about expression and gene characteristics can give hints, the exact function of this gene is yet unknown. The aim of this thesis is to get a better understanding of the function of *AT4G18395,* by the characterization of phenotypic traits by means of different T-DNA knock-out and overexpression lines. Plants overexpressing *AT4G18395* show a different phenotype in terms of rosette shape (observation by eye). Thus the gene can initiate changes in tissues other than pollen when overexpressed in the whole plant. These changes in rosette shape were characterized and possible involvement of the gene in germination, root architecture and ion content were tested, with the following research questions:

1. What is changed regarding to rosette shape and chlorophyll content in *AT4G18395* knock-out or overexpression lines? How does heat stress change the rosette characteristics in these lines?

2. Has germination changed in knock-out or overexpression lines of *AT4G18395*? How does heat stress affect the germination of these lines?

3. Does *AT4G18395* play a role regarding root architecture?

4. Are ion contents changed in *AT4G18395* knock-out or overexpression lines?

Materials and methods

Plant material and growth

Two knock-out mutants were ordered for *AT4G18395*, one with a predicted insertion in the promotor area (T28) and one with a predicted insertion in the coding sequence (T108). Furthermore, overexpression lines were made in Col-0 background, CS background and in one of the knock-out backgrounds. Detailed descriptions of these lines can be found in Table 2. Different subsets of these lines are grown in the different experiments. This is indicated per experiment.

Table 2: OVERVIEW OF GENOTYPES. Arabidopsis wild type, knock-out and overexpression lines that were used in the experiments.

Type	code		Detail
Wild type	WT Col	2	Arabidopsis ecotype Columbia (Col-0)
	WT CS	1	Arabidopsis ecotype CS, background from which mutants were made
	WT T28	4	Arabidopsis CS background of T28 mutant
	WT T108	25	Arabidopsis CS background of T108 mutant
Knock-out	T-DNA T28	3	T-DNA knockout line insertion T28 (in promotor)
	T-DNA T108	24	T-DNA knockout line insertion T108 (in coding sequence)
Overexpression	Oe T28 - Col	17-23	7 overexpression lines with 35S promotor insertion fused to *AT4G18395* in Col-0 background
	Oe T28 - CS	11-16	6 overexpression lines with 35S promotor insertion fused to *AT4G18395* in CS background
	Oe T28 - T28	5-10	6 overexpression lines with 35S promotor insertion fused to *AT4G18395* in T-DNA knockout T28 background

In all experiments, seeds first received a cold treatment at 4°C for 4 days followed by one day of pre-germination at room temperature to release dormancy before sowing them on rockwool saturated with Hyponex nutrient solution. Three times a week the rockwool blocks were saturated with this nutrient solution using an automated flooding system. Plants were grown in a climate chamber at 22°C during day and 18°C during night. The lights are set to 16h of light and 8h of darkness a day. In the experiments plants were sown following a complete random design or random block design.

Verification of T-DNA knock-out insert

Three leaves were collected from three-week-old plants of WT CS, T-DNA knock-out lines T28 and T108, WT T28 and WT T108. For DNA extraction, the protocol of Cheung et al. (1993) was adapted as follow: 175 µL extraction buffer was added to the plant material, which was then grinded mechanically. After grinding, the samples were incubated at 60°C for an hour. 75µL iso-propanol and 30µL NH_4Ac were added to 75 µL supernatant to precipitate DNA. Other than the protocol of Cheung describes, the pellet was dissolved in MQ water after washing with ethanol. The gene region was amplified in these samples by PCR. The PCR protocol was as following: 94°C for 4 min., followed by 30 cycles of 94°C for 15 sec., 55°C for 25 sec. and 72°C for 1 min 30, and after these cycles another 10 minutes at 72°C. Thereafter, the PCR product was put on 1% agarose gel to visualize the result.

Rosette characterization

a. Rosette shape and biomass

All lines from Table 2 were grown and examined for the characterization of rosette shape, in the first repetition five plants per genotype were used, and in the second repetition ten biological replicates per line were used. The plants were grown for four weeks and thereafter harvested, because after four weeks the plants switch to the generative phase. The different rosette characteristics that were measured are rosette area, perimeter, solidity and biomass. Rosette area, perimeter and solidity were calculated from photo material taken twice a week, using Photoshop and ImageJ. Pictures were taken 13, 17, 20 and 24 days after sowing. From these pictures, the background was removed in Photoshop by color range selection (Figure 2). In ImageJ, the picture was made binary after which the area, perimeter and solidity (in shape descriptors, figure 3) of each individual rosette were measured.

Rosette fresh weight was measured during harvest 28 days after sowing for all ten replicates of the second repetition. Half the plants were used to measure rosette dry weight, while the other five replicates were used to measure chlorophyll content. The fresh weight to dry weight ratio was calculated afterwards.

Figure 2: First picture shows the non-edited photo, second picture shows the same plant after removing the background in Photoshop and the third picture is made binary in ImageJ after which area, perimeter and solidity are measured.

Figure 3: Solidity is calculated as the ratio between filled surface (rosette) and empty surface (blue) of the total area, within the black line (drawn as if an elastic would be stretched around the leaves)

b. Chlorophyll extraction

To extract chlorophyll from the leaves as described in the protocol of Gibon (nd), rosettes were freeze-dried and grinded. There were two biological replicates per line. Two mg dried material was taken from the sample and washed three times with 250 μl 80% ethanol, 150μl 80% ethanol and 250 μl 50% ethanol respectively. After each washing step, the suspension was mixed well, incubated at 80°C for 20 minutes and centrifuged for 5 minutes at 14000 rpm. Supernatant was collected together on ice after each step. 50 μl of the supernatant was transferred to a 96-wells-microplate with 120 μl 98% ethanol, in technical duplicate. The fluorescence of each well was determined with Infinite 200Pro microplate reader at 645 and 665 nm. From these fluorescence values, chlorophyll A and B contents were calculated with the following formula's after correcting.

Chlorophyll A = $5.48A_{665} - 2.16A_{645}$
Chlorophyll B = $9.67A_{645} - 3.04A_{665}$

c. Single leaves characterization

For WT CS and five Oe T28 – CS lines, rosettes were dissected and detached leaves were photographed. From this photo material, leaf area, perimeter and solidity were calculated with Photoshop and ImageJ as described earlier in Figure 2. Furthermore, petiole length and blade width were calculated with the ImageJ plug-in LeafJ (Maloof et al., 2013). For quantification, only the values of the middle 5 leaves of each line and at least two replicates were used in the statistical analysis (Figure 4).
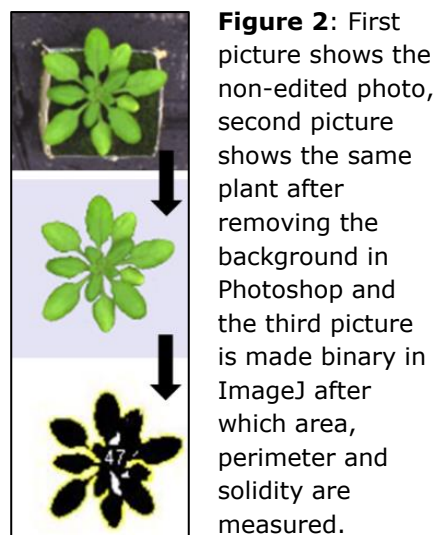
Figure 4: Indication of dissected leaves, with on the upper photo the rosette leaves of a representative WT CS plant and in the lower photo line a representative plant of Oe T28 – CS (line 15). The orange box indicates the middle five leaves, on which the analysis was done.

d. Heat stress during vegetative phase

To follow the influence of heat on rosette growth, WT CS, WT T28, T-DNA T28, Oe T28 - CS (5x) plants received a heat stress treatment one week after sowing, consisting of 35°C for 12 hours in a Weiss cabinet. Control plants remained in the climate chamber during the experiment. Five replicates per line were sown for each treatment. Rosette area, perimeter and solidity were calculated from pictures taken 13, 17, 20 and 24 days after sowing. Fresh and dry weights were measured at harvest, 28 days after sowing.

Germination assay

Germination was analyzed for the following 8 lines: WT CS, WT T28, T-DNA T28, Oe T28 – CS (5x). The germination assay was performed with the germinator and according to the published protocol (Joossen et al, 2010). After cold treatment, for control conditions, the seeds were placed at 22°C for five days, while for heat stress conditions the seeds were placed at 35°C for the first 24 hours and then moved to 22°C for the remaining time both under constant light. There were three replicates for each line. Germination was determined by the maximum percentage of seeds to germinate and how fast seeds initiate germination. The effect of heat stress during germination was additionally studied by analyzing rosette development on rockwool. Five replicates of control and heated treated seeds were sown directly after the first 24 hours of heat treatment. For these plants the rosette area, perimeter and solidity were measured 8, 13, 17, 20 and 24 days after sowing. Fresh and dry weights were measured at harvest, 28 days after sowing.

Pilot assay for root architecture

In the pilot experiment the following six lines were grown: WT CS, WT T28, T-DNA T28, Oe T28 - CS (3x). Seeds were grown *in vitro* onto half Murashige & Skoog (MS) medium supplemented with 1.5% daishin agar. Seeds were vapor-sterilized for three hours in a desiccator using 100 mL bleach and 3 mL HCl. Following the sterilization, the seeds were sown on the agar plate, and placed at 4°C for cold treatment. After cold treatment, the plates were transferred to the climate cell. Scans were made of the plates at 7, 10 and 14 days after transfer to the climate cell. After cropping these scanned images in Photoshop the length of the primary root, number of lateral roots and length of the lateral roots were measured using the RootNav software package (Pound et al., 2013) (Figure 5). Root and shoot fresh weight was weighted 18 days after transferring to the climate cell.
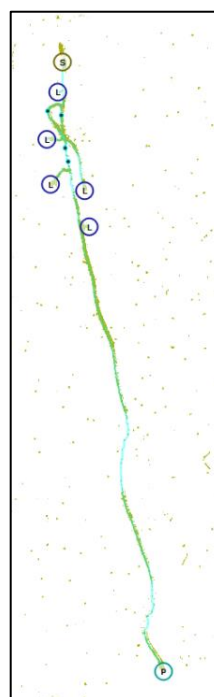
Figure 5: Image of root length measured using RootNav software (Pound et al., 2013). First, the source of the root is selected (yellow S), then the primary root tip (blue P) is indicated and the root is drawn automatically. Thereafter lateral root tips (purple L) are selected and lateral roots drawn. (colours on this image are inverted).

Ion content measurements

The anions that were measured are mentioned in Table 3. First a pilot experiment was carried out with three-week-old plants of the following six lines: WT CS, WT T28, T-DNA T28, Oe T28 - CS (3x). Two to three plants per line were pooled, and two pools per genotype were used for ions contents measurements. In the next experiment, material was taken from both three-week-old plants and four-week-old plants. Furthermore, in this experiment two more lines of Oe T28 – CS were included and there were three pools per line, with three plants per pools. The material was freeze-dried and grinded. To 3-5 mg of the leaf material, 1mL 0.5N HCl + 50 mg/L trans-aconitate (internal standard) was added followed by 15 minutes at 100°C and centrifugation for five minutes at maximum speed. 200µL of the supernatant was analyzed on the HPLC. Dionex HPLC (High-Performance Liquid Chromatography) was used with a Dionex AS11-HC column and AG11-HC guard column and ATC ion trap column at a temperature of 30°C. All peak areas of different anions were translated to the amount of anion per liter with the standard concentration data. Lastly, the data was corrected for with the ratio of trans-aconitate in the blanc and other samples and corrected for the exact weight of leaf material.

Table 3: Overview of the different anions measured with HPLC.

Anions
Na_2SO_4 (Sodium sulfate)
$C_2H_2O_4 2H_2O$ (oxalic acid)
$NaNO_3$ (sodium nitrate)
$Na_2HPO_4.2H_2O$ (di-sodium phosphate)
$C_6H_8O_7.1H_2O$ (citric acid)
IP6 (phytic acid)

Data analysis

For statistical analyses Genstat's and SPSS's Anova test was used with α=0.05 and LSD values to discriminate significances between wild type and different mutant lines. In the Germinator package, statistical analysis of the data was included. The student T-test was used in this package, with α =0.05. Furthermore the student T-test was used for data analysis from other heat stress experiments. For the root assay, the number of replicates was not equal for the different lines in the end. Therefore, a regression analysis with α =0.05 was used to discriminate significant differences.

