Master Thesis

Functional characterization of an unknown gene involved in heat stress tolerance

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ABSTRACT

As a consequence of global warming, global mean temperatures increase and can form a threat to food security because plants are often not able to cope with heat stress. Although the whole plant is often affected by heat stress, the reproductive phase in flowering plants is one of the most sensitive stages during plant growth. Because food is often the product of sexual reproduction in flowering plants, it is critical to understand underlying plant mechanisms during the reproductive plant phase when experiencing heat stress. During a GWAS study, a few interesting genes possibly involved in the heat stress response during flowering were found by measuring silique lengths after heat stress. In a subsequent study, the gene *AT4G18395* was selected. To investigate the function of this gene, wild type Colombia-0 and *AT4G18395* knock out and overexpression lines were used to quantify phenotypic differences under control and heat stress conditions, such as plant architecture and fertilization efficiency. Under control conditions, overexpression lines showed drastic changes in plant morphology. Compared to wild type lines, knock out lines were not phenotypically different, but overexpression lines had a smaller main stem length, secondary branch length and internode length. Furthermore, overexpression lines had a wider stem base diameter and a reduced fertility. After heat stress application, both knock-out and overexpression lines had reduced silique lengths, but this reduction was more severe in overexpression lines. This suggests that they were more susceptible to heat stress conditions than wild type plants. Overall, these results indicate a function of *AT4G18395* in fertility during heat stress, but the gene needs to be further investigated to understand its function.

Keywords: *Arabidopsis Thaliana*; *AT4G18395;* fertility; heat stress; plant architecture; plant reproduction; pollen; seed set; silique size

TABLE OF CONTENTS

INTRODUCTION

As a consequence of climate change, plants have to face more severe and more frequently occurring weather events. One of the likely consequences of climate change is an increasing global mean temperature and more frequent temperature extremes such as heat waves (Hirabayashi *et al.,* 2013). High temperatures are a major limiting factor for crop productivity, because many plants are not adapted to cope with extreme temperatures (Driedonks, Rieu & Vriezen, 2016). Consequently, heat stress can cause irreversible damage to plants, especially in combination with other stresses such as drought. However, the impact of heat stress depends on the duration, intensity and rate of the temperature change (Wahid *et al.,* 2007). Overall, cellular functioning is disturbed during high temperatures. For example, membrane fluidity is affected, as well as nucleic acids and protein structures. Furthermore, photosynthesis is inhibited due to stress on photosynthetic tissues (Taiz & Zeiger, 2010).

Although the whole plant is affected by heat stress, development of the reproductive tissues seems to be the most sensitive process. While heat stress during the vegetative stage can accelerate flowering, it leads to less developed seeds due to the lack of accumulated resources (Zinn, Tunc-Ozdemir & Harper, 2010). Heat stress during flower development causes floral asynchrony and can defect parental tissues. In addition, floral stigmas are receptive to pollen for a shorter period, resulting in a decrease of successful fertilization (Müller & Rieu, 2016). Because food is often the product of sexual reproduction in flowering plants, it is critical to understand underlying plant mechanisms during the reproductive plant phase when experiencing heat stress. This knowledge can contribute to breeding for stress tolerance in crop plants (Driedonks, Rieu & Vriezen, 2016).

To identify genes involved in heat stress tolerance, a genome-wide association mapping (GWAS) was performed by Bac-Molenaar *et al.* (2015) in a natural population of *Arabidopsis thaliana*. By measuring the length of the siliques, various developmental reproduction phases could be investigated and a few interesting genes possibly involved in heat stress response during flowering were found. In subsequent research (Fradin et al., unpublished) one of these genes, *AT4G18395*, with a yet unknown predicted function was selected. Interestingly, analysis of a T-DNA insertion mutant, where this gene is knocked out, suggests the mutant is more susceptible to heat stress because of a smaller silique size after heat stress compared to the wild type. Based on the expression profiling, the gene appears to be involved in a number of biological processes, including pollen development, pollen germination, flowering, petal differentiation and expansion stage, trichomes and plant embryo globular stage (Arabidopsis eFP browser).

A preliminary assay on the function of the gene *AT4G18395* using overexpression lines showed drastic changed in plant morphology. Overall, plants were more vigorous. To further characterize the function of *AT4G18395*, knock-out plants, overexpression lines and wild-type plants were used in phenotyping essays. The T-DNA lines were genotyped prior to this thesis. Because the gene is expressed in flowering parts of plants and due to the reduced silique size after heat stress, pollen viability, silique length and fertilization efficiency were assessed. Due to changed morphology in plants overexpressing the gene, plant properties concerning plant main stem length and diameter, length of the axillary branches and the number of basal branches were also assessed. Finally, because the gene is expressed in trichomes, trichome properties were recorded.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* wild-type Colombia-0 (CS60000), a T-DNA insertion mutant (T28) and the associated segregating wild-type line (WT-T28) and five independent transgenic lines overexpressing *At4G18395* in the background Columbia-0 CS60000 (oeT28-CS) (**Fout! Verwijzingsbron niet gevonden.**) were sown on wet filter paper and stratified at 4°C for two to four days. Stratified seeds were pre-germinated at room temperature for one day and subsequently transferred to rockwool blocks saturated with nutrient solution (Hyponex, 1mM N, 1.1 mM P, and 5.9 mM K) in a climate room (125 µmol m^{-2} s^{-1}) on a 22°C/18°C, 16-h day/night cycle. Plants were automatically watered with the Hyponex solution three times per week.

Table 1. Code and abbreviations used for wild-types, T-DNA insertion mutant and overexpression lines.

Code*	Abbreviation	Description
1	CS60000	Wild type Colombia-0
3	T28.2	T-DNA insertion mutant
4	T28.3	Wild type T-DNA insertion mutant
12	oeT28.13.1	Transgenic line constitutively overexpressing *At4G18395*
13	oeT28.11.2	Transgenic line constitutively overexpressing *At4G18395*
14	oeT28.9.2	Transgenic line constitutively overexpressing *At4G18395*
15	oeT28.7.1	Transgenic line constitutively overexpressing *At4G18395*
16	oeT28.6.2	Transgenic line constitutively overexpressing *At4G18395*

*All in the background CS60000

Heat stress treatment

To assess the effect of heat stress on pollen viability and germination, fertilization in the pistils and silique size, heat treatment was applied to half of the plants after all plants started to flower. One day prior to the heat stress, the first bud about to open on the day of the heat stress (flower 0) was marked with a red thread. The bud of control plants was tagged with a blue thread and the control plants were kept in the climate room under control conditions. Plants that received heat treatment were moved to a climate cabinet, where the temperature was raised from 18 to 35°C in 2 hours for the transition from night to day. After a 13,5 hour heat treatment at 35 degrees, the temperature was decreased to 18°C in a period of 2 hours as a transition from day to night. after which the next day the growing condition at 22°C resumed and the plants were moved back to the climate room.

Branching experiments

To assess the branching phenotypes, the genotypes 1, 3, 4 and 12 to 16 were sown as previously described and arranged in a completely randomized design. The flowering time was recorded for each plant and two weeks after flowering the following traits were measured on the main stem: (1) distance up to first silique, (2) average internode length, (3) basal main stem diameter; on the axillary branches (4) total number, (5) length; and finally total number of basal branches (6). At the end of the life cycle, the main stem length was measured. The experiment was repeated three times. During the first experiment, five replicates per

genotype were used and traits 1, 2 and 3 were assessed. During the second repetition, ten replicates per genotypes were used and all traits but (6) were measured, finally in the third repetition ten replicates per genotype were used and all traits were measured. During the third repetition, also axillary branch length was measured at the end of the life cycle.

Trichomes measurements

To assess the number and type of trichomes (2- 3- or 4-branched) on genotypes 1, 3, 4 and 12 to 16, five plants per genotype were sown as previously described and arranged in a completely randomized design. After two weeks, when the fifth or sixth rosette leaf appeared, the first two true leaves were studied for trichome number and type of branching. The leaves were either still attached to plant and directly studied under the binoculars, or harvested (repetition 1 and 2, respectively). In the case of harvested leaves, repetition 2, leaves were taped to microscope slides and scored under the binoculars after overnight drying.

Silique size

To assess the silique size, the genotypes 1, 3 and 16 were sown as previously described and arranged in rows organised by genotype. After all plants started flowering, heat stress was applied and after 25 days, silique six to twenty-one were harvested from heat stressed plants and control plants. Siliques were incubated in ethanol-acetic acid (3:1) overnight (Ojangu et al., 2012), washed in distilled water and organised by position on the plant by taping them to transparent tape on top of plastic sheets. The sheets were scanned by using an Epson Perfection V330 Photo scanner and analysed for silique size using ImageJ.

