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Abstract

Ethylene has myriad roles as a plant hormone, ranging from senescence and defending against pathogen attacks to fruit ripening and interactions with other hormones. It has been shown to increase cambial activity in poplar, but the effect on wood formation in *Arabidopsis* hypocotyl has not previously been studied. The Auxin-Regulated Gene involved in Organ Size (*ARGOS*), which increases organ size by lengthening the time for cell division, was found to be upregulated by ethylene. We tested the effect of ethylene treatment at 10 and 100 µM ACC on three genotypes of *Arabidopsis*, Col0 (wild-type), an *ARGOS* deficient mutant (*argos*), and *ein3-1*, an ethylene insensitive mutant. *ARGOS* expression analysis with qPCR indicated that ACC does induce *ARGOS* and *ARGOS-LIKE* (*ARL*) in the hypocotyl. As seen in poplar, ethylene also decreases stem elongation.Histochemical staining, showed that ethylene changes the way secondary xylem lignifies, causing gaps in lignification around the outer edge of secondary xylem. Our results also implied that ethylene treatment changes the proportion of secondary to total xylem, resulting in less secondary, whereas in poplar, ethylene treatment caused an increase.

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1 Introduction

1.1 Ethanol production

In traditional ethanol production starch (from corn), or other simple sugars (sucrose in sugar cane or sugar beets) are fermented to ethanol. Second generation ethanol makes use of the plant cellulose, providing another source for biofuels and helping to reduce dependence on dwindling fossil fuel resources. In the cell walls of xylem cells (the wood) the cellulose is arranged in crystalline aggregates of microfibrils, which are built up of long glucose chains. A major bottleneck to use this resource for ethanol production is the breakdown of the cellulose crystals to individual glucose molecules to be fermented by microorganisms. Hemicellulose, composed of 5-carbon sugars, is intertwined between the microfibrils and lignin is cross-linked throughout the cellulose/hemicellulose matrix (Hori and Elbein, 1985). While there is research into fermenting hemicellulose, cellulose is found in much higher concentrations in wood with about 45% in *Populus* spp. compared to 25% hemicellulose (McDougall et al., 1993).

When trees are harvested, the crowns are not generally used, and are often left in place to decompose naturally. The unused parts of the trees can be used as a resource of sugars for ethanol production. If real headway is to be made in lessening dependence on fossil fuels, however, more biomass will be needed. *Populus* spp. or hybrids, as well as *Salix* spp, are the trees of preference to grow in plantations in North America and Europe. *Populus* hybrids have long been used in laboratory settings because of their relatively easy genetic transformation, and as such, the genome of *Populus tricocarpa* has been entirely sequenced. After sequencing, gene expression can be investigated in different tissues, for different treatments, and developmental stages. Thanks to those transcriptome studies, genes have been identified that are key players in wood development (e.g Sterky et al., 1998; Hertzberg et al., 2001; Dharmawardhana et al., 2010).

Since wood is built up of xylem cells, it is valuable to learn more about the process the cells go through to become mature wood, and how they change over the life of the tree. If we wish to change their development and/or

chemical composition, whether for increased biomass or cellulose, diminished lignin content, or to boost the growth rate, knowledge of the molecular, biochemical and physiological processes involved will allow those changes to be realized. There are differences in early wood and late wood (formed in different seasons), juvenile and mature wood, normal wood and tension wood, and by harnessing the information gathered by studying wood formation, it is possible to change the way plants grow. Biofuel production can be improved in two ways; by modifying the extraction and fermentation methods, and/or changing the raw material.

1.2 Wood development in trees

Wood is formed from the vascular cambium. In xylem development, six stages can be distinguished that are observable as distinct, radially oriented zones in stem cross-sections: cell division, differentiation (which includes expansion, cell wall thickening, lignification, and programmed cell death), and heartwood formation (Plomion et al., 2001). The vascular cambium, which encircles the stem, is made up of undifferentiated initials, as well as phloem- and xylem mother cells. The mother cells are formed when the initials divide periclinally. The xylem mother cells are on opposite sides of the initials, with phloem mother cells towards the periphery of the stem, and xylem mother cells toward the stem's center. The middle lamella is the first cell wall layer to be developed after the cells divide, consisting of primary walls of neighboring cells, and adheres each cell to its neighbors.