Results

To get a better understanding of the function of *AT4G18395*, the following phenotypic traits were characterized for *AT4G18395* overexpression lines and T-DNA knock-out lines. Changes in rosette are characterized by shape descriptors like area, perimeter and solidity, rosette biomass and chlorophyll content. Germination time and maximum germination percentage are determined and a pilot is set up to measure root architecture. Lastly, differences in leaf ion content are measured. Additionally the effects of heat stress on rosette growth and germination were studied.

Verification of T-DNA knock-out insert
Figure 6 shows the PCR product of *AT4G18395* for lines WT CS, WT T108 and T-DNA T108 put on gel. The bands for WT CS, WT T108 and positive control with the gene's forward and reverse primer combination show the entire gene is amplified for these lines. The band for T-DNA T108 for the primer combination SALK and reverse shows the gene is amplified from the T-DNA insert until the end of the gene, while no product where obtained with the primer pair annealing to the gene itself. Similar results were found for the T-DNA knock-out line T28, using the respective primer pairs (data not shown).
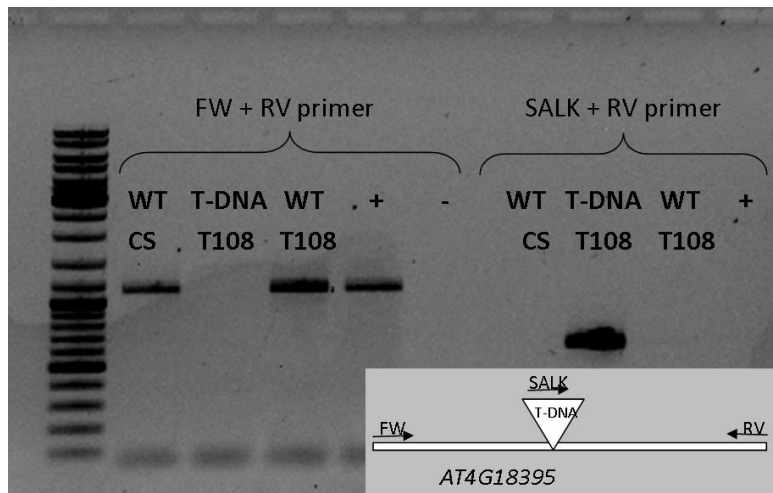
Figure 6: VERIFICATION OF KNOCK-OUT INSERT. Gel image of PCR amplification of WT CS, T-DNA T108, WT T108, positive control DNA (+) and absence of DNA as negative control (-) with two primer combination. FW = forward primer *AT4G18395*, RV= reverse primer *AT4G18395*, SALK = primer in T-DNA. In the corner a schematic representation is drawn of *AT4G18395* with T-DNA knock-out insert (triangle) and the position of the primers with arrows.

Rosette characterization
a. Rosette shape and biomass

A prominent phenotypic difference between plants overexpressing *AT4G18395* and wild type plant is the altered rosette shape (Figure 7 (1-6)). Overall the plants seemed to have a lighter green colour and rosette leaves looked larger and wider. To quantify this, rosette area and perimeter were measured at four different time points with two repetitions. The effectiveness of *AT4G18395* overexpression differs between different lines, and a trait is seen as significantly different if at least half of the lines show a similar significant phenotype. Rosette area was larger for *AT4G18395* overexpression lines in Col-0 background for both repetitions (Figure 7a). For the first experiment, five to six of the seven *AT4G18395* overexpression lines in Col-0 background had a significantly larger rosette area for all time points (Figure 7a, Table A1 appendix) and in the second experiment 20 and 24 days after sowing (DAS), four of the seven Oe T28-Col lines had a larger area (Figure 7a, Table A2 appendix). However, this larger area was not seen for *AT4G18395* overexpression lines in CS background for both repetitions (Table A1 and A2 appendix). The perimeter showed only a significant difference for the second repetition at 24 DAS, and not for other moments in time or the other repetition (Table A1 and A2 appendix). Thus it looks like perimeter is not changed upon *AT4G18395* overexpression. Next to rosette area and perimeter, the shape descriptor solidity was measured for the individual rosettes (Figure 7b). This trait was generally higher for overexpression lines, which indicates these rosettes cover more area (Figure 7b). The difference with rosette area is that this is a calculated ratio (Figure 3), which makes it more stable during changes in plant growth. For the first repetition, more than half of the Oe T28-Col lines and all Oe T28-CS lines had a significantly higher solidity compared to wild type plants at 17, 20 and 24 DAS. For the second repetition, almost all Oe T28-CS lines had a significantly higher solidity at 17, 20 and 24 DAS, but in Col-0 background this was not the case. Thus solidity seems to capture the different rosette appearance that is seen for *AT4G18395* overexpression lines well (Figure 7 (1-4)), except at 13 days after sowing.
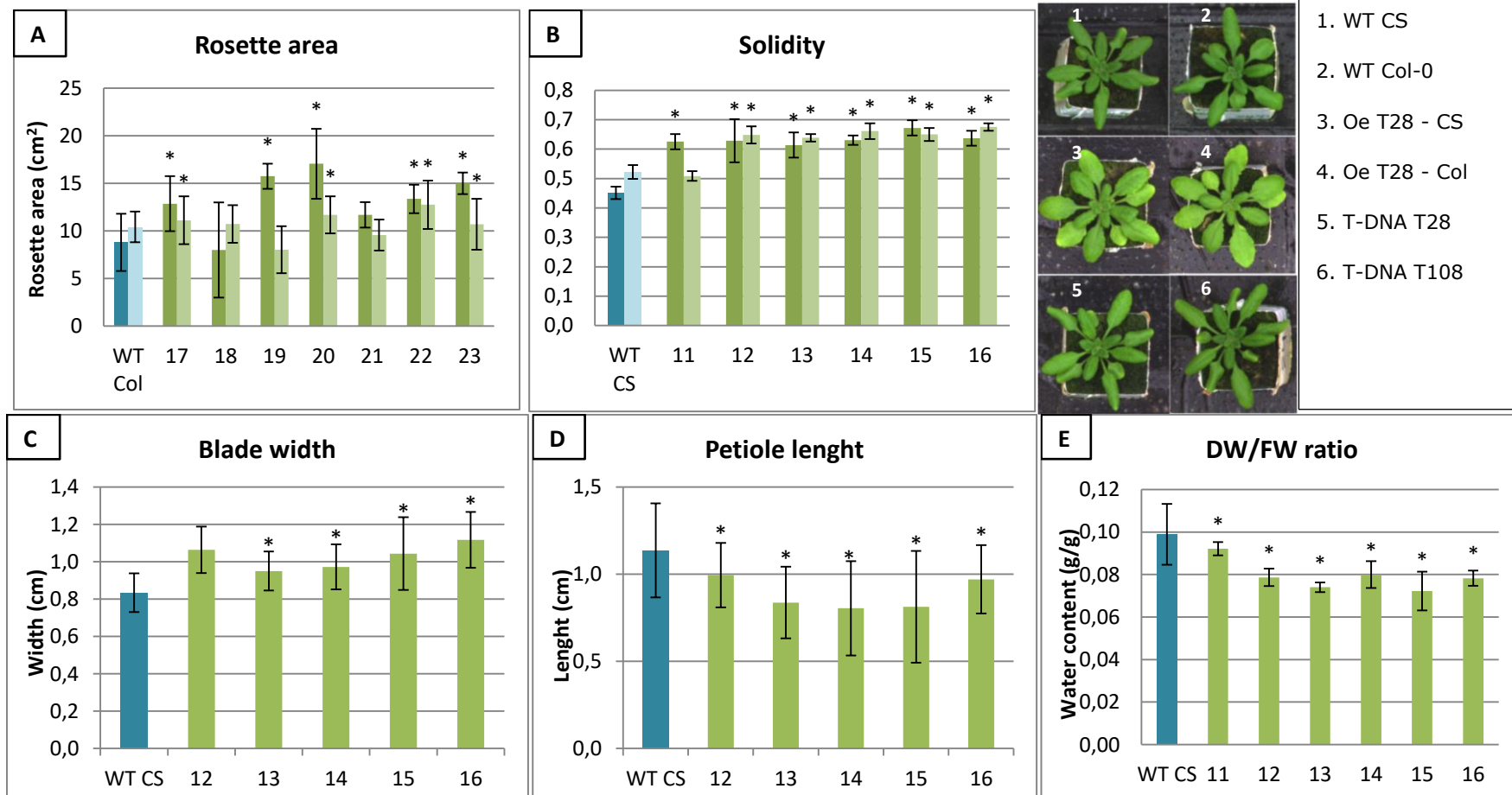
Figure 7: OVEREXPRESSION OF *AT4G18395* ALTERS ROSETTE DEVELOPMENT. Overview of rosette measurements, with the different lines (wild types in blue, overexpression lines in green). Photos (1-6): Picture of a representative plants for wild type, knock outs, and overexpression lines. **A)** Average rosette area measured 24 days after sowing (DAS). In green are seven *AT4G18395* overexpression lines in Col-0 background. The first repetition (with 5 replicates) is indicated with dark-coloured bars and the second repetition (with 10 replicates) with light-coloured bars. **B)** Average solidity measured 24 DAS. In green are six *AT4G18395* overexpression lines in CS background. The first repetition (with 5 replicates) is indicated with dark-coloured bars and the second repetition (with 10 replicates) with light-coloured bars. **C)** Average blade width of 20 leaves (10 leaves for line 15), measured at harvest (28 DAS). In green are five *AT4G18395* overexpression lines in CS background. **D)** Average petiole length of 20 leaves (10 leaves for line 15), measured at harvest. In green are five *AT4G18395* overexpression lines in CS background. **E)** Average rosette dry weight to fresh weight ratio from five replicates, calculated from dry weights and fresh weights measured at harvest 28 DAS. In green are six *AT4G18395* overexpression lines in CS background. Significant differences are indicated by an asterisk (*) (details can be found in Table A1 and A2, appendix).

16

Since the leaf shape differed for *AT4G18395* overexpression lines from wild type lines, leaves were dissected from the rosette and the middle 5 leaves of each rosette were compared in the analysis (Figure 4). To describe the leaves, the following characteristics were measured: average leaf area, perimeter, solidity, blade width and petiole length. Leaf area was larger for only two of the five tested *AT4G18395* overexpression lines and also perimeter was smaller for just two of these overexpression lines (Table 4). The trait solidity showed no significant difference (Table 4). The traits blade width and petiole length showed a significant difference for respectively four lines and all five lines(Table 4, Figure 7cd). Thus, leaves overexpressing *AT4G18395* had a wider blade and smaller petiole.

Table 4: CHARACTERISATION OF LEAF SHAPE. Overview of individual leaf traits of overexpression lines in the CS background. Averages are given of the 5 middle leaves of 4 rosettes (2 replicates in case of line 15). Significances are calculated with Genstat's Anova (p- and LSD values are indicated per trait below) and indicated with an asterisk.

Line		Area (cm^2)		Perimeter (cm)		Solidity		Blade width (cm)		Petiole length (cm)	
WT CS	1	1.68		8.78		0.82		0.83		1.14	
Oe T28 CS (5x)	12	2.08	*	8.65		0.84		1.06		0.99	*
	13	1.73		8.20		0.85		0.95	*	0.84	*
	14	1.63		7.49	*	0.84		0.97	*	0.80	*
	15	1.90		7.95	*	0.86		1.04	*	0.81	*
	16	2.18	*	8.86		0.83		1.12	*	0.97	*
P-value		<0.001		<0.001		0.203		<0.001		<0.001	
LSD		0.27		0.70		0.03		0.09		0.16	

The rosettes were further characterized by measuring total rosette fresh weight, dry weights and water content for one repetition. Both fresh weight and dry weight did not show a significant increase or decrease for more than three *AT4G18395* overexpression lines for both backgrounds. Since only such a low number of overexpression lines showed a difference, it looks like biomass is not changed upon *AT4G18395* overexpression. The ratio between dry weight and fresh weight on the other hand is lower for all *AT4G18395* overexpression lines in the CS background (Figure 7e, Table A2 appendix) and for five *AT4G18395* overexpression lines in Col-0 background compared to the corresponding wild types (Table A2 appendix). In other words, plants overexpressing *AT4G18395* had a higher leaf water content.