Pollen germination

In order to obtain a method that resulted in the highest percentage of pollen germination, stage 13 flowers, the stage during which the petals elongate and become visible between the sepals (Alvarez-Buylla *et al.,* 2010), were collected for pollen germination experiments. The collected pollen we germinated on pollen germination medium (PGM), which consisted out of sucrose (18%), Boric acid (0.01%), $CaCl_2$ (1mM), $Ca(NO_3)_2$ (1 mM), and $MgSO_4$ (1 mM) and an adjusted pH of 7.0. For solidified PGM, 0.5% Noble agar was added. Germination percentage was recorded for genotypes 1, 3, 4 and 12 to 16.

Flowers were harvested and collected in either Petri dishes containing wet filter paper, 24-well plates or in Eppendorfs containing liquid PGM. Pollen were collected by dipping the opened flower onto PGM or by means of centrifugation. During the dipping method, one to three flowers with or without petals were dipped directly into liquid or solid PGM. Centrifugation was executed as described by Boavida and McCormick (2007). In short, multiple flowers (ranging from 1 to 20) were collected in liquid PGM and briefly vortexed, after which the flower parts were removed and pollen grains were pelleted using the centrifuge for 30 seconds at 800g. Pollen were resuspended in 250µl fresh PGM and after germination, a 10µl aliquot was placed on a microscope slide and under the light microscope for observation. An adjusted method of Boavida and McCormick (2007) was also used, in which the petals were removed from the flowers before collecting them in liquid PGM. Furthermore, centrifugation was adjusted to 45 seconds, resuspension of the pellet was in 120µl fresh liquid PGM, and the 10µl aliquot was placed on a microscope slide containing solid PGM.

In all methods of pollen collection, pollen grains were incubated for four hours to overnight incubation in the light or dark. Incubation was performed at 22°C degrees or at 35°C in case of heat stress.

Furthermore, slides were kept under humid conditions in closed 100mm square Petri dishes containing wet paper.

Pollen viability and number of pollen

To test pollen viability, pollen grains were stained using either Alexander (Alexander, 1969) or Peterson staining (Peterson, Slovin & Chen, 2010) (**Fout! Verwijzingsbron niet gevonden.**). Two pollen viability experiments were conducted, with five and four replicates per genotype per condition (experiment 1 and 2, repectively). Plants were grown under control conditions and received heat treatment as described in paragraph 2. Starting from flower 6, three closed flowers were harvested per day for a period of five days and collected in 96-well plates to keep track of the flower number. On the day of the harvesting, one closed anther per flower was selected and placed on a microscope slide. A 10µl droplet of Alexander or Peterson staining solution was added, covered with a coverslip and slightly agitated to release the pollen from the anther. Coverslips were sealed with commercial nail polish and slides were incubated overnight at 50°C. After incubation, pollen were visualised under a light microscope and images were captured.

A pilot experiment was conducted to assess the number of pollen in one anther. One closed anther from a closed flower was taken from two different 43-days old plants per genotype. The anther was stained with Peterson's staining solution (**Fout! Verwijzingsbron niet gevonden.**), covered with a cover glass and broken to release the pollen. After a few hours of incubation, slides were visualised under the light microscope and pictures were taken to assess the number of pollen.

Table 2. Ingredients of Alexander and Peterson staining for pollen viability. Ingredients are added in the order indicated in the table.

Ingredient	Alexander	Peterson
95 % ethanol	10 mL	10 mL
Malachite green (1 % solution in 95 % ethanol)	1 mL	1 mL
Distilled water	52.5 mL	54,5 mL
Glycerol	25 mL	25 mL
Phenol	5 mL	-
Chloral hydrate	0.5 g	-
Acid fuchsin [1 % (w/v) solution in water]	5 mL	5 mL
Orange G [1 % (w/v) solution in water]	0.5 mL	0.5 mL
Glacial acetic acid	1 mL	4 mL

Aniline blue staining

To assess pollen tube growth and fertilization inside the pistils, pistils of flower at positions six to twenty-one were collected 24 hours after pollination and processed based on Ishiguro et al. (2001). In short, anthers, petals and sepals were removed from the flower. The dissected flower, mainly consisting of the pistil, was fixed for one hour in ethanol/acetic acid (3:1), softened overnight in 8M NaOH and washed with distilled water. Pistils were subsequently incubated for three hours in the dark in aniline blue solution consisting out of 0,1% aniline blue dissolved in 0.1 M K_2HPO_4-KOH buffer with a final pH of 11. Aniline blue stains callose, which is lacking in the surrounding style tissue. It therefore only stains the pollen tubes, which in turn exhibit fluorescence when illuminated with UV light (Dumas & Knox, 1983). The stained pistils were placed on a microscope slide in 50% glycerol and observed under the microscope using UV light and DAPI filter.

Statistics

The statistical analysis was performed using SPSS 22.0 for Windows. Analysis of Variance (ANOVA) was carried out in a completely randomized design.

RESULTS

Plant morphology is altered in *AT4G18395* overexpression lines

Different phenotype characteristics between wild type (WT) and overexpression (OE) lines were observed during plant growth under control conditions (Figure 1). Knock-out (KO) plants did not seem different than the WT. Overexpression lines had an overall broader plant appearance (Figure 1B) and seemed to have stiffer branches and leaves compared to wild type plants. Furthermore, leaves seemed to be more yellow (Figure 1C), 'shiny' and seemed to have a more distinct venation patterning (Figure 1D). Analyzation of the venation patterning was attempted using cleared six-week old first two true leaves, but a reliable method to score the pattern was not found before the end of this thesis.

Another phenotype characteristic was the growth angle of axillary branches. To quantify this, three representative WT plants (line 1) and OE plants (line 16) grown under control conditions were selected for a pilot experiment. The growth angle of the axillary branches was measured and was of a higher degree in overexpression lines, resulting in a more horizontal growth of the axillary branches (Figure 1E, F).

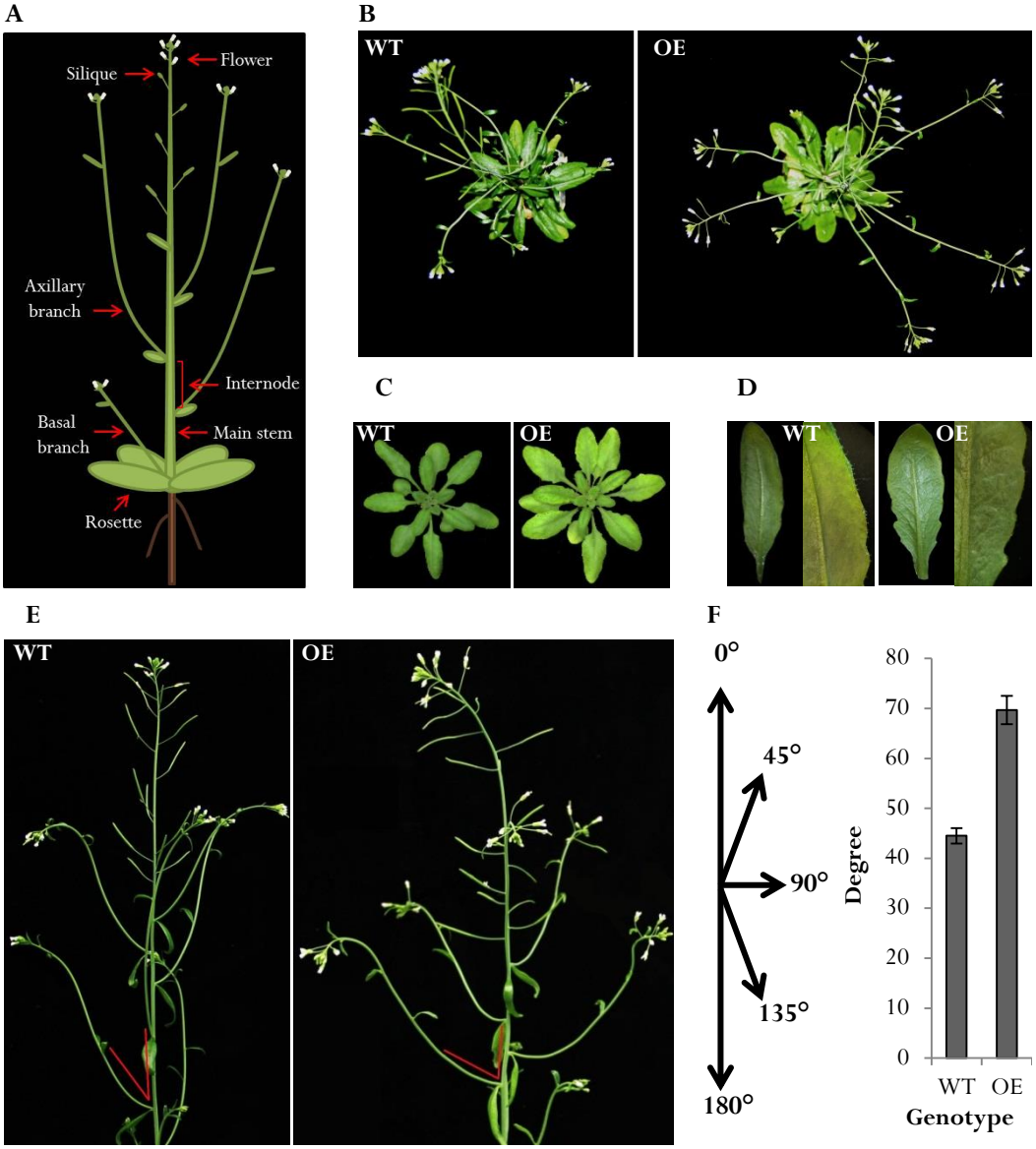

Figure 1. *AT4G18395* overexpression results in altered plant morphology. (A) Illustration of an adult Arabidopsis plant architecture, showing tissues such as rosette leaves and siliques. **(B)** Top view from 39-day-old WT

and OE plants. **(C)** Rosette phenotypes of 32-day-old WT and OE lines. **(D)** Leaf phenotypes of 45-day-old WT and OE plants. Close-up pictures were obtained using binoculars. **(E)** Side view of 39-day-old WT and OE plants. Red line indicates the growth angle of axillary branches. **(F)** Diagram illustrates possible axillary branch growth angles. Graph is the quantification of the growth angle of all axillary branches present on the main stem. Bars represent average growth angle of axillary branches (n=11) of three WT and three OE plants. Error bars represent SEM**.** WT: wild type Columbia-0 (line 1); OE: *AT4G18395* overexpression (line 16).

Morphological changes during control conditions

To determine the effect of the *AT4G18395* knock-out and overexpression in Arabidopsis more precisely, main stem diameter, distance between internodes, length of axillary branches, and the number of axillary and basal branches (Figure 1A) was recorded two weeks after flowering of each individual plant. The time until the plants started flowering varied over experiments and was often not significantly different from WT (1), although KO plants flowered significantly faster during two out of three experiments (Table 3).

Table 3. Average flowering time differs between repetitions. Columns represent average flowering time of wild type Columbia-0 (1) and *AT4G18395* knock-out (3), overexpression lines (12-16), and wild type segregant (4).

line	Average flowering time per experiment		
	1[a]	2[b]	3[b]
1	32	32	36
3	31*	31	34*
4	33	33	36
12	32	32	34*
13	32	33	35*
14	33	33	34*
15	32	34	34*
16	31	33	34*

[a] n=5; [b] n=10

* Significant difference between indicated genotype and WT (1)

Overall plant length is decreased and stem diameter is increased in overexpression lines

On average, the distance between main stem base and first silique was significantly shorter in the five independent overexpression lines (12-16) compared to the wild type line (1). Knock-out line (3) and the segregating wild type line (4) were not significantly different from the wild type line (1) (Figure 2). This result was in line with results found in the other repetitions and main stem length measurements at the end of the growth period of the third experiment (SUPPLEMENTARY DATA).

Recordings of the length of axillary branches on the main stem revealed that the average length of axillary branches was decreased in the five *AT4G18395* overexpression lines (12-16) compared to the wild type line (1). Axillary branches of the knock-out plants (3) were significantly longer than those of the wild type plants (1) (Figure 3A). However, the ratio between the length of the axillary branches and the length of the main stem is higher in most OE lines (12-15), and the KO line and one OE line (16) are not different (Figure 3B). This means that although the axillary branches are shorter compared to those of the WT plants, they are longer when their length is compared to the main stem.

Measurements of the diameter of the base of the main stem revealed that overexpression of *AT4G18395* results in wider stems. In the overexpression lines, the main stem diameter was on average up to 26% broader than for that of the wild type plants (1) (Figure 3C). This result was in line with results found in the other repetitions (SUPPLEMENTARY DATA).

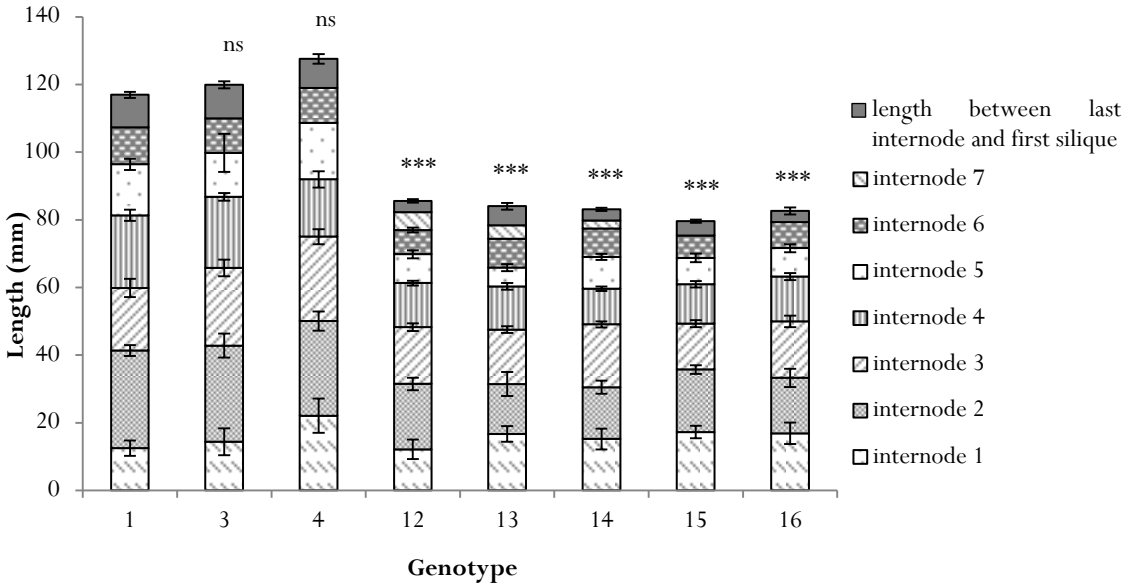

Figure 2. Overexpression of *AT4G18395* induces shorter plants. Bars represent average length from base to first silique and internode lengths of wild type Columbia-0 (1) and *AT4G18395* knock-out (3), overexpression lines (12-16), and wild type segregant (4), two weeks after flowering (n=10). Error bars represent the standard error of the mean (SEM); asterisks indicate statistical significance (*** p<0.001, One-way ANOVA with LSD test). Data is from repetition 3.