Overall, *AT4G18395* knock-out mutants (T-DNA T28 and T-DNA T108) did not show a different rosette phenotype from their corresponding wild type. Rosette area, perimeter and solidity did not show significant changes for the knock-out mutants for both repetitions, except solidity for one time point (Table A1 and A2 appendix). Moreover, both knock-out mutants showed no difference from WT CS in weight or dry to fresh weigh ratio (Table A2 appendix). WT T108 showed no significant differences to WT CS, except perimeter for one time point (Table A1 and A2 appendix). WT T28 however differed in multiple traits from WT CS. This line had a larger rosette area in the second repetition (Table A2 appendix) and a higher solidity value in the first repetition at 17 and 20 DAS (Table A1 appendix). Its perimeter was lower in the first repetition for the last two time points, but higher for almost all time points in the second repetition (Table A1 and A2 appendix).

b. Chlorophyll extraction

When comparing *AT4G18395* overexpression lines with wild type plants, the difference in colour is also striking as *AT4G18395* overexpression lines seem brighter in colour (Figure 7 (1-4)). To check whether this brighter green colour results from a difference in chlorophyll content, chlorophyll A and B content were calculated from fluorescence measurements (Figure 5). Though *AT4G18395* overexpression lines in CS background seemed to have lower chlorophyll A and B content than WT CS, this could not be significantly proven (Figure 5, Table A3 appendix). There was also no significant difference between *AT4G18395* overexpression lines in Col-0 background and WT Col-0 or *AT4G18395* knock-out lines and WT CS (Table A3 appendix). However, when comparing *AT4G18395* overexpression lines in CS background with *AT4G18395* overexpression lines in T28-knock-down background, the Oe T28 - CS group had a significantly lower chlorophyll A content compared to Oe T28 - T28 (t-test, p=0.005). For chlorophyll B, Oe T28 – CS was not significantly different from Oe T28 – T28 (t-test, p= 0.36).
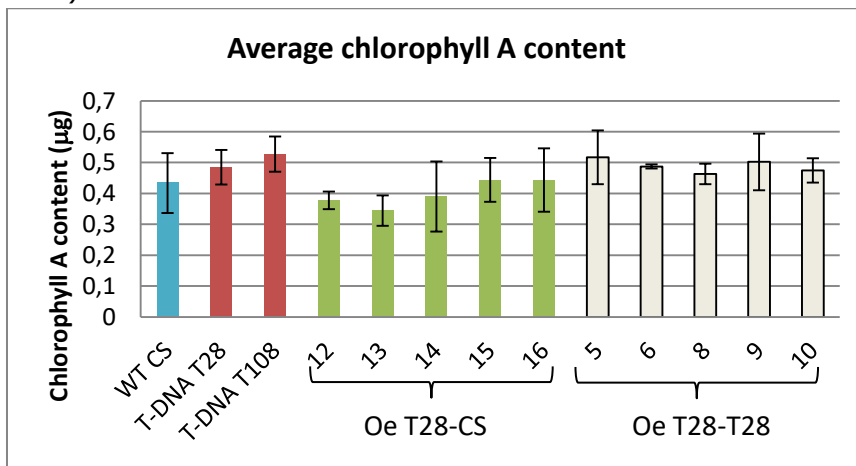
Figure 5: CHLOROPHYLL A CONTENT. Average chlorophyll A content of two replicates in μg for CS wild type (blue), knock-out lines T28 and T108 (red) and five overexpression lines in CS (green) and T28 knock-out backgrounds (white). Chlorophyll A is calculated as Chlorophyll a = $5.48A_{665} - 2.16A_{645}$, where A is fluorescence. Oe T28 - CS differs significantly from Oe T28 - T28 (Student t-test; p=0.005).

c. Heat stress during vegetative phase

Plants were exposed to a heat treatment one week after sowing to get insight in the effect of heat stress on *AT4G18395* overexpression and knock-out mutants during the vegetative phase. In general, plants that were exposed to heat stress showed a reduction in rosette area compared to plants grown under control conditions (Figure 8a), and this could be proven significantly for *AT4G18395* overexpression lines for all time points and WT CS for 24 DAS (Table A4 appendix). Three of the five *AT4G18395* overexpression lines in CS background had a significantly smaller rosette area after heat stress for the first three time points and two were significantly smaller 24 days after sowing (Table A4 appendix). The rosette perimeter was not significantly different for heat stressed plants, except two *AT4G18395* overexpression lines 20 DAS and WT CS 24 DAS (Table A4 appendix). At 13 DAS, solidity was significantly lower after heat stress treatment for three *AT4G18395* overexpression lines and WT T28, but this is not seen back at later time points (Table A4 appendix). In addition, fresh weight and dry weight were reduced upon heat stress for WT CS, T-DNA T28 and two *AT4G18395*

overexpression lines (Figure 8b, Table A2 appendix). Except for WT T28, no significant changes were seen in DW/FW ratio upon heat stress (Table A4 appendix).
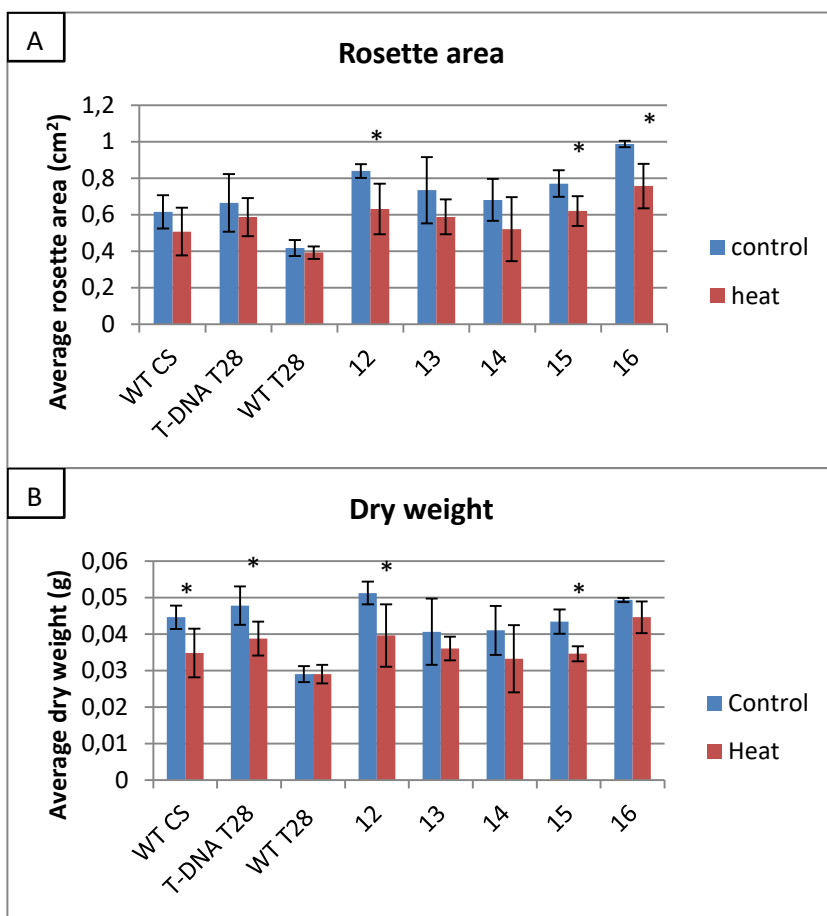
Figure 8: EFFECT OF HEAT STRESS DURING ROSETTE GROWTH. Differences in rosette area (A) and dry weight (B) between WT CS, T-DNA T28, WT T28 and five Oe T28 – CS lines grown under control conditions of 22 ^{0}C (blue bars) and plants grown after exposure to heat stressed 35 ^{0}C for one day (red bars). Significances between heat treatments and respective control treatments are indicated with an asterisk (student T-test, α=0.05) **A)** Average rosette area of five replicates, measured 13 DAS. **B)** Average dry weight of five replicates, measured at harvest 28 DAS.

Germination assay

The influence of *AT4G18395* overexpression and knock-out on maximum germination percentage and germination time was studied for seeds grown under control conditions and heat stress conditions.

Table 5: GERMINATION TIME AND PERCENAGE. Germination assay under control conditions (22 ^{O}C) and heat stress (35 ^{O}C for the first 24h). Average time until 10% and 50% of the total amount of seeds germinated (t10, t50) and average percentages of seeds that germinated (gMAX) of three replicates are given for CS wild type (blue), overexpression lines in CS background (green) and a T-DNA knock-out line (red). Significances are calculated with Student T-test (α=0.05) by the Germinator package and lines that differ from WT CS are indicated with an asterisk (*).

Line	Control						Heat					
	t10 (hr)		t50 (hr)		gMAX (%)		t10 (hr)		t50 (hr)		gMAX (%)	
WT CS	127.9		132.7		99		137.4		144.1		94	
Oe T28 CS (5x)	131.2	*	138.8	*	97		153.1	*	164.3	*	85	
	133.1	*	139.6	*	94	*	157.1	*	166.1	*	77	*
	132.0	*	137.6	*	100		157.7	*	165.9	*	77	*
	132.3	*	138.0	*	99		154.1	*	161.9	*	91	
	131.6	*	138.6	*	99		154.9	*	163.2	*	86	*
WT T28	132.3	*	137.8	*	98		145.7	*	156.3	*	84	*
T-DNA T28	129.3		133.0		99		140.4		146.2		96	

Under control conditions, all five lines overexpressing *AT4G18395* (Oe T28 – CS) required more time until 10% and 50% of the seeds were germinated (t10 and t50) compared to WT CS (Table 5, Figure 9a). This indicates *AT4G18395* overexpression lines had a slower germination rate. The germination time of the T28 knock-out line did not differ from CS wild type, but WT T28 showed a slower germination than WT CS. When heat stress was applied, these differences in germination time are even stronger (Table 5, Figure 9b). At first, the difference t10 between wild type and overexpression lines was around 4 hours under control conditions. This difference in germination time between WT CS and *AT4G18395* overexpression lines increased to around 17 hours (Table 5). In addition, the maximum percentage of seeds germinating was lower for three *AT4G18395* overexpression lines and WT T28 (Table 5, Figure 9b). WT CS had an average maximum germination of 94%, but the germination percentage of these mutant lines decreased with 8-17%.
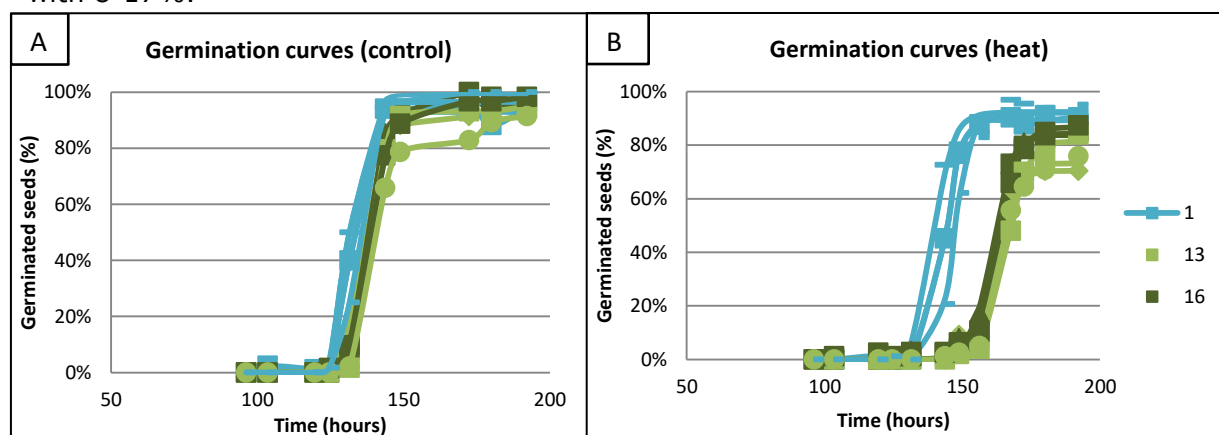
Figure 9: GERMINATION OF SEEDS GROWN UNDER HEAT AND CONTROL CONDITIONS. Germination curves with on the y-axis the percentage of germinated seeds and on the x-axis time in hours, for both control conditions (22 ^{O}C) and heat stress (35 ^{O}C for 24h). Three replicates of CS wild type are indicated in blue (1) and three replicates of two representative overexpression lines in CS background are indicated in different shades of green (13 and 16). Data was obtained by the Germinator package.

Thereafter, an experiment was set up to examine rosette development of seeds that received this heat stress treatment. Seeds that were sown on rockwool after exposure to heat seemed to have a smaller rosette area compared to plants grown under control conditions. However, this was only significant for overexpression line 12 at 8 DAS (Figure 10). After 13 days there was no distinct difference in rosette area between rosettes grown from heat treated seeds or seeds under control conditions (Table A5 appendix). Perimeter and solidity were not different for rosettes grown from heat treated seeds or seeds grown under control conditions, except one line had a lower perimeter 8 days after sowing and WT CS had a lower solidity 13 days after sowing for heat stress conditions (Table A5 appendix). Moreover, no difference in rosette weight was found at harvest between rosettes grown from heat treated seeds or seeds under control conditions (Table A5 appendix).

Rosette area

Average rosette area (cm²)

(graph with values 0 to 0,14; categories: WT CS, T-DNA T28, WT T28, 12, 13, 14, 15, 16; Control (blue) and Heat (red); asterisk above line 12)
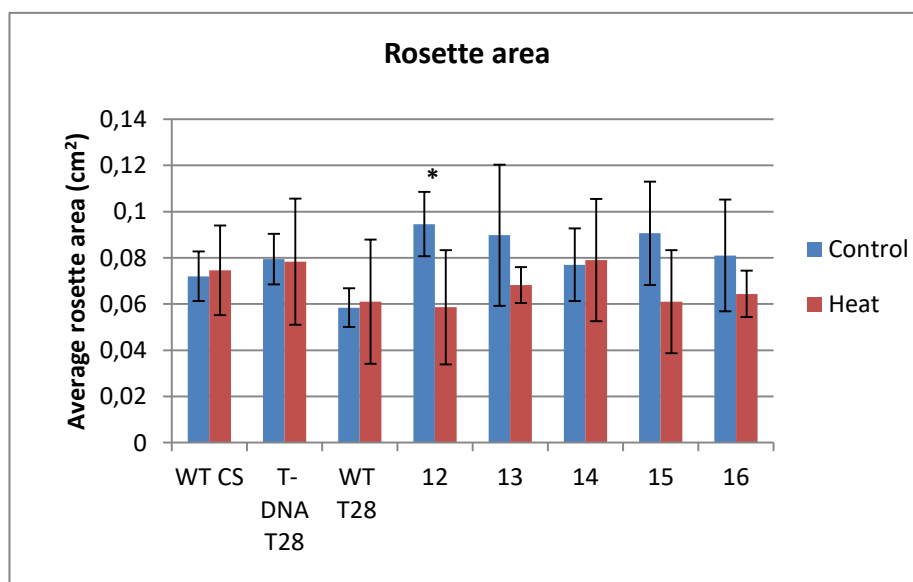
Control
Heat

Figure 10: ROSETTE DEVELOPMENT OF HEAT-STRESSED SEEDS. Difference in rosette area between WT CS, T-DNA T28, WT T28 and five Oe T28 – CS lines grown under control conditions of 22 ^{0}C (blue bars) and seeds grown after exposure to heat stressed 35 ^{0}C for one day (red bars). Average rosette area of five replicates, measured 13 days after sowing. Significances between control and heat are indicated with an asterisk (*) (student T-test, α=0.05).

Pilot assay for root architecture

A pilot experiment was set up to study the possible role of *AT4G18395* on root architecture. All three *AT4G18395* overexpression lines (Oe T28 – CS) tested in the pilot experiment showed a root growth deviating from WT CS (Table 6). The length of the primary root seemed smaller for *AT4G18395* overexpression lines. However, the primary root length of one of the *AT4G18395* overexpression lines (12) was longer than the wild type root length. This line 12 also had more and longer lateral roots compared to CS wild type (Table 6). Knock-out line T28 did not show any significant differences in root growth and architecture from WT CS. However, WT T28 performed worse than WT CS, as WT T28 had less lateral roots and also smaller primary and lateral roots (Table 6).

Table 6: ROOT ARCHITECTURE ASSAY. Averaged root traits of WT CS (in blue) and overexpression lines (green) and knock-out lines (orange) in the CS background. The traits root length and number were measured 7, 10 and 14 days after sowing (DAS). Significances are calculated with Genstat's regression analysis, since the number of replicates varied between five and seven replicates (p- and LSD values are indicated per trait below) and significances are indicated with asterisk (*).

Line	Root biomass (g)		Primary root length (mm)						Number of lateral roots						Lateral root length (mm)				
			7 DAS		10 DAS		14 DAS		7 DAS	10 DAS		14 DAS		7 DAS	10 DAS		14 DAS	
WT CS	0.0087		23.43		46.75		75.33		0.17	9.18		29.99		1.94	23.34		144.6	
12	0.0092		18.55	*	38.96	*	97.84	*	1.57	12.59	*	28.56		1.99	35.20	*	164.8	
13	0.0072		15.60	*	35.73	*	64.23	*	0.86	8.81		25.61		0.86	18.05		99.3	*
14	0.0073		15.36	*	35.37	*	63.89	*	1.13	9.38		26.14		1.14	23.30		112.5	
WT T28	0.0038	*	18.08	*	36.89	*	61.87	*	0	1.74	*	15.21	*	0	1.57	*	37.6	*
T-DNA T28	0.0063		22.53		45.37		73.72		0.25	7.64		27.56		0.65	17.19		109.4	
F-value	0.009		<0.001		<0.001		0.004			<0.001		0.003			<0.001		<0.001	
LSD	0.0026		2.05		3.67		7.54			2.96		6.38			9.32		36.22	

Ion content measurements

A pilot experiment helped to determine the amount of leaf material that was needed for HPLC analysis. Though, due to lack of material there was only one replicate for most of the lines in this pilot and ion content results could not be used. After this pilot experiment, ion content was measured for CS wild type, five lines overexpressing *AT4G18395* in the CS background and the T28 knock-out mutant and wild type. Leaves were taken from these lines three weeks and four weeks after sowing. Five of the seven anions showed significant differences between lines (Table A6 appendix). Five of the seven anions showed a significantly difference in ion content for three-week-old leaves and four-week-old leaves (data not shown). But the interaction between these traits was never significant (Table A6 appendix), meaning that the difference between plants and difference between time points is not correlated and should be seen separate from each other. Only for phytic acid (IP6) a significant difference in ion content was found for T-DNA T28 compared to the CS wild type (Table 7, table A6 appendix). The wild type of T-DNA T28 showed a significant decrease in phytic acid, citrate and sulphate content (Table A6 appendix). When four of the *AT4G18395* overexpression lines showed a similar phenotype in ion content compared to WT CS this response is seen as significantly different. For the anions phytic acid (IP6), nitrate and sulphate, four of the five tested *AT4G18395* overexpression lines showed a difference in ion content (Table 7). Phytic acid was less abundant in *AT4G18395* overexpression lines compared to the CS wild type concentration. Nitrate and sulphate was more abundant in *AT4G18395* overexpression lines compared to CS wild type concentration of these anions.

Table 7: LEAF ION CONTENT. Phytic acid, nitrate and sulphate content is shown for CS wild type (blue), overexpression lines in CS background (green) and a T-DNA knock-out line (red), averaged for leaves harvested 3 and 4 weeks after sowing with 3 replicates for each date. Significances are calculated with Genstat's Anova, $\alpha=0.05$ and LSD as post-hoc test.

Line		Phytic acid (IP6)		Nitrate		Sulphate	
WT CS	1	0.914		116.20		14.35	
Oe T28 CS (5x)	12	0.755		144.25	*	17.55	*
	13	0.668	*	150.60	*	16.55	*
	14	0.661	*	130.20		13.83	
	15	0.566	*	151.95	*	17.76	*
	16	0.567	*	144.80	*	17.31	*
WT T28	4	0.625	*	117.25		13.54	
T-DNA T28	3	0.57	*	114.60		11.36	*
p-value		0.007		<.001		<.001	
LSD		0.184		18.55		2.832	

Discussion

In order to get more insight in the function of *AT4G18395*, the phenotype of different overexpression and knock-out lines was studied for the following traits: rosette shape and chlorophyll content, germination, root architecture and ion content. First of all, the response of these different overexpression and knock-out lines will be discussed, to see which lines give a reliable and stable phenotype for the different traits. Thereafter, the deviating phenotype will be discussed per trait and related to possible functions of *AT4G18395*.

AT4G18395 overexpression lines show deviating phenotypes

When comparing lines overexpressing *AT4G18395* in different backgrounds (CS and Col-0) to their respective wild type, these results first of all confirm successful 35S promotor insertion, since plants overexpressing *AT4G18935* show a clearly distinct phenotype. The difference in phenotype between overexpression lines and wild type was strong in some overexpression lines and was hardly seen in other lines, which can be due to the position of the 35S promotor insert. Lines 11, 18 and 21 seem to have a less strongly deviating phenotype, in terms of solidity and water content (Table A1 appendix). The phenotype of lines 17,19,20 and 23 is most deviating from Col-0 wild type in terms of solidity, rosette area and water content. For the CS background, line 13 and 16 show a consistent overexpression phenotype for rosette area, solidity, petiole length, blade width and leaf water content, germination time and maximum germination percentage (for control and heat stress conditions), and sulphate and nitrate content. This could mean that in these six *AT4G18395* overexpression lines, expression is higher, maybe due to the position of the insert. It would be interesting to check *AT4G18395* gene expression for the overexpression lines to confirm this hypothesis. If this is the case, it would be wise to use these lines in further experiments rather than growing all lines, to reduce the number of lines.

Both knock-out lines (T28 and T108) often do not show a difference in phenotype from CS wild type. T-DNA T108 showed only once a deviating phenotype from WT CS, namely an increase in solidity for one time point and one repetition. T-DNA T28 deviates from WT CS in solidity for one time point and repetition, rosette weight after heat stress treatment applied to one-week-old plants and phytic acid content in the leaves. The PCR amplification of the gene shows the whole gene was amplified for WT CS, WT T28 and WT 108, while only the fragment from the T-DNA insert until the end was amplified for T-DNA knock-out lines T28 (Figure 9) and T108 (data not shown). This indicates that the T-DNA insertion is present in both knock-out mutants of *AT4G18395*. In addition, earlier expression studies confirm these are indeed knock-out lines for *AT4G18395*, since T-DNA insertion resulted in no expression of the gene (in case of T28) or partial gene expression (in case of T108) (unpublished). The reason no deviating phenotype is seen for *AT4G18395* knock-out lines for traits as rosette shape, germination and root architecture could be that in wild type plants the gene is only highly expressed in pollen. So gene knock-out would not matter for these traits that are not related to pollen. There was another group of overexpression lines, in a background of the T28 T-DNA knock-out line. This group was expected to show a phenotype similar to overexpression lines, since there is a 35S overexpression insert in the whole plant. Surprisingly, overexpression of *AT4G18395* in the T28 knock-out background did not lead to significant changes in solidity or changes in water content, unlike overexpression lines in the CS and Col background. Thus the knock-out background in these lines led to a phenotype similar to wild type plants, overruling the overexpression.

The wild type background line of T28 knock-out differs from the CS wild type in rosette area and perimeter, in root length and number of lateral roots and in germination time. These lines are expected be similar though, since there is no *AT4G18395* knock-out mutation in this line. If the seeds are assumed to be healthy and the genotype is as expected, it could be that by coincidence, some seedlings of this line grew worse than normal and gave an incorrect representation of line WT T28. However, that is unlikely, since the line was sown for multiple assay with similar results. Alternatively, the seeds might not be as healthy and vigorous as they should be. In the germination assay, WT T28 does show a delay in time to germinate compared to WT CS, which could support this hypothesis. Though delayed germination would also lead to delayed rosette growth and rosette area and perimeter were larger for WT T28. Thus maybe another mutation is present in this genotype, which results in these unexpected results.