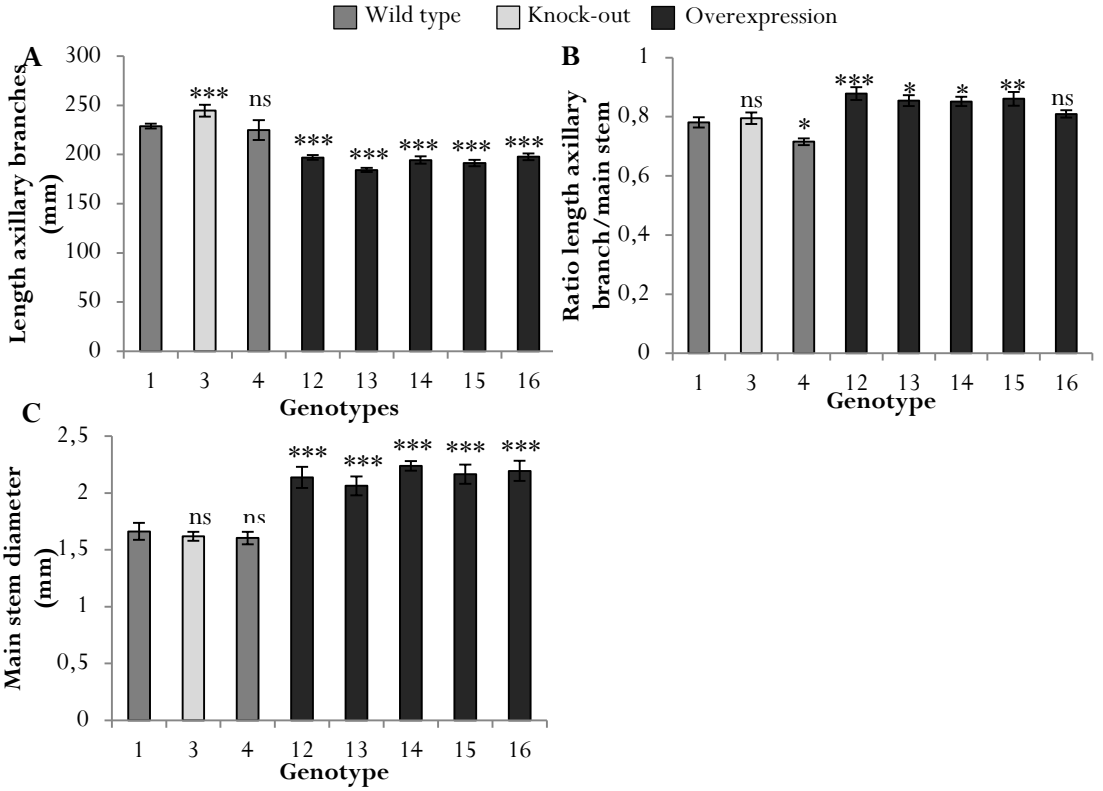

Figure 3. Overexpression of *AT4G18395* induces shorter axillary branches but with a higher ratio of side branch length relative to the main stem length and a wider base. (A) Bars represent average length of axillary branches of wild type Columbia-0 (1) and *AT4G18395* knock-out (3), overexpression lines (12-16), and wild type segregant (4), two weeks after flowering (n=10). **(B)** Bars represent ratio of side branch length relative to the main stem length, at the end of the growth period (n=5). **(C)** Bars represent the diameter of the main stem at the base, two weeks after flowering (n=10). Error bars represent SEM; asterisks indicate statistical significance (* p<0.05, ** p<0.01, *** p<0.001, One-way ANOVA with LSD test). Data is from repetition 3.

Significant differences between the number of axillary or basal branches remain unconfirmed

Phenotypic data on the number of axillary branches indicated no significant change in the average number of axillary branches during the experiments (Figure 2). However, only OE lines had a seventh internode during the first (line 12 to 16) and third experiment (line 12, 13 and 14), which did not occur in WT or KO plants. However, there were also no 7th internodes in the second repetition. The number of basal branches was not measured during the first experiment. During the second experiment, buds within the rosette were not recorded, these results are therefore not discussed. During the third experiments, basal branches were categorised in two groups: shoots and dormant buds. Dormant buds can be activated and develop into a branch, or may remain dormant. The number of shoots was significantly higher for the wild-type segregant (4) and most OE lines (12, 14, 15 and 16) compared to the WT (1). Furthermore, the number of dormant buds was significantly lower in all lines (3-15) except for OE line 16. Overall, it seems that in WT Colombia-0 more basal buds are dormant, while in OE lines more basal buds have developed into shoots.

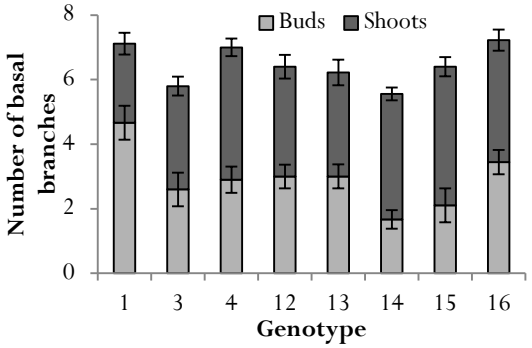

Figure 4. Buds are more often dormant in WT Colombia-0. Bars represent the ratio of dormant buds and buds that developed into shoots of wild type and *AT4G18395* knock-out (3), overexpression lines (12-16), wild type segregant (4), and wild type Columbia-0 (1), two weeks after flowering (n=10).

Most lines overexpressing *AT4G18395* have fewer trichomes

Trichome number and branching were recorded to determine if the overexpression of *AT4G18395* gene had influence on density and type of trichomes (Figure 5). During the first repetition, both the knock-out (3) and the overexpression lines (12-16) had fewer trichomes than the wild type line (1) (Figure 6A). However, during the second repetition, plants from the knock-out line and one overexpression line were not significantly different (Figure 6B). However, overexpression lines 12, 14 15 and 16 have significantly fewer trichomes during both repetitions.

Figure 5. Overexpression lines have fewer trichomes. Photograph of two-week-old first true leaves of wild type Columbia-0 (left) and AT4G18395 overexpression line 14 (right) taken under the binoculars.

Overexpression or knock-out of *AT4G18395* did not seem to have an effect on trichome type. When the amount of three- and four-branched trichomes was analysed, no significant differences were found during the first experiment. During the second, *AT4G18395* KO line (4) and OE line (13) had significantly fewer four-branched trichomes, but none of the other OE lines had the same result (SUPPLEMENTARY DATA).

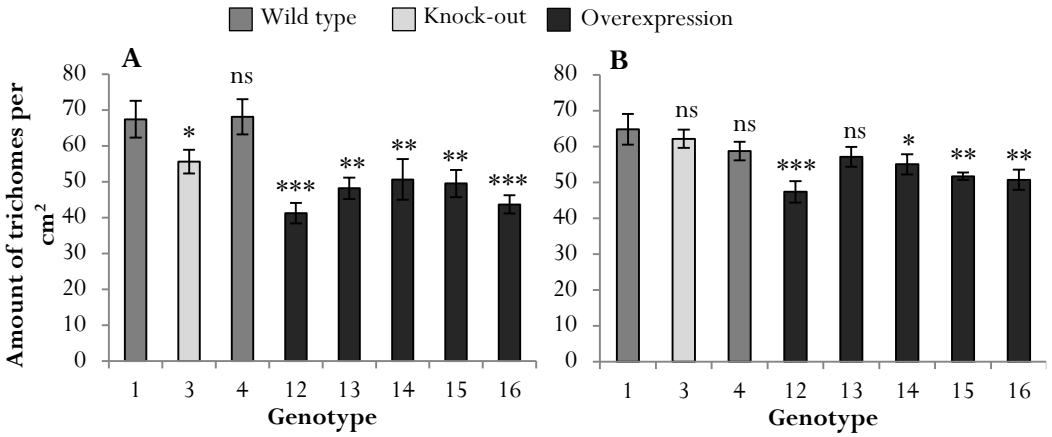

Figure 6. Overexpression of *AT4G18395* reduces the amount of trichomes. (A) Bars represent the amount of trichomes per cm^2 during the first experiment of wild type and *AT4G18395* knock-out (3), overexpression lines (12-16), wild type segregant (4), and wild type Columbia-0 (1), two weeks after sowing (n=5 plants). **(B)** Bars represent the amount of trichomes per cm^2 during the second experiment of wild type, knock-out and overexpression lines as described above (n=5 plants). Error bars represent SEM; asterisks indicate statistical significance (* $p<0.05$, ** $p<0.01$,*** $p<0.001$, One-way ANOVA with LSD test).

Pollen optimisation experiments

The effect of gene knock-out and overexpression was investigated in relation to pollen development. To do so, the number of pollen grains between lines grown under control conditions was compared, as well as pollen viability, germination and fertilization efficiency was examined under heat stress and control conditions. To examine pollen grain numbers, unopened anthers from stage 12 of flower buds were collected. However, no reliable results were obtained due to few repetitions and how pollen distributed on the microscope slide.

In order to obtain a reliable quantification of pollen viability, unopened anthers from stage 12 of flower buds were collected and pollen were tested for viability using either Alexander or Peterson staining. Solutions were diluted 10x and 50x, but the distinction between aborted and non-aborted pollen grains was most evident in non-diluted staining solutions. Alexander's staining yielded more 'transparent'-looking stained pollen but the difference between aborted and non-aborted pollen grains was similarly clear in both Alexander's and Peterson's staining solutions. Because Peterson staining solution is an adaptation of the Alexander's solution not containing chloral hydrate, mercuric chloride and phenol, Peterson staining solution was selected for pollen viability experiments. However, results of the pollen viability assays have not been processed.

In order to obtain a method that resulted in the highest percentage of pollen germination, freshly opened flowers were collected and pollen was collected by means of dipping- or centrifugation-methods, and germinated on solid and/or liquid PGM. Of all methods tested, the most effective method for pollen collection was by means of centrifugation with attached petals, in liquid PGM (Table 4). However, the standard error of the mean was very high (11.1) and the amount of pollen that were collected were low compared to other methods. The same was observed for dipping in liquid medium, the method with the second highest germination percentage. The method with the smallest variation in the percentage of pollen germination and yielding a descend amount of pollen during collection, was the centrifugation method with solid and liquid media. This method was therefore applied for all other germination experiments.

Pollen germination was also tested for incubation time, between 3 hours and overnight incubation, and germination in the light or dark. Visually, the time of incubation and light or dark conditions did not seem to affect germination, therefore overnight incubation in the dark was selected as most suitable method.

After optimisation, two pollen germination experiments were performed. The plants from the second experiment were stressed during growth and are therefore not discussed. The first experiment revealed that on average, more pollen germinated at 35 degrees than at 22 degrees (Figure 9). Pollen germination of wild type lines rose by 11.4 and 12.1 percent (line 1 and 4, respectively), knock out (3) pollen germination percentage rose by 16.1 percent and pollen of overexpression lines rose by 4.2, 7.9, 3.3, 2.1 and 2.6 percent (line 12-16 respectively), a far lower increase than in WT and KO plants. At 22 degrees, *AT4G18395* knock-out plants yielded no different germination percentage than wild type plants, but at 35 degrees 10,5% more pollen were germinated. The highest germination percentage, 80%, was of an OE line (14) at 35 degrees Celsius. Overall, during both temperatures, significantly more pollen grains germinated from the *AT4G18395* overexpression lines compared to the WT plants.

Table 4. Centrifugation method with solid and liquid medium results in the most equal distribution of germinated pollen. Three different media were used, petals were either attached or removed from the flower and two methods were tested: dipping and centrifugation.

		Methods					
		Dipping			Centrifugation		
Medium	**Petals**	**Germination (%)**	**Standard error**	**Pollen number**	**Germination (%)**	**Standard error**	**Pollen number**
Solid	**Attached**	8.7%	2.1	291	n/a		
Liquid	**Attached**	23.8%	3.8	44	0.0%	0	0.3
Solid&liquid	**Attached**	n/a			24.0%	11.1	24
	Removed	n/a			13.3%	1.6	120

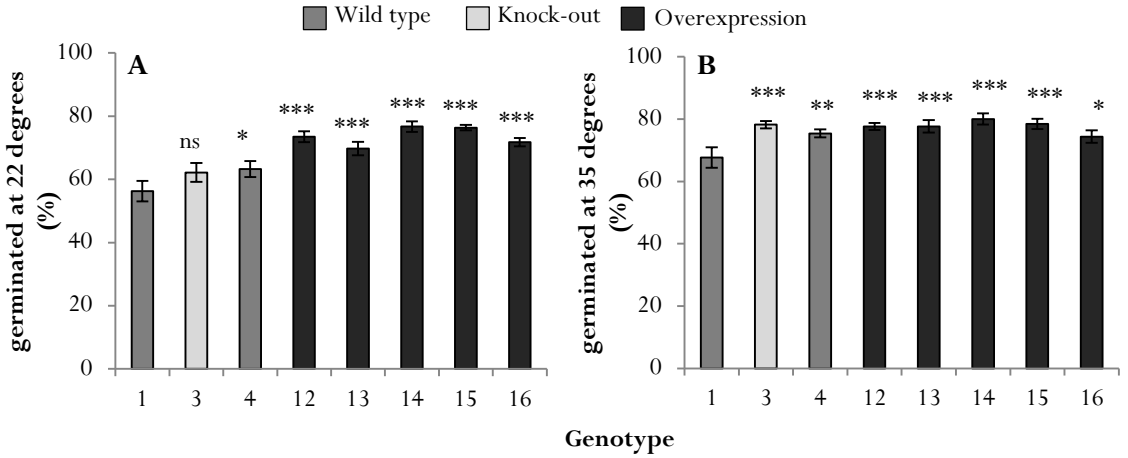

Figure 7. Overexpression of *AT4G18395* increases pollen germination percentage. Average pollen germination percentage (n=5 plants) of *AT4G18395* knock-out (3), overexpression lines (12-16), wild type segregant (4), and wild type Columbia-0 (1), at 22 degrees **(A)** and at 35 degrees Celcius **(B).** Error bars represent SEM; asterisks indicate statistical significance (* $p<0.05$, ** $p<0.01$,*** $p<0.001$, One-way ANOVA with LSD test).

Fertilization seems to be less efficient in overexpression plants

To examine fertilization efficiency, pistils were harvested 24 hours after fertilization and pollen tubes were stained with Aniline blue (Isahiguru et al., 2001). Samples were not analysed, but pollen grain attachment and tube growth were scored when the pistils were visualised under the microscope. Under control conditions, pollen attached to the stigma seemed to grow into the pistil and pollen tubes seemed to elongated to the ovaries. However, compared to WT (1), fewer pollen seemed to be attached to the

stigma of OE plants (16) and pollen tubes more often did not reach the end of the pistil (Figure 8). These results were intensified after application of heat stress, especially for line 16 where much less or no pollen at all were attached to the stigma (data not shown). In addition, in OE plants, after flower number 12, all or most buds were arrested in their development, so no siliques were formed in these plants. This was not the case in WT plants and to a lesser extend in most KO plants.

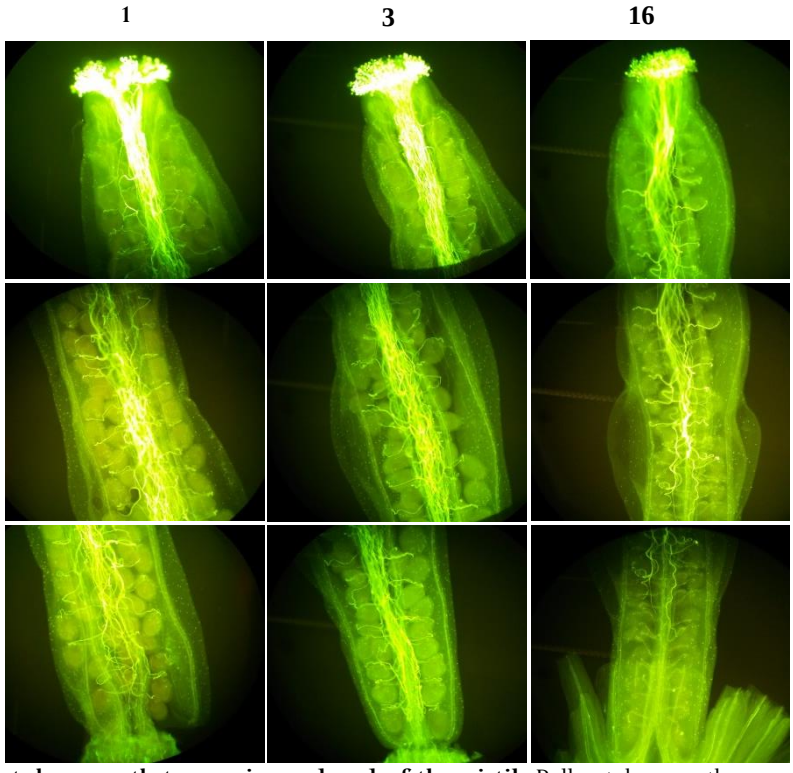

Figure 8. Pollen tube growth to ovaries and end of the pistil. Pollen tube growth was monitored in self-pollinated pistils of wild type Columbia-0 (1), *AT4G18395* knock-out (3) and overexpression line (16).

Overexpression of *AT4G18395* results in irregular silique length

To examine the effect of *AT4G18395* overexpression or knockout during control and heat stress conditions, 25 days after applying stress siliques 6 to 21 were harvested and cleared to see the seeds in the siliques. Silique length was measured and difference in silique length were observed in wild type lines compared to *AT4G18395* overexpression lines. During control conditions, wild type siliques had an average length of 15.4 varying up to 1.4 millimetres between all averaged silique sizes of all plants. Knock-out lines had an average of 15.3 millimetres, varying up to 2.3 millimetres. These variances were mainly caused by a naturally decreasing silique size. On the contrary, overexpression line 16 had irregular silique length (Figure 9A), varying up to 10 millimetres between all average silique sizes. When WT silique length was considered as 'normal', normal silique length was between 14.7 and 16.0 mm. In this case, silique lengths of OE lines were abnormal, because all average silique sizes were below 14,7 or above 16.0 mm (SUPPLEMENTARY DATA). After heat stress, all lines had high variance in silique length, although these differences were still bigger in OE lines (Figure 9B).

Seed content was not scored, but it was visible that under control conditions (Figure 9C) seed formation in WT line (1) and KO line (3) was similar in all siliques. However, in overexpression line (16), the small siliques contained few or no seeds. Furthermore, more 'holes' appeared in OE siliques, most likely due to unfertilized ovaries. Plants that received heat stress treatment (Figure 9D) had more variance in seed

content for all genotypes. All genotypes produced less seeds under heat stress than under control conditions, more often with 'holes' in-between seeds. Interestingly, OE line 16 seemed to have few fertilized ovules, but within the longer siliques barely any holes were visible and they appeared larger than siliques of WT and KO lines.