Phenotypic changes in relation to gene function
AT4G18395 *overexpression lines deviate in leaf shape and heat tolerance during early growth stage*
Larger and wider rosette leaves and a lighter green colour are two prominent differences between *AT4G18395* overexpression lines and wild type plants. These and more rosette related changes were quantified.

A larger rosette area was found for more than half of *AT4G18395* overexpression lines in Col-0 background, but only few *AT4G18395* overexpression lines in CS background had this larger rosette area. Few lines overexpressing *AT4G18395* showed a difference in perimeter compared to their corresponding wild types, thus it looks like perimeter is not changed upon *AT4G18395* overexpression. Certain is the change in rosette compactness, measured by solidity when *AT4G18395* is overexpressed, because this is significant in both backgrounds, and accurately translate visual observation into a quantifiable trait. The reason the trait solidity is more consistent than rosette area is that it is a calculated ratio (Figure 3), which makes it more stable during rosette development.

The change in rosette shape can be defined in more detail by the differences in detached leaf shape. *AT4G18395 o*verexpression lines have a significantly wider leaf blade and a smaller petiole compared to the wild type plant. Leaves act primarily as photosynthetic organ and a change in leaf shape can be initiated to adapt photosynthesis. Larger leaves capture more sunlight, petioles can grow longer to escape shade or leaves can curl up to reduce the leaf area, to adjust the amount of sunlight received by leaves. In general, change in the shape of leaves is a response of natural selection on function, because photosynthesis is vital to plant growth and survival (Nicotra et al., 2011). Small leaves are often associated with stress conditions(Nicotra et al., 2011). Thus photosynthesis is an important factor to take into account. When chlorophyll pigments are in the light, photosystem II has a certain fluorescence emission that relates to the efficiency of the photosynthetic reaction and therefor, chlorophyll fluorescence is often used as measure of photosynthesis (Baker, 2008). Leaf colour is determined by chlorophylls, and leaves with higher chlorophyll content show a deeper green colour (Inada, 1963). Thus, leaf colour can be estimated by chlorophyll content (Madeira et al., 2007). By eye, *AT4G18395* overexpression lines seem to be brighter green compared to wild type plants, but chlorophyll content did not differ significantly. This could be caused by the large standard deviation between measurements (especially in WT CS) and the low number of replicates. Furthermore, the leaf material was stored for some weeks before extracting and measuring chlorophyll, which could have led to degradation. Though, the results hint there might be actual difference in chlorophyll content. When comparing the *AT4G18395* overexpression lines in CS background with

lines where the *AT4G18395* overexpression effect is compensated by T-DNA knock-out, a lower chlorophyll content for Oe T28 - CS was found at least for chlorophyll A. I would recommend to repeat this experiment with more repetition to examine the difference in chlorophyll content. If indeed chlorophyll content is reduced in *AT4G18395* overexpression lines, this indicates photosynthesis is reduced. Thus another experiment to measure chlorophyll content might give more clarity.

The effect of *AT4G18395* overexpression on heat stress was studied for rosette growth, by means of rosette area, perimeter and solidity. In earlier experiments it was found that heat stress during reproduction caused reduced silique sizes in the *AT4G18395* knock-out line. Heat stressed *AT4G18395* overexpression lines show a smaller rosette area compared to plants grown under control conditions for all time points. This can be caused by delayed growth due to heat stress, which could indicate these overexpression lines are more susceptible to heat stress than wild type plants, for which the reduced growth was only significant for the last time point. Thus reduced growth was seen for all time points and rosette weight is also reduced in heat stressed overexpression lines. Furthermore, WT CS heat stressed plants also had reduced rosette weight compared to plants grown under control conditions. This indicates all these lines were not yet able to recover from the heat stress three weeks after stress was applied. Perimeter and solidity did not show a strong or consistent heat stress phenotype for any of the tested lines. Normally, reproduction and emergence are seen as sensitive stages for heat (Hasanuzzaman et al., 2013, Wahid et al., 2007), and not rosette growth. But since more than half *AT4G18395* overexpression showed a significantly lower rosette area and this phenotype lasted until harvest, three weeks after stress was applied, maybe these *AT4G18395* overexpression lines are rather heat sensitive, also in this developmental stage. Though is seemed that also wild type plants still showed reduced growth three weeks after plants were stressed.

Higher water content in AT4G18395 *overexpression lines might be explained by enhanced uptake or water use efficiency*

A pilot experiment was set up in order to study the possible role of *AT4G18395* in root architecture. When water content was calculated, it appeared that leaf water content was higher for plants overexpressing *AT4G18395*. Though, the root system is not likely to cause this higher water content, since primary root length is smaller for *AT4G18395* overexpression lines in the pilot experiment. However, in one of the *AT4G18395* overexpression lines, the number and length of lateral roots is larger than in CS wild type plants. From this pilot experiment it is not possible to say whether the overexpression of *AT4G18395* causes this increase in lateral roots or whether it is coincidence. A hypothesis could be that *AT4G18395* overexpression lines invest more resources in lateral roots. It would be interesting to perform a larger experiment, with more *AT4G18395* overexpression lines and more replicates to check whether the phenotype of this one line represents the effect of *AT4G18395* overexpression or not. In relation to water uptake, research to the amount of root hairs in overexpression lines would be interesting.

In literature, a possible crosstalk between the hormonal regulation of root and shoot architecture has been described (Leyser, 2009). Hormone activity in roots influences branching of shoots and changes in leaf photoreceptors can change gene expression related to auxin in roots (Leyser, 2009). This makes it interesting to look for similarities between root data and shoot branching data. In *AT4G18395* mutants (T28 knock-out line and Oe T28 - CS) the number of branches does not differ, but branches are smaller for *AT4G18395* overexpression lines compared to CS wild type plants (unpublished). Furthermore, the total length from base to first silique is smaller for

overexpression lines compared to wild type plants (unpublished). If there would be crosstalk between root and shoot branching and these branching results correlate with root branching, the hypothesis of more and longer lateral roots would be unlikely. This crosstalk is also found in a research in peach trees, namely compact trees with more lateral shoot branches also had more and longer lateral roots (Tworkoski and Scorza, 2001).

Another possible explanation for the higher leaf water content could be that the water use efficiency is higher in *AT4G18395* overexpression lines. Interestingly, overexpression lines are more drought tolerant (unpublished), which correlates with the effect of a more efficient water use. Higher relative water content is often correlated with species that are more drought tolerant (Jarvis and Jarvis, 1963). A high concentration of osmolytes helps the plant to maintain a good water balance (Yordanov et al., 2000). And both drought stress and heat stress involves osmolyte production (Yordanov et al., 2000; Wahid et al., 2007). Maybe *AT4G18395* overexpression mutants have an enhanced osmolyte production and consequently higher leaf water content and drought tolerance. However, *AT4G18395* overexpression mutants seem to be less tolerant to heat instead of more tolerant, which an enhanced osmolyte production would suggest.

AT4G18395 *overexpression lines show delayed germination under control and heat stress conditions*

The influence of *AT4G18395* overexpression on germination was studied for seeds grown under control conditions and heat stress conditions. *AT4G18395* overexpression lines show a delayed germination time under control conditions compared to wild type plants of around 4 hours before 50% of the seeds germinated. Upon heat stress, this delayed germination time increased to around 17 hours and maximum germination percentage is lower for *AT4G18395* overexpression lines compared to CS wild type plants. Thus in terms of germination, it seems that plants overexpressing *AT4G18395* perform worse than wild type plants under normal conditions and elevated temperatures. When seeds were sown after heat stress was applied, this difference between performance of *AT4G18395* overexpression lines and CS wild type plants was hardly seen, which might indicate a relatively fast recovery of rosette growth for *AT4G18395* overexpression lines. A pollen germination assay for *AT4G18395* overexpression mutants showed contrasting results. Pollen of plants overexpressing *AT4G18395* have a higher germination percentage than wild type plants, both under control conditions and under heat stress (unpublished). Even though it is a matter of germination in both cases, the physiological processes and genes involved could differ, which seems to be the case in this situation.

Higher sulphate and nitrate content in AT4G18395 *overexpression lines*

Ion content of seven different anions was measured with HPLC analysis. There were three anions for which the ion content differed for at least four *AT4G18395* overexpression lines from CS wild type plants. Four of the five tested *AT4G18395* overexpression lines showed a lower phytic acid content and a higher sulphate and nitrate content.

Phytic acid, however, is mostly found in seed tissue as phosphorus storage (Urbano et al., 2000). Thus, since phytic acid is mostly functional in seeds and not in leaves and concentrations are low in leaves, the difference between these lines is biologically less meaningful. Nitrate and sulphate are both mineral anions, taken up by the roots and distributed via the xylem to other plant organs. Nitrate and sulphate are the sources of the macronutrients nitrogen and sulfur for the plant (Crawford, 1995; Leustek and Saito, 1999).

The uptake of nitrate from the soil is regulated by intern and environmental factors and involves transporters from two different gene families. Genes encoding these transporters are either constitutively expressed or nitrate-inducible (Crawford and Glass, 1998). Nitrate is then further distributed via different pathways. The cellular nitrate influx is regulated by controlled uptake and release of nitrate to the apoplasm. Nitrate can also be converted into nitrite by nitrate reductase and stored in plasmids. Lastly, nitrate is also stored for future use. In these three distribution pathways, nitrate is also acting as a regulating signal (Geelen et al., 2000). Thus a higher nitrate concentration probably means that either more nitrate is taken up by the transporters in root hairs, or that less nitrate is converted into nitrite.

Sulphate is taken up in the roots by active transport and thereafter distributed further to other plant organs. The major function of sulphate is providing sulfur for two amino acids and multiple plant metabolites, of which cysteine is the most important (Leustek and Saito, 1999). Like nitrate, the higher sulphate concentration could possibly be caused by an enhanced sulphate uptake or lower assimilation of sulfur-containing amino acids or metabolites from the available sulphate. Interestingly, the protein AT4G18395 contains four cysteines, which makes lower assimilation the least likely hypothesis.

Conclusion

The aim of this thesis is to characterize phenotypic differences in mutant lines of *AT4G18395,* in order to get an idea of the cellular function of this gene. In wild type Arabidopsis, the gene is only expressed in pollen. When the gene is overexpressed in the whole plant, the phenotype changes notably, which is quantified in this thesis research. This shows that, although normally *AT4G18395* is expressed just in pollen, the protein can function and interact in other tissue when the gene is present with 35S promotor.

In lines overexpressing *AT4G18395* the rosette shape deviates from wild type, measured as solidity, caused by wider leaves with a smaller petiole. Furthermore, *AT4G18395* overexpression mutants might have a lower chlorophyll content, but a more extensive experiment is needed to confirm this hypothesis. Leaf water content is higher for *AT4G18395* overexpression lines compared to wild type plants, which is probably caused by a more efficient water use, since *AT4G18395* overexpression lines are also more drought tolerant. Primary root length seems to be shorter for *AT4G18395* overexpression lines, but an elaborate root experiment is needed to confirm or reject the results found in this pilot experiment. Seeds with *AT4G18395* overexpression take more time to germinate. Under heat stress the difference in germination time for *AT4G18395* overexpression lines is even larger and also less seeds germinate. The delay in germination time however is hardly seen in further plant development. Unlike heat stress applied during the rosette growth stage, for which *AT4G18395* overexpression lines clearly have a delayed development, from the moment of heat stress until harvest (three weeks after stress). Thus *AT4G18395* overexpression lines seem to be more sensitive to heat stress than wild type plants. This coincides with the heat sensitive phenotype found in the knock-out line during reproduction, namely a reduction in silique size. Lastly, lines overexpressing *AT4G18395* seem to have higher nitrate and sulphate contents.