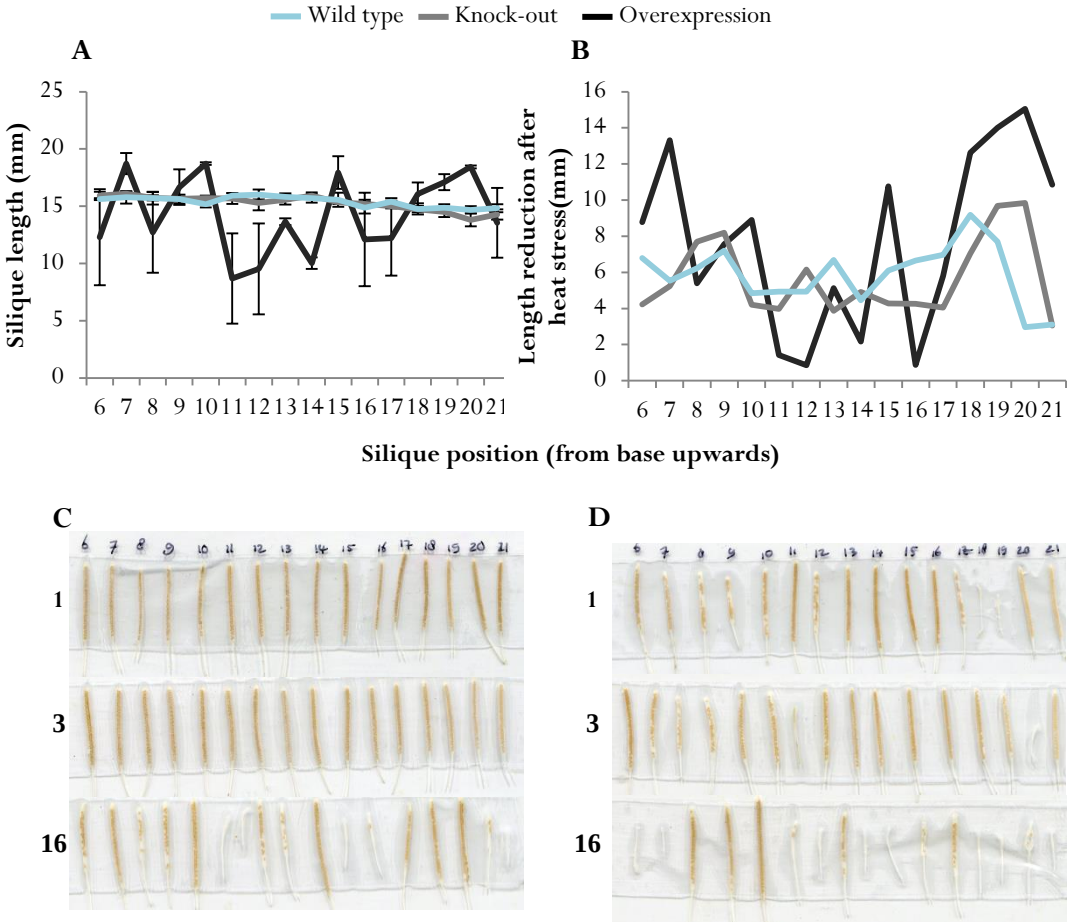

Figure 9. Overexpression of *AT4G18395* causes high variance in silique length. (A) Lines represent silique length of silique at positions 6 to 21 of wild type Columbia-0 (1), *AT4G18395* knock-out (3) and overexpression line (16), during control conditions (n=3). **(B)** Graph represent reduction in silique length of wild type, knock out and overexpression lines as explained above, after applying heat stress. Position numbers are relative to the flower that opened first at the day of the heat stress, flower number 0.

DISCUSSION

In an attempt to understand the function of the gene *AT4G18395,* wild-type Colombia-0 and *AT4G18395* knock-out and overexpression lines were used to quantify phenotypic differences under control and heat stress conditions, such as plant architecture and fertilization efficiency. Under control conditions, overexpression lines showed drastic changes in plant morphology, including a decreased overall plant length and reduced fertility. Furthermore, both knock-out and overexpression lines were more susceptible to heat stress conditions than wild type plants, resulting in reduced silique lengths. These results indicate a function of *AT4G18395* in fertility.

Roles of phytohormones in plant architecture

To quantify phenotypic differences in plant architecture when the gene *AT4G18395* was knocked-out (KO) or overexpressed (OE), a variety of plant characteristics under control conditions were recorded. One of the results that was found was the more horizontal growth of axillary branches of OE lines compared to wild type (WT) and KO plants. So far, little is known about the mechanism controlling side branch growth angle, but an aspect that is involved is the gravitropic set-point angle (GSA). The GSA is a defined angle from the gravity vector, usually resulting in vertical growth of roots and inflorescence stems (Blancaflor & Masson, 2003). Lateral roots and shoots often have a non-vertical growth and are maintained at their GSA due to an antigravitropic offset mechanism (AGO), which operates in tension with the gravitropic response and results in a net symmetry between these two (Roychoudhry & Kepinski, 2015). Branch GSA depends the magnitude of AGO, because a strong AGO results in a more horizontal growth. Both gravitropic growth and AGO are regulated by high and low levels of auxin, respectively (Roychoudhry et al., 2013). Higher AGO activity could be the reason that axillary branches of OE plants in this thesis grew more horizontal, which would suggest lower levels of auxin in OE lines.

Another phenotypic trait that is influenced by lower levels of auxin is an increased bud outgrowth. Early research already showed that removal of the main auxin source, the apical meristem, resulted in outgrowth of axillary and rosette buds (Thimann & Skoog, 1933). Furthermore, an experiment using auxin-insensitive mutants conducted by Hayward et al (2009) showed that mutation of *bodenlos-2* (*bdl*-2), a semi-dominant auxin response mutant in the Columbia-0 background, caused increased rosette bud outgrowth. Mutation of *bdl*-2 also reduced shoot height (Hayward et al., 2009). Interestingly, during this thesis an increased outgrowth of rosette buds and reduced stem length was also found in OE lines two weeks after flowering. It was evident that under control conditions, overexpression of *AT4G18395* resulted in a shorter main stem, shorter internodes and shorter axillary branches. KO plants did not have these phenotypic differences compared to the WT, except for longer axillary branches compared to the WT. However, when the ratio of axillary branch length to main stem length was compared, KO plants were not different from WT plants. This suggests that only overexpression of *AT4G18395* results in a changed phenotype concerning axillary branch length. Furthermore, knock-out and overexpression of *AT4G18395* seemed to have an effect on basal bud outgrowth. Significantly more basal buds had developed into shoots in KO and OE lines, while basal buds seemed more dormant in WT. Although the amount of axillary branches was not significantly different across the five independent OE lines, interestingly, a seventh axillary branch was produced only in a few OE lines, but never in the WT or KO lines.

These results could all be related to a decreased level of auxin in OE lines. However, it should to be noted that the mechanisms behind branching are more complex than only a reduced expression of auxin, because the hormones strigolactones and cytokinin are important regulators of branching as well. Auxin and strigolactones are known to suppress axillary bud outgrowth, whereas cytokinin promotes axillary bud

outgrowth (Al-Babili & Bouwmeester, 2015). Cytokinin has been demonstrated to induce bud outgrowth when directly applied exogenously (Sachs & Thimann, 1967), but also to induce bud outgrowth endogenously when overexpressed (Medford et al. 1989). Furthermore, cytokinin biosynthetic gene mutants have strongly reduced axillary branching initiation, showing that normal axillary branching initiation requires cytokinin biosynthesis and signalling (Müller et al., 2015). Therefore, not only lower auxin levels could explain the increased bud outgrowth in OE lines, increased cytokinin could explain this as well. It could be either of them or a combination of both, because auxin inhibits cytokinin biosynthesis by suppressing the IPT gene, a cytokinin biosynthetic gene (Cheng, Ruyter-Spira & Bouwmeester, 2013). Thus, lower auxin levels may increase cytokinin biosynthesis. However, cytokinin has been suggested to overcome this auxin-mediated bud inhibition under favourable conditions such as a high nitrate availability (Müller et al., 2015), so cytokinin could also act alone, even with regular auxin levels present.

Besides the promotion of axillary buds, cytokinin regulates the initiation of the vascular cambium, a lateral meristem that gives rise to a secondary xylem, xylem fibers and xylem parenchyma cells (Taiz & Zeiger, 2010). An increased stem diameter (thickening of the shoot), also called secondary growth, is mediated by the vascular cambium (Agusti et al., 2011). The involvement of cytokinin in secondary growth is proven in the *ipt* mutant, a mutant in which cytokinin levels are severely decreased due to the function of IPT as a cytokinin biosynthetic gene (Matsumoto-Kitano et al., 2008). Decreased cytokinin levels results in a greatly reduced stem diameter and reduced number and size of the vascular bundles, together with a dwarf phenotype. Furthermore, external application of cytokinins or overexpression of IPT genes increases secondary growth (Matsumoto-Kitano et al., 2008). Increased cytokinin levels could explain the increased stem diameter measured in OE lines, as results on the indicate that compared to the WT, the diameter is increased in OE lines, but is not significantly different in KO lines.

Although increased cytokinin levels could explain the increased stem diameter, it does not explain the measured reduction in number of trichomes in OE lines, as cytokinins act as positive regulators of trichome development in Arabidopsis (Pattanaik et al., 2014). Application of cytokinin in the form of benzylaminopurine (BAP) resulted in an increased number of trichomes per rosette leaf, while the trichomes became shorter and DNA content was reduced compared to untreated plants. Although cytokinin has an effect on trichomes in rosette leaves, it is more involved in trichome formation on inflorescence organs (Matías-Hernández et al., 2015; Pattanaik et al., 2014). Phytohormones that are involved in trichome formation on Arabidopsis rosette leaves are salicylic acid, gibberelin and jasmonic acid (Maes, Inzé, & Goossens, 2008), but the knowledge on molecular mechanisms used by phytohormones to influence trichome formation is still limited and will require further research.

Although OE lines had reduced trichome numbers, the number of trichomes were inconsistent across the two repetitions. During the first repetition, KO and all OE lines had significantly fewer trichomes than the WT Colombia-0, while during the second experiment the KO line and one OE line (13) were comparable to WT line. The differences between these experiments may be due to unconscious selection during the first repetition, where five plants were picked out of a group of ten plants per genotype, while during the second repetition there was a total of five replicates for the experiment. However, during both experiments, the majority of OE lines had significantly fewer trichomes, suggesting that overexpression of *AT4G18395* results in fewer trichomes. Another aspect of trichomes was also quantified in this study, the number of branches. There were no significant differences between WT, KO and OE lines, as all genotypes had three to four trichomes, the most common amount of branches of Arabidopsis trichomes (Marks, 2014).

Many factors influence fertility

The gene *AT4G18395* is highly expressed (over a 100 fold change) in mature and dry pollen, pollen germination, and the stamen during flower stage 12, during which stamens and gynoecium lengthen co-ordinately (Alvarez-Buylla, et al., 2010). *AT4G18395* appears to mainly be related to flowering, because according to its expression patterning it is expressed during pollen development, petal differentiation and expansion stage and stamen during flowering stage 15 (Arabidopsis eFP browser). For this reason, pollen number, viability and germination was recorded in wild type and *AT4G18395* knock-out and overexpression lines. In addition, fertilization efficiency and silique lengths were assessed.

Pollen grain number quantification was attempted by taking one closed anther and releasing the pollen. However, no reliable results were obtained due to few repetitions and the pollen distribution of pollen themselves on the microscope slide. In some cases pollen would distribute relatively uniformly on the slide, while in other cases pollen seemed to remain close to the anther. Because pollen grain number was assessed using pictures taken from the slides it may not have captured a realistic representation of the true amount. In the future, it is suggested to stain the pollen grains to count the total number directly under the microscope. Staining the pollen is recommended, as it will allow recognition of pollen that remain in the anther. The method that was used for pollen germination using centrifugation of flowers could also be used to assess the number of pollen. However, during the viability assay it was observed that non-viable pollen often remain in the anther while viable pollen were easier released. Therefore, the downside of the centrifugation method is that if non-viable pollen stay attached to the anther, it can give an incorrect impression of the number of pollen, as there will be fewer pollen released in the liquid.

To assess the viability of WT and *AT4G18395* KO and OE pollen, pollen were stained using Peterson's staining solution (Peterson, Slovin & Chen, 2010). During staining, viable pollen grains yield magenta-red colour and non-viable pollen grains show blue-green colouration, and are translucent. The stain contains malachite green and acid fuchsin, which stain the cellulose in pollen walls and the pollen protoplasm respectively. Non-viable pollen grains lack protoplasm and therefore do not stain with acid fuchsin (Peterson, Slovin & Chen, 2010). However, during the experiments performed during this thesis, all pollen coloured solid red or translucent red if they were aborted. Since the anther also coloured red, and the non-viable pollen often remain inside the anther, this made it difficult to reliably quantify the amount of non-viable pollen. After adding more malachite green this difficulty was solved and non-viable pollen grains showed the blue-green colouration that was expected. Although the pollen viability scoring did not go as expected, visual observations suggest that KO and OE lines always had viable pollen under control conditions. To confirm these observations and to determine the effect of KO and OE of *AT4G18395* on pollen viability under heat conditions, a new viability assay should be performed.

Although pollen viability experiments yielded less results than expected, pollen germination can give a representation of the viability of the pollen. Even though it is not possible to know if the pollen that did not germinate are viable or aborted, the percentage of germinated pollen grains can indicate the minimum of viable pollen. Pollen grains were germinated on pollen germination medium at 22 or 35 degrees Celsius. At both temperatures, *AT4G18395* overexpression resulted in more germinated pollen compared to WT pollen. Interestingly, at 35 degrees Celsius, germination percentages of all genotypes were higher than at 22 degrees Celsius. Although KO pollen germination was comparable to that of WT at 22 degrees, significantly more KO pollen germinated at 35 degrees Celsius. The increased germination at 35 degrees Celsius differs from the findings by to Boavida et al. (2007), where it is stated that the optimal temperature for pollen germination is 22 degrees Celsius, and higher or lower temperatures resulted in a reduced pollen germination (Boavida et al., 2007). Therefore, it is suggested to repeat the germination experiments at least once, to assess if the results are reproducible. Furthermore, the pollen germination

measurements were all performed with flowers of plants grown under control conditions, so the effect on pollen germination when heat stress is applied to the whole plant is unclear. Heat stress is known to have a negative effect on many stages of flower development, including pollen development (Müller & Rieu, 2016). Because the gene is highly expressed during pollen development an pollen germination (Arabidopsis eFP browser), and KO and OE lines had a more decreased silique size than WT lines after heat stress, it is hypothesized that pollen viability and germination is also negatively affected. A new viability assay would reveal how many pollen are viable or non-viable after heat stress, but it may also be interesting to assess if germination and germ tube length are affected.

Results demonstrate that overexpressing *AT4G18395* induces abnormal and irregular silique lengths, which is in line with previously obtained data (Fradin, unpublished). Seed content was not scored, but from the silique experiment it was clearly visible that OE lines had irregular seed formation with 'holes' between seeds, and small siliques contained few or no seeds under control conditions. This result was not expected because it was evident that pollen grains germinate better than WT when grown on pollen germination medium. For this reason, pollen germination is most likely not the limiting factor for the reduced fertilization in OE lines, but other factors, such as a reduced stamen length or anther dehiscence, may play a role. To assess fertilization efficiency, the staining of pollinated pistils with aniline blue was performed. Results of plants grown under control conditions suggest that the pollen of WT and OE lines have no problems entering the stigma and reaching and fertilizing the ovules, as it was clearly visible that the pollen tubes grew into the pistil and in most cases reached the ovules at the base of the pistil. Seed content of these lines confirm successful fertilization of the ovules. However, fewer OE pollen attach to the stigma, which may result in a reduction in fertilized ovules, due to fewer pollen tubes growing into the pistil. This would also explain the 'holes' between seeds and the shorter siliques, because not all ovules are fertilized. After heat stress, pollen adhesion, silique length and seed content varied and was decreased in all lines, and siliques more often contained 'holes' in-between seeds. However, KO and OE siliques seemed to be even more sensitive to heat stress, due to a greater seed content reduction and in OE often empty siliques. This was probably caused by the reduced or absent pollen adhesion after heat stress, something that was clearly visible in the aniline blue experiment. Interestingly, the few well-fertilized siliques in OE lines had barely any holes and were larger than siliques of WT and KO lines. This is most likely to compensate with the reduced seed number in the other siliques.

A likely reason for reduced pollen attachment under control conditions is a mismatch between the ripening of the stigma and the anthers, that can be caused by a reduced stamen length or the wrong timing of anther dehiscence. An important phytohormone that regulates these traits is auxin (Cecchetti et al., 2008), as it is necessary for both male and female floral organ development. Disruption of either auxin-associated signalling, biosynthesis or transport genes leads to flowers with diverse abnormalities (Smith & Zhao, 2016). Example are mutations in Auxin Response Factor 6 (ARF6) and ARF8, genes that play a key role in regulating the expression of auxin response genes. The *arf6 arf8* double mutants have many flower defects, including shortened petals, immature gynoecium and shortened stamen filaments. Furthermore, these double mutants develop abnormal ovules and have undehisced anthers, therefore failing to release their pollen (Smith & Zhao, 2016). Flowers are less defected in *arf6* and *arf8* single mutants, but still have delayed stamen filament elongation and decreased seed set (Reeves et al., 2012). Again, lower auxin levels may explain the reduced pollen adhesion of OE lines found under control conditions during this thesis, if the reason for the reduced adhesion is a mismatch between the ripening of the stigma and anthers.