The next challenge would be to find the common factor between all these changes to deduce the function *AT4G18395* from these phenotypic changes. The fact that the overexpression of *AT4G18395* in other tissue led to significant changes in phenotype points to a more regulatory function, in for example signaling pathways. Apparently, the protein is able to interact and influence different processes in the plant in leaf tissue, seed tissue and maybe root tissue, when transcribed. It would be very interesting to check for changes in hormone levels between *AT4G18395* overexpression and control plants. Moreover, an experiment is being done to check the interaction of AT4G18395 with other proteins. This is still on-going, but there is a possible interaction with a peptide transporter. Research to this and possibly other proteins can give another clue of what kind of protein would fit with these outcomes and thus what kind of protein AT4G18395 is. From this thesis it seems that *AT4G18395* plays a role during heat stress, and when transcribed in tissue other than pollen, possibly influencing the plants water use efficiency, chlorophyll content and leaf shape, germination and nitrate and sulphate content. This shows that AT4G18395 has a very diverse impact on different tissues and processes.

References

1 Arabidopsis eFP Browser: http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Data from Expression Map of Arabidopsis Development: Schmid et al., 2005 Nat.Gen. 37:501).

2 Bac-Molenaar, J.A. Fradin, E.F. Becker, F.F.M. Rienstra, J.A. Van der Schoot, J. Vreugdenhil, D. and Keurentjes, J.J.B. (2015) *Genome-Wide Association Mapping of Fertility Reduction upon Heat Stress Reveals Developmental Stage-Specific QTLs in Arabidopsis thaliana.* The Plant Cell **27**: 1857-1874

3 Baker, N.R. (2008) *Chlorophyll Fluorescence: A Probe of Photosynthesis In Vivo.* Annual Review of Plant Biology **59**: 89-113

4 Bita, C.E. and Gerats, T. (2013) *Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops.* Frontiers in Plant Science **4**:1-18

5 Bita, C.E. Zenoni, S. Vriezen, W.H. Mariani, C. Pezzotti, M. Gerats, T. (2011) *Temperature stress differentially modulates transcription in meiotic anthers of heat-tolerant and heat-sensitive tomato plants.* BMC Genomics **12**: 384

6 Cheung, W. Hubert, N. and Landry B.S. (1993) *A Simple and Rapid DNA Microextraction Method for Plant, Animal, and Insect SuiTable for RAPD and Other PCR Analyses.* Genome Research **3**:69-70

7 Crawford, N.M. (1995) *Nitrate: Nutrient and Signal for Plant Growth.* The Plant Cell **7**:859-868

8 Crawford, N.M. and Glass, A.D.M. (1998) *Molecular and physiological aspects of nitrate uptake in plants.* Trends in Plant Science **3**:389-395

9 Fahey, R.C. Hunt, J.S. and Windham, G.C. (1977) *On the Cysteine and Cystine Content of Proteins: Differences between Intercellular and Extracellular Proteins*. Journal of Molecular Evolution **10**:155-160

10 Firon, N. Shaked, R. Peet, M.M. Pharr, D.M. Zamski, E. Rosenfeld, K. Althan, L. Pressman, E. (2006) *Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions.* Scientia Horticulturae **109**:212-217

11 Frank, G. Pressman, E. Ophir, R. Althan, L. Shaked, R. Freedman, M. Shen, S. Firon, N. (2009) *Transcriptional profiling of maturing tomato (*Solanum lycopersicum *L.) microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones, and sugars in the heat stress response.* Journal of Experimental Botany **60**:3891–3908

12 Geelen, D. Lurin, C. Bouchez, D. Frachisse, J. Lelièvre, F. Courtial, B. Barbier-Brygoo, H and Maurel, C. (2000) *Disruption of putative anion channel gene AtCLC-a in Arabidopsis suggests a role in the regulation of nitrate content.* The Plant Journal **21**: 259-267

13 Gibon, Y. (nd) *AG Stitt Basic Analysis Platform*

14 Hasanuzzaman, M. Nahar, K. Alam, M.M. Roychowdhury, R. and Fujita, M. (2013) *Physiological, Biochemical and Molecular Mechanisms of Heat Stress Tolerance in Plants.* International Journal of Molecular Sciences **14**: 9643-9684

15 Inada, K. (1963) *Studies on a Method for Determining the Deepness of Green and Color Chlorophyll Content of Intact Crop Leaves and Its Practical Applications. 1. Principle for estimating the deepness of green color and chlorophyll content of whole leaves.* Japanese Journal of Crop Sciences **32**: 157-162

16 Intergovernmental Panel on Climate Change (IPCC) (2007) *Climate change 2007–The physical science basis.* In: Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press; Cambridge, UK

17 Jarvis, P.G. and Jarvis, M.S. (1963) *The Water Relations of Tree Seedlings. IV. Some Aspects of the Tissue Water Relations and Drought Resistance* Physiologia Plantarum **16**: 501-516

18 Joossen, R.V.L. Kodde, J. Willems, L.A.J. Ligterink, W. Van der Plans, L.H.W. and Hilhorst, H.W.M. (2010) *Germinatior: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination*. The Plant Journal **62**: 148-159

19 Kim, S.Y. Hong, C.B., Lee, I (2001) *Heat shock stress causes stage-specific male sterility in* Arabidopsis thaliana. Journal of Plant Research **114**: 301-307

20 Larkindale, J. Hall, J.D., Knight, M.R. and Vierling, E. (2005) *Heat Stress Phenotypes of Arabidopsis Mutants Implicate Multiple Signaling Pathway in Acquisition of Thermotolerance*. Plant Physiology **138**: 882-897

21 Leustek, T. and Saito, K. (1999) *Sulfate Transport and Assimilation in Plants.* Plant Physiology **120**:637-643

22 Leyser, O. (2009) *The control of shoot branching: an example of plant information processing.* Plant, Cell and Environment **32:** 694-703

23 Madeira, A.C., Ferreira, A. De Varennes, A. and Vieira, M.I. (2007) *SPAD Meter Versus Tristimulus Colorimeter to Estimate Chlorophyll Content and Leaf Color in Sweet Pepper.* Communications in Soil Science and Plant Analysis **34**: 2461-2470

24 Maloof, J.N. Nozue, K. Mumbach, M.R. and Palmer, C.M. (2013) *LeafJ: an ImageJ plugin for semi-automated leaf shape measurement.* Journal of Visualized Experiments **71**: 1-6 (e50028)

25 Mittler, R. Finka, A. and Goloubinoff, P. (2012) *How do plants feel the heat?* Trends in Biochemical Sciences **37**: 118-125

26 Nicotra, A.B. Leight, A. Boyce, C.K. Jones, C.S. Niklas, K.J. Royer, D.L. and Tsukaya, H. (2011) *The evolution and functional significance of leaf shape in the angliosperms.* Functional Plant Biology **38**: 535-552

27 Pound, M.P. French, A.P. Adkinson, J.A. Wells, D.A. Bennett, M.J. and Pridmore, T. (2013) *RootNav: Navigating Images of Complex Root Architectures.* Plant Physiology **162**: 1802-1814

28 Song, J. Nada, K. and Tachibana, S. (2002) *Suppression of S-adenosylmethionine Decarboxylase Activity is a Major Cause for High-Temperature Inhibition of Pollen Germination and Tube Growth in Tomato (*Lycopersicon esculentum *Mill.).* Plant and Cell Physiology **43**:619-627

29 Tworkoski, T. and Scorza, R. (2001) *Root and Shoot Characteristics of Peach Trees with Different Growth Habits.* Journal of the American Society for Horticulture Science **126**: 785-790

30 Urbano, G. López-Jurado, M. Aranda, P., Vidal-Valverde, C. Tenorio, E. and Porres, J. (2000) *The role of phytic acid in legumes: antinutrient or beneficial function?* Journal of Physiology and Biochemistry **56**: 283-294

31 Wahid, A. Gelani, S. Ashraf, M. and Foolad, M.R. (2007) *Heat tolerance in plants: An overview*. Environmental and Experimental Botany **61**: 199-223

32 Wang, J.Z. Cui, L.J. Wang, Y. Li, J.L. (2009) *Growth, lipid peroxidation and photosynthesis in two tall fescue cultivars differing in heat tolerance*. Biologia Plantarum **53**: 247–242.

33 Warren, C.R. (2008) *Rapid Measurement of Chlorophylls with a Microplate Reader*. Journal of Plant Nutrition **31**: 1321-1332

34 Wise, R.R. Olson, A.J. Schrader, S.M. Sharkey, T.D. (2004) *Electron transport is the functional limitation of photosynthesis in field-grown Pima cotton plants at high temperature.* Plant Cell Environment **27**: 717–724

35 Yordanov, I. Velikova, V. and Tsonev, T. (2000) *Plant responses to drought, acclimation, and stress tolerance*. Photosynthetica **38**: 171-186

Appendix

Table A1: ROSETTE MEASUREMENTS REPETITION ONE. Overview of rosette measurements for the first repetition and the different lines (wild types in blue, overexpression lines in green and T-DNA knock-out lines in orange). The measured traits are an average of five replicates. For all traits the number of days after sowing the measurement was taken is indicated below (13, 17, 20 and 24 days after sowing (DAS)). Overexpression lines and knock-out lines are compared to the wild type background in which mutants are made (Oe T28 - Col vs WT Col; Oe T28 - CS vs WT CS and all knock-out lines and WT's vs WT CS). All traits were significant different (SPSS Anova, α=0.05 with LSD) and p-values are given (p<0.05 are red).

Line	Time point (DAS)	Area (cm^2) 13	sign.	17	sign.	20	sign.	24	sign.	Perimeter (cm) 13	sign.	17	20	sign.	24	sign.	Solidity 13	17	sign.	20	sign.	24	sign.
WT Col	2	0.52		1.81		4.03		8.79		5.02		12.86	20.99		34.70		0.65	0.56		0.52		0.48	
Oe T28 Col (7x)	17	0.86	0.01	3.02	0.01	6.25	0.01	12.86	0.02	7.05	0.01	16.24	24.29	0.29	36.32	0.72	0.66	0.58	0.21	0.61	0.00	0.66	0.00
	18	0.51	0.97	1.88	0.88	3.86	0.84	7.98	0.64	5.24	0.79	13.33	22.07	0.73	35.58	0.85	0.64	0.51	0.05	0.50	0.40	0.47	0.72
	19	0.95	0.00	3.08	0.00	6.98	0.00	15.74	0.00	7.61	0.00	16.55	25.02	0.20	41.29	0.15	0.67	0.60	0.05	0.62	0.00	0.66	0.00
	20	1.01	0.00	3.35	0.00	7.39	0.00	17.06	0.00	7.77	0.00	17.02	26.36	0.09	38.69	0.38	0.66	0.60	0.03	0.62	0.00	0.67	0.00
	21	0.76	0.08	2.80	0.03	6.06	0.03	11.69	0.11	7.08	0.02	17.62	30.17	0.01	48.19	0.01	0.64	0.49	0.01	0.48	0.10	0.42	0.01
	22	0.82	0.02	2.84	0.02	6.06	0.02	13.36	0.01	6.58	0.06	14.86	22.06	0.73	31.18	0.44	0.69	0.62	0.01	0.67	0.00	0.68	0.00
	23	0.98	0.00	3.48	0.00	7.54	0.00	15.00	0.00	7.39	0.00	17.81	27.89	0.03	37.44	0.55	0.63	0.56	0.75	0.63	0.00	0.67	0.00
WT CS	1	0.64		2.43		5.24		11.09		6.08		16.33	27.34		44.46		0.62	0.51		0.49		0.45	
Oe T28 CS (6x)	11	0.92	0.03	3.14	0.10	6.93	0.05	14.93	0.03	7.71	0.05	15.74	26.68	0.83	40.11	0.34	0.65	0.57	0.00	0.63	0.00	0.63	0.00
	12	0.87	0.07	3.02	0.17	6.31	0.21	13.99	0.09	6.76	0.41	15.89	25.58	0.57	35.73	0.06	0.67	0.61	0.00	0.61	0.00	0.63	0.00
	13	0.99	0.01	3.22	0.07	6.73	0.08	14.31	0.06	7.21	0.17	15.35	23.93	0.27	36.20	0.07	0.67	0.61	0.00	0.63	0.00	0.61	0.00
	14	0.61	0.83	2.31	0.77	5.18	0.95	12.02	0.58	5.53	0.50	12.90	19.19	0.01	34.81	0.04	0.66	0.60	0.00	0.63	0.00	0.63	0.00
	15	0.67	0.80	2.38	0.90	5.19	0.95	11.82	0.67	5.82	0.75	13.65	20.78	0.04	29.80	0.00	0.68	0.59	0.00	0.63	0.00	0.67	0.00
	16	1.01	0.00	3.28	0.05	7.36	0.02	16.16	0.00	8.02	0.02	16.88	28.07	0.82	41.37	0.50	0.67	0.58	0.00	0.62	0.00	0.64	0.00
T-DNA T28	3	0.79	0.23	2.99	0.20	5.76	0.55	12.45	0.42	6.90	0.32	18.74	26.74	0.85	46.94	0.58	0.65	0.50	0.76	0.48	0.65	0.46	0.52
WT T28	4	0.51	0.32	1.67	0.08	3.62	0.06	7.82	0.06	5.11	0.24	12.37	20.47	0.03	34.10	0.02	0.67	0.57	0.01	0.54	0.04	0.49	0.06
T-DNA T108	24	0.55	0.52	2.07	0.43	4.01	0.18	8.67	0.18	5.77	0.72	14.37	22.38	0.13	40.25	0.38	0.62	0.53	0.27	0.46	0.14	0.44	0.54
WT T108	25	0.54	0.45	1.98	0.29	4.11	0.19	8.94	0.21	5.27	0.33	12.92	20.00	0.02	37.94	0.15	0.65	0.54	0.12	0.54	0.07	0.46	0.61
Oe T28 T28(6x)	5	0.76	0.34	2.67	0.57	5.48	0.78	11.81	0.67	6.81	0.38	17.35	28.01	0.83	48.58	0.36	0.64	0.51	0.93	0.50	0.67	0.45	0.93
	6	0.71	0.55	2.44	0.99	4.96	0.74	10.89	0.91	6.55	0.57	16.07	25.84	0.63	46.82	0.60	0.63	0.50	0.68	0.46	0.22	0.45	0.80
	7	0.68	0.74	2.43	0.99	5.05	0.82	10.47	0.72	6.49	0.62	16.04	25.52	0.56	41.78	0.55	0.66	0.51	0.80	0.52	0.32	0.45	0.88
	8	0.65	0.94	2.28	0.72	4.60	0.45	9.86	0.47	6.00	0.92	14.95	23.10	0.17	37.68	0.14	0.67	0.53	0.25	0.54	0.07	0.48	0.13
	9	0.84	0.11	2.98	0.20	6.20	0.27	12.87	0.30	7.37	0.12	18.33	27.46	0.97	48.03	0.43	0.65	0.51	0.88	0.52	0.30	0.48	0.17
	10	0.50	0.30	1.90	0.21	4.42	0.34	9.19	0.27	5.28	0.33	13.28	23.69	0.24	39.25	0.25	0.66	0.52	0.52	0.52	0.23	0.46	0.79
significance		<.001		<.001		<.001		<.001		0.001		0.051	0.024		<.001		0.66	<.001		<.001		<.001	

Table A2: ROSETTE MEASUREMENTS REPETITION TWO. Overview of rosette measurements for the second repetition and the different lines (wild types in blue, overexpression lines in green and T-DNA knock-out lines in orange). The measured traits are an average of ten replicates (five replicates for dry weight and DW/FW ratio). The traits solidity and fresh- and dry weight are in the table on the next page. For the traits area, perimeter and solidity, the number of days after sowing the measurement was taken is indicated below (13, 17, 20 and 24 days after sowing (DAS)). Overexpression lines and knock-out lines are compared to theCol-0 or CS wild type background in which mutants are made. All traits were significant different (SPSS Anova p<0.001, with LSD) and p-values are given (p<0.05 are red).

		Area (cm2)								Perimeter (cm)							
Time point (DAS)		13	sign.	17	sign.	20	sign.	24	sign.	13	sign.	17	sign.	20	sign.	24	sign.
WT Col	2	0.69		2.21		4.55		10.41		6.74		14.79		24.64		40.61	
Oe T28 Col (7x)	17	0.75	0.16	2.36	0.08	5.12	**0.02**	11.12	**0.00**	7.14	0.88	16.11	0.81	26.92	0.58	43.55	**0.00**
	18	0.75	0.45	2.33	0.17	4.97	0.14	10.73	0.17	6.62	0.97	15.27	0.19	25.77	0.92	40.64	0.11
	19	0.50	0.17	1.59	0.11	3.53	0.10	8.02	0.10	5.54	0.81	12.20	0.65	20.31	0.33	34.25	**0.00**
	20	0.79	**0.05**	2.54	**0.01**	5.18	**0.00**	11.67	**0.00**	6.88	0.70	16.84	0.95	26.01	0.83	44.07	0.05
	21	0.57	0.57	1.91	0.25	4.25	0.16	9.56	0.15	5.89	0.94	14.38	0.20	22.75	0.50	40.32	0.07
	22	0.91	**0.00**	2.88	**0.01**	5.73	**0.00**	12.75	**0.01**	7.88	**0.00**	18.18	**0.00**	28.87	**0.00**	45.34	**0.00**
	23	0.77	**0.03**	2.36	**0.00**	4.69	**0.00**	10.70	**0.00**	6.81	0.80	15.92	0.43	23.46	0.69	38.25	**0.01**
WT CS	1	0.69		2.19		4.90		11.31		6.39		15.04		25.86		40.39	
Oe T28 CS (6x)	11	0.66	0.24	2.06	0.42	4.54	0.14	10.49	0.13	6.24	0.95	14.61	0.46	22.83	0.25	38.92	**0.03**
	12	0.78	0.99	2.40	0.63	5.26	0.10	12.07	**0.02**	6.77	0.27	13.88	0.46	22.56	0.53	34.88	0.10
	13	0.69	0.96	2.33	0.93	5.34	0.67	12.96	0.53	6.16	0.17	13.87	0.17	23.50	**0.03**	36.23	**0.00**
	14	0.69	0.22	2.19	0.25	4.76	0.51	11.09	0.89	6.02	0.06	13.07	0.13	20.62	**0.01**	31.21	**0.00**
	15	0.60	0.24	1.93	0.38	4.23	0.16	10.26	0.14	5.75	0.95	12.89	0.78	19.86	0.16	31.19	**0.00**
	16	0.78	0.55	2.42	0.31	5.23	**0.04**	12.02	**0.01**	6.77	0.88	14.45	0.86	22.11	0.25	30.70	0.15
T-DNA T28	3	0.73	0.44	2.46	0.61	5.55	0.39	13.15	0.77	6.66	0.82	15.01	0.70	22.57	0.53	36.73	0.99
WT T28	4	0.86	**0.02**	2.78	**0.01**	6.25	**0.04**	14.45	**0.03**	7.22	**0.02**	15.81	**0.04**	25.93	**0.02**	35.42	**0.02**
T-DNA T108	24	0.81	0.67	2.69	0.65	5.84	0.60	12.59	0.70	7.17	0.81	17.73	0.58	27.09	0.59	47.83	0.74
WT T108	25	0.86	0.94	2.74	1.00	5.92	0.88	12.88	0.76	7.02	0.88	15.55	0.74	25.17	0.77	34.50	0.86
Oe T28 T28(6x)	5	0.90	0.18	2.95	0.17	6.76	0.19	15.41	0.24	7.34	0.78	16.19	0.10	27.31	0.45	38.34	0.20
	6	0.79	0.12	2.63	0.20	5.80	0.54	12.68	0.43	7.18	0.11	17.69	0.74	28.13	0.30	48.37	0.91
	7	0.51	**0.00**	1.66	**0.01**	3.65	**0.02**	8.17	**0.03**	4.81	**0.03**	10.19	**0.01**	16.75	**0.02**	26.62	0.08
	8	0.92	0.27	3.04	0.54	6.93	0.78	15.71	0.79	7.28	0.89	17.08	0.38	27.63	0.52	36.75	0.39
	9	0.72	0.97	2.32	0.93	4.80	0.47	10.82	0.41	6.87	0.50	15.48	0.84	25.61	0.50	41.51	0.93
	10	0.68	0.69	2.21	0.53	4.62	0.99	10.74	0.94	6.66	0.35	15.21	0.88	24.11	0.32	41.10	0.53

Table A2 continuation

		Solidity								FW		DW		DW/FW	
Time point (DAS)	13	13	sign.	17	sign.	20	sign.	24	sign.		sign.		sign.		sign.
WT Col	2	0.63		0.54		0.52		0.52		0.61		0.07		0.10	
Oe T28 Col (7x)	17	0.63	0.10	0.51	**0.00**	0.47	**0.00**	0.47	**0.00**	0.71	**0.03**	0.07	0.17	0.10	**0.00**
	18	0.66	0.40	0.55	0.73	0.53	**0.02**	0.50	0.27	0.63	0.19	0.07	0.07	0.11	0.17
	19	0.66	0.05	0.56	**0.00**	0.52	0.00	0.52	0.00	0.50	0.16	0.05	0.80	0.10	**0.02**
	20	0.65	0.07	0.52	**0.00**	0.50	**0.00**	0.49	**0.00**	0.74	**0.00**	0.07	**0.03**	0.10	**0.00**
	21	0.65	0.52	0.54	0.30	0.51	0.80	0.49	0.77	0.62	0.05	0.07	**0.03**	0.10	0.70
	22	0.64	**0.00**	0.52	**0.00**	0.50	**0.00**	0.50	**0.00**	0.80	0.08	0.08	0.06	0.10	**0.01**
	23	0.65	**0.01**	0.54	0.00	0.53	**0.00**	0.53	**0.00**	0.71	**0.00**	0.06	**0.04**	0.10	**0.00**
WT CS	1	0.65		0.53		0.52		0.52		0.69		0.06		0.10	
Oe T28 CS (6x)	11	0.65	0.08	0.54	**0.00**	0.52	**0.00**	0.51	0.00	0.64	0.06	0.07	0.97	0.09	**0.00**
	12	0.66	0.05	0.59	**0.00**	0.61	**0.00**	0.65	**0.00**	0.76	**0.00**	0.07	0.22	0.08	**0.00**
	13	0.66	**0.01**	0.61	**0.00**	0.61	**0.00**	0.64	**0.00**	0.84	0.30	0.05	0.80	0.07	**0.00**
	14	0.67	**0.00**	0.60	**0.00**	0.62	**0.00**	0.66	**0.00**	0.69	0.54	0.06	**0.04**	0.08	**0.00**
	15	0.68	0.41	0.60	**0.00**	0.60	**0.00**	0.65	**0.00**	0.66	0.10	0.05	0.18	0.07	**0.00**
	16	0.64	0.16	0.59	**0.00**	0.61	**0.00**	0.68	**0.00**	0.74	**0.00**	0.05	0.97	0.08	**0.00**
T-DNA T28	3	0.65	0.05	0.60	0.72	0.62	0.27	0.64	**0.03**	0.89	0.76	0.07	0.93	0.07	0.76
WT T28	4	0.65	0.05	0.58	0.25	0.60	0.59	0.67	0.78	0.87	0.17	0.08	0.20	0.09	0.45
T-DNA T108	24	0.64	0.73	0.52	0.12	0.50	0.06	0.48	**0.00**	0.81	0.17	0.09	0.19	0.11	0.32
WT T108	25	0.65	0.81	0.58	0.36	0.60	0.50	0.68	0.06	0.82	0.50	0.07	1.00	0.09	0.11
Oe T28 T28(6x)	5	0.65	0.21	0.56	0.11	0.60	0.22	0.66	**0.02**	1.02	0.11	0.09	1.00	0.09	0.63
	6	0.63	0.15	0.50	0.63	0.47	0.55	0.47	**0.00**	0.86	0.84	0.09	0.87	0.10	0.12
	7	0.70	0.71	0.66	0.08	0.67	0.24	0.66	**0.04**	0.56	**0.01**	0.05	0.09	0.09	0.23
	8	0.66	0.28	0.57	1.00	0.62	0.18	0.69	0.40	1.02	0.21	0.09	0.53	0.08	0.11
	9	0.64	0.16	0.52	0.27	0.49	0.75	0.48	0.95	0.72	0.30	0.08	0.83	0.10	0.22
	10	0.63	0.21	0.53	0.70	0.51	0.99	0.50	0.23	0.66	0.71	0.07	0.93	0.10	**0.01**
Replicate		10		10		10		10		10		5		5	