Interestingly, during heat stress in Arabidopsis, endogenous auxin levels are suppressed in developing anthers. When auxin is exogenously applied, this male sterility is completely reversed, which suggests a role for auxin in maintaining pollen fertility under heat stress (Sakata et al., 2008). This may be the reason behind the decreased fertility that was found for all lines after heat stress during this thesis. The possibly

already deceased auxin levels in OE lines may also explain the extreme sensitivity of this line to heat stress, which leads to an even greater reduction of auxin. Although reduced stamen length and anther dehiscence may explain the reduced pollen adhesion, other factors such as the receptiveness of the stigma or pollen adhesion may also play a role. It is therefore suggested to study stamen length and anther dehiscence under control conditions and after heat stress application, to determine if either or both of these are the reason for a reduced pollen number attached to the stigma in OE lines. If not, stigma receptiveness and pollen adhesion (Zinkl et al., 1999) can be tested by for example pollinating the stigma of OE lines with WT pollen and vice versa.

Conclusions and recommendations

Overexpression of *AT4G18395* has a significant effect on plant architecture and floral organs. Interestingly, knock-out plants were visually comparable to wild type plants under control conditions. The reason behind this may be because *AT4G18395* is mainly involved in flowering according to its expression patterning. Therefore, a knock-out may not affect the whole plant, because it is specifically expressed in a few plant parts and not the whole plant. However, when it is overexpressed with a 35S promotor, the gene is activated throughout the plant and not as tightly regulated as in wild type plants.

With the main focus on hormones as an explanation for changed plant architecture and pollen qualities, some interesting results come to light. As was discussed before, lower auxin levels may explain the more horizontal growth of the axillary branches and the fact that there seem to be less dormant buds in OE lines, because auxin is known to suppress branching. Furthermore, it could explain the decreased fertility in OE lines. However, strigolactones are also known to inhibit bud growth, even though strigolactones have been proven to promote a wider main stem diameter through cambium development, a phenotypic property that was clearly visible in OE lines. The increased bud outgrowth in OE lines and an increased stem diameter could therefore be explained by cytokinins as they have promoting activity on vascular cambium and bud outgrowth. However, apical auxin is known to suppress cytokinins in shoots, and also strigolactones and cytokinin are known to have synergistic interaction. Overall, it is a complicated interaction with possibly multiple hormones at play, and it is unclear if the gene *AT4G18395* may affect all these hormones, few of them, one or maybe even none. For this reason, it is suggested to test hormone levels in different plant tissues, for example by means of hormone extraction, qPCR or promotor-GFP fusion (hormone-GFP construct), with a main focus on auxin because it seems to be involved in almost all processes that are changed in OE lines.

As regards to fertilization efficiency, it is important to do a pollen viability stain with the adjusted amount of malachite green, to get a precise overview of fertilization efficiency by combining pollen viability data with aniline blue staining, silique length and seed content results. Furthermore, it is suggested to look at the length of the anthers and anther dehiscence at the day the flowers open, to see if a mismatch between ripening of the stigma and anthers is the reason for a reduced number of pollen attached to the stigma. If this is not the case, reduced stigma receptiveness or pollen adhesion might be the reason for the number of pollen that attach to the stigma, so these properties would be interesting to assess.

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SUPPLEMENTARY DATA

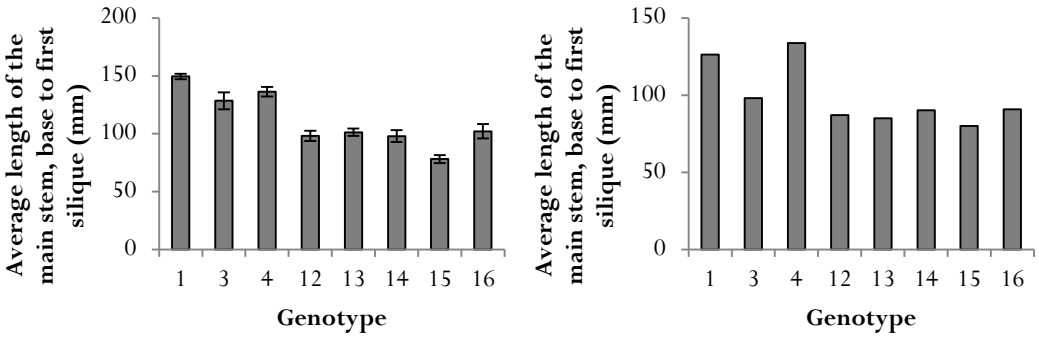

Figure 10. Average length of the main stem, base to the first silique. Graph on the left is from branching experiment 1 (n=5), graph on the right is from experiment 2 (n=10).

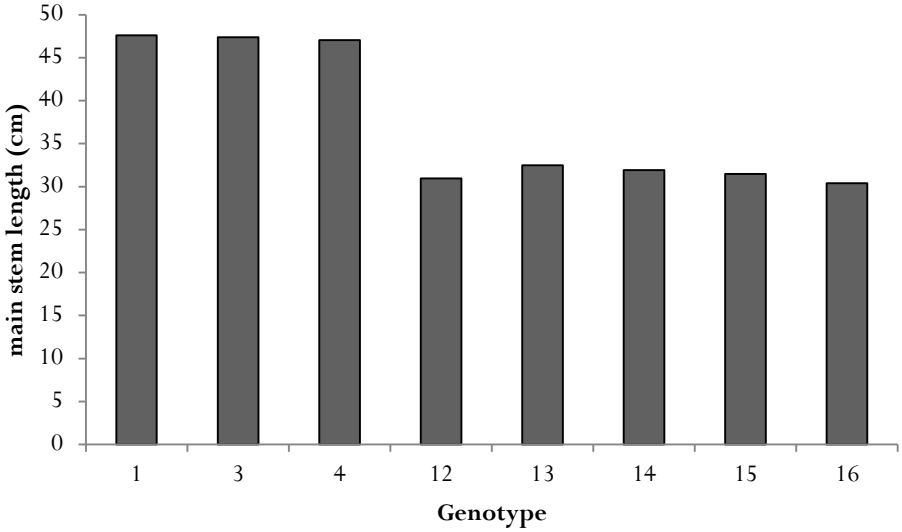

Figure 11. Main stem length at the end of the growth period of branching experiment 3.

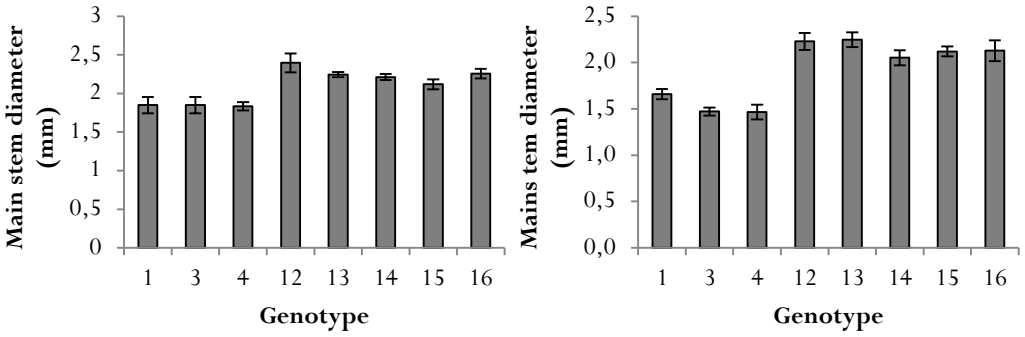

Figure 12. Main stem diameter. Graph on the left is from branching experiment 1, graph on the right from experiment 2.

Table 5. Number of four-branched trichomes. Results are from branching experiment 2. Asterisks indicate a significant difference compared to the wild type (1).

Genotype	Trichomes with 4 branches (%)
1	28,4
3	23,6
4	17,3*
12	23,4
13	17,3*
14	25,5
15	29,1
16	25,0

Table 6. Silique size under control conditions. Silique size of the silique experiment. Colours represent siliques that are considered abnormal (Bigger or smaller than the biggest and smallest WT siliques); yellow if less than 14.7, red if higher than 16.

Silique	WT	KO	OE
6	15.6	15.9	12.3
7	15.8	16.1	18.7
8	15.7	15.7	12.7
9	15.6	15.6	16.7
10	15.2	15.7	18.7
11	15.9	15.7	8.7
12	16.0	15.3	9.5
13	15.8	15.6	13.7
14	15.7	15.9	10.0
15	15.6	15.4	17.9
16	14.9	15.2	12.1
17	15.4	15.0	12.2
18	14.8	14.7	16.1
19	14.9	14.5	17.1
20	14.7	13.8	18.4
21	14.8	14.3	13.6