Table A3: CHLOROPHYLL CONTENT. Average chlorophyll A and B content of two replicates in µg for CS wild type (blue), knock-out lines T28 and T108 (red) and five overexpression lines in CS (green) and T28 knock-out backgrounds (white). Calculations can be found in materials and methods. No significant differences were found between mutant lines and their respective wild types (student t.test, α=0.05)

		Chlorophyll A		Chlorophyll B	
		Average	t.test	Average	t.test
WT Col-0	2	0.53		0.53	
Oe T28 Col-0	17	0.43	0.16	0.30	0.33
	19	0.37	0.11	0.24	0.42
	21	0.52	0.73	0.63	0.26
	22	0.36	0.22	0.29	0.19
WT CS	1	0.43		0.43	
Oe T28 CS	12	0.38	0.56	0.47	0.92
	13	0.34	0.40	0.37	0.91
	14	0.39	0.72	0.56	0.84
	15	0.44	0.92	0.68	0.28
	16	0.44	0.93	0.69	0.60
T-DNA T28	3	0.48	0.60	0.54	0.49
WT T28	4	0.50	0.52	0.51	0.45
T-DNA T108	24	0.53	0.96	0.74	0.51
Oe T28 T28	5	0.52	0.46	0.49	0.36
	6	0.49	0.58	0.53	0.45
	8	0.46	0.74	0.60	0.54
	9	0.50	0.54	0.52	0.42
	10	0.47	0.66	0.57	0.55

Table A4: ROSETTE GROWTH AFTER HEAT STRESS DURING ROSETTE DEVELOPMENT. Difference in average rosette area, perimeter, solidity, dry and fresh weight and DW/FW ratio (of five replicates) between WT CS, WT T28 (blue), T-DNA T28 (red), and five Oe T28 – CS lines (green) grown under control conditions of 22 ^{0}C and plants grown after exposure to heat stressed 35 ^{0}C for one day, measured 13, 17, 20, 24 days after sowing and at harvest 28 days after sowing. Significances are calculated with student T-test, α=0.05 and P-values are shown. Significant differences are marked red.

Days after sowing		13			17			20			24			Weight (g)		
		Area (cm^2)												FW		
Line		control	heat	p-value	control	heat	p-value	control	heat	p-value	control	heat	p-value	control	heat	p-value
WT CS	1	0.62	0.51	0.17	1.90	1.60	0.23	3.93	3.24	0.12	8.99	7.22	**0.03**	0.48	0.37	**0.02**
Oe T28 CS (5x)	12	0.84	0.63	**0.03**	2.64	1.95	**0.03**	5.51	4.22	**0.03**	12.97	10.34	**0.03**	0.74	0.57	**0.04**
	13	0.73	0.59	0.16	2.22	1.76	0.14	4.50	3.69	0.18	10.26	8.88	0.24	0.59	0.51	0.29
	14	0.68	0.52	0.13	1.98	1.63	0.19	4.17	3.41	0.20	9.23	8.51	0.62	0.52	0.47	0.54
	15	0.77	0.62	**0.02**	2.30	1.82	**0.01**	4.71	3.73	**0.00**	10.74	8.86	**0.00**	0.61	0.50	**0.02**
	16	0.99	0.76	**0.01**	2.94	2.28	**0.01**	6.14	4.82	**0.00**	12.70	11.35	0.08	0.70	0.63	0.08
WT T28	4	0.42	0.39	0.38	1.24	1.16	0.42	2.63	2.46	0.22	5.79	5.84	0.87	0.31	0.31	0.76
T-DNA T28	3	0.66	0.59	0.41	1.99	1.97	0.94	4.15	3.63	0.26	9.38	7.99	0.06	0.53	0.41	**0.02**
		Perimeter (cm)												DW		
		control	heat	p-value	control	heat	p-value	control	heat	p-value	control	heat	p-value	control	heat	p-value
WT CS	1	6.13	5.80	0.46	13.8	12.0	0.23	23.5	20.4	0.08	40.9	35.1	**0.03**	0.045	0.035	**0.03**
Oe T28 CS (5x)	12	7.30	6.75	0.30	15.4	13.3	0.14	23.1	21.8	0.57	37.9	36.3	0.65	0.051	0.040	**0.04**
	13	6.76	6.40	0.59	13.6	11.5	0.14	21.1	18.7	0.26	33.8	32.5	0.68	0.041	0.036	0.33
	14	6.62	5.95	0.31	13.2	11.5	0.15	22.8	18.9	0.10	34.8	30.0	0.11	0.041	0.033	0.17
	15	7.06	6.68	0.11	13.5	11.8	0.07	22.6	19.3	**0.00**	36.5	31.9	0.10	0.043	0.035	**0.00**
	16	7.95	7.41	0.15	16.2	14.5	0.15	28.1	22.8	**0.00**	41.9	37.9	0.38	0.049	0.045	0.07
WT T28	4	5.23	5.20	0.88	10.5	9.6	0.10	19.2	18.4	0.18	31.7	30.8	0.68	0.029	0.029	1.00
T-DNA T28	3	6.38	6.28	0.86	14.1	13.7	0.79	23.7	22.8	0.70	41.7	38.9	0.28	0.048	0.039	**0.03**
		Solidity												DW/FW		
		control	heat	p-value	control	heat	p-value	control	heat	p-value	control	heat	p-value	control	heat	p-value
WT CS	1	0.61	0.58	0.12	0.54	0.56	0.25	0.45	0.47	0.11	0.45	0.45	0.74	0.09	0.09	0.59
Oe T28 CS (5x)	12	0.64	0.57	**0.01**	0.59	0.56	**0.04**	0.58	0.55	0.10	0.61	0.59	0.27	0.07	0.07	0.34
	13	0.62	0.59	0.12	0.60	0.60	0.93	0.55	0.57	0.29	0.61	0.59	0.26	0.07	0.07	0.50
	14	0.62	0.58	**0.01**	0.58	0.61	0.15	0.54	0.56	0.59	0.56	0.58	0.65	0.08	0.07	0.11
	15	0.59	0.58	0.37	0.61	0.61	0.89	0.58	0.58	0.88	0.62	0.58	0.06	0.07	0.07	0.27
	16	0.63	0.57	**0.00**	0.58	0.57	0.47	0.58	0.56	0.18	0.60	0.59	0.42	0.07	0.07	0.79
WT T28	4	0.60	0.58	0.14	0.57	0.57	0.74	0.47	0.48	0.30	0.46	0.46	0.76	0.09	0.09	0.49
T-DNA T28	3	0.64	0.58	**0.00**	0.53	0.56	0.08	0.46	0.48	0.43	0.45	0.45	0.88	0.09	0.10	**0.05**

35

Table A5:ROSETTE DEVELOPMENT OF HEAT-STRESSED SEEDS Difference in average rosette area, perimeter, solidity, dry and fresh weight and DW/FW ratio (of five replicates) between WT CS, T-DNA T28, WT T28 and five Oe T28 – CS lines grown under control conditions of 22 ^{0}C and seeds grown after exposure to heat stressed 35 ^{0}C for one day, measured 8 and 13 and at harvest 28 days after sowing. Significances are calculated with student T-test, α=0.05 and P-values are shown. Significant differences are marked red.

Line		Area (cm2)						Weight (g)		
		8			13			FW		
		control	heat	p-value	control	heat	p-value	control	heat	p-value
WT CS	1	0.072	0.075	0.81	0.51	0.56	0.57	0.34	0.46	**0.04**
Oe T28 CS (5x)	12	0.095	0.059	**0.03**	0.65	0.47	0.11	0.52	0.48	0.66
	13	0.090	0.068	0.17	0.68	0.57	0.19	0.48	0.39	0.28
	14	0.077	0.079	0.89	0.63	0.58	0.57	0.45	0.49	0.18
	15	0.091	0.061	0.07	0.66	0.54	0.24	0.50	0.42	0.08
	16	0.081	0.064	0.21	0.64	0.54	0.18	0.47	0.49	0.80
WT T28	4	0.058	0.061	0.82	0.41	0.46	0.96	0.28	0.32	0.39
T-DNA T28	3	0.079	0.078	0.19	0.58	0.58	0.21	0.38	0.34	0.52

Line		Perimeter (cm)						DW		
		8			13			DW		
		control	heat	p-value	control	heat	p-value	control	heat	p-value
WT CS	1	1.29	1.39	0.33	5.46	5.90	0.36	0.032	0.041	**0.02**
Oe T28 CS (5x)	12	1.58	1.22	0.05	6.04	5.05	0.19	0.037	0.032	0.40
	13	1.55	1.36	0.21	6.13	5.63	0.19	0.036	0.036	0.97
	14	1.42	1.45	0.87	5.74	5.48	0.60	0.033	0.035	0.56
	15	1.62	1.26	**0.05**	6.15	5.21	0.16	0.041	0.031	0.29
	16	1.49	1.32	0.14	5.76	5.59	0.71	0.033	0.041	0.22
WT T28	4	1.21	1.19	0.59	4.54	5.12	0.69	0.026	0.032	0.17
T-DNA T28	3	1.37	1.38	0.24	5.66	6.11	0.38	0.036	0.031	0.42

Line		Solidity						DW/FW		
		8			13			DW/FW		
		control	heat	p-value	control	heat	p-value	control	heat	p-value
WT CS	1	0.77	0.73	0.07	0.63	0.60	**0.00**	0.10	0.09	0.42
Oe T28 CS (5x)	12	0.75	0.72	0.19	0.64	0.63	0.56	0.07	0.07	0.28
	13	0.72	0.71	0.92	0.65	0.65	0.36	0.07	0.09	0.20
	14	0.75	0.74	0.62	0.66	0.64	0.26	0.07	0.07	0.53
	15	0.74	0.74	0.91	0.64	0.64	1.00	0.08	0.08	0.71
	16	0.74	0.72	0.49	0.67	0.63	0.10	0.07	0.08	0.14
WT T28	4	0.75	0.77	0.52	0.66	0.63	0.09	0.09	0.10	0.16
T-DNA T28	3	0.79	0.77	0.22	0.65	0.60	0.48	0.10	0.09	0.60

Table A6:LEAF ANION CONCENTRATIONS. Anion content in leaf material of WT CS, T-DNA T28, WT T28 and five Oe T28 – CS lines averaged for three and four weeks after sowing with three replicates for each date. The following seven anions were measured: phytate (IP6), citrate, isocitrate, nitrate, oxalate, phosphate and sulphate. Significances are calculated with Genstat's Anova, α=0.05 and LSD as post-hoc test. P values are shown for the interaction between line and date (not significant) and for the differences between lines, with corresponding LSD value. Significances are indicated with an asterisk (*).

Line		IP6		Citrate		Isocitrate		nitrate		Oxalate	Phosphate	Sulphate	
WT CS	1	0.914		21.74		0.48		116.20		1.76	23.02	14.35	
Oe T28 CS (5x)	12	0.755		19.33		0.38	*	144.25	*	1.41	22.38	17.55	*
	13	0.668	*	20.10		0.44		150.60	*	0.74	22.12	16.55	*
	14	0.661	*	17.56	*	0.34	*	130.20		0.88	19.63	13.83	
	15	0.566	*	19.25		0.39	*	151.95	*	0.86	21.87	17.76	*
	16	0.567	*	19.95		0.45		144.80	*	0.86	21.81	17.31	*
T-DNA T28	3	0.625	*	23.17		0.50		117.25		1.59	23.62	13.54	
WT T28	4	0.57	*	19.03	*	0.44		114.60		1.56	22.87	11.36	*
F line		0.007		0.007		0.005		<.001		0.134	0.476	<.001	
LSD		0.184		2.66		0.07839		18.55		0.893	3.519	2.832	
F interaction		0.551		0.865		0.806		0.174		0.978	0.424	0.729	