The differentiation of the cells begins with expansion and simultaneously, the further development of the primary cell wall as the cell expands. The primary cell wall consists of randomly oriented cellulose microfibrils, pectic molecules and hemicelluloses found in between the microfibrils (Plomion et al., 2001). This primary cell wall is elastic to allow the cells to grow to their mature size and become functional. Once the new cells reach their ultimate size, the deposition of the secondary cell wall begins.

The secondary cell wall is composed of three layers, S_1, S_2, and S_3, each having different properties and chemical compositions. The S layers are

made up of cellulose, hemicellulose, and lignin, along with cell wall proteins, and account for the majority of woody biomass The composition of cell walls changes between species, and even between cells within the same plant, due to environmental stresses and stimuli, but most of the time they are 40-50% cellulose, 25% hemicelluloses, and 25-35% lignin within the S-layers (Plomion et al., 2001).

The next step that xylem cells undergo in their differentiation is lignification of the cell walls. After cellulose, lignin is the second most abundant biological polymer on earth, making up nearly 30% of the organic carbon, and is what allows trees to withstand the gravitational forces that come with growing tall (Boerjan et al., 2003). Lignin is composed of chains with differing amounts of three cinnamyl alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohol). These alcohols are combined to create *p*-hydroxyphenyl (H), guaniacyl (G), and syringyl (S) monolignol units (Baucher et al. 2003). Angiosperm trees consist of mostly S and G units, with H units present in the lowest amounts (Boerjan et al., 2003).

After lignification is complete, the water conducting xylem cells must undergo programmed cell death in order to become functional. Programmed cell death employs the use of hydrolases, which are stored in the vacuole until they are needed (McCann and Roberts, 2000). A change in calcium concentrations causes the vacuole to burst and release the hydrolases, which then begin the process of breaking down the cellular contents to render the cell a hollow passage, capable of transporting water from the roots to the top of the tree (Groover and Jones, 1999).

These water conducting tubes are then functional for a number of years and continue transporting water until they suffer damage, or undergo water stress and go through cavitation. The last alteration that wood cells undergo, is the conversion of sapwood (functioning in water transport) to heartwood (non-functioning in water transport). Sapwood is sometimes called the “living wood” but in fact, the majority of the cells in sapwood are non-living tracheids, vessels, or fibers. The cells that remain alive are the ray parenchyma cells that transport nutrients radially throughout the sapwood

(Higuchi, 1997). The heartwood accumulates phenolic compounds as a deterrent to rot, and assists in structural support.

Vessel elements and fibers make up the majority of xylem in angiosperms and there are many similarities between the secondary xylem in trees and in the herb *Arabidopsis. Arabidopsis thaliana* has long been used as a model organism in experimental plant biology to determine the functions of genes, owing to its small size, short generation time, and the fact that it was the first plant genome to be sequenced (Somerville, 2002). Like trees, the hypocotyl of *Arabidopsis* forms secondary xylem. Chaffey et al. (2002) compared the anatomy and the structure of vessel elements and fibers in the secondary xylem of poplar stem and *Arabidopsis* hypocotyls, through sectioning or maceration and observation under a microscope. What they found was that the conformation of the xylem and cells were very similar, although cells differed in size (with poplar cells being much larger than *Arabidopsis*) (Chaffey et al. 2002). Fiber cells in both species are long and narrow, with tapered ends, while the vessel elements were much wider and had many pits in the walls. The hypocotyl of *Arabidopsis* has xylem I and xylem II, with the differences being that fibers and vessels in xylem II are lignified, whereas only the vessels are lignified in xylem I. Because of the similarities in *Arabidopsis* secondary xylem to poplar xylem, it can also be used to study basic principles of wood formation.

1.3 The role of plant hormones in wood formation

Plant hormones are small signaling molecules that regulate a diverse range of functions and development in plants. Even at very low concentrations, they are essential for plants to maintain the correct organization of cells and tissues. Hormones allow plants to adjust to their changing environments. There are many plant hormones, including, but not limited to, auxin, cytokinins, gibberellin, brassinosteroids, and ethylene.

Plant hormones regulate wood development, in the presence and absence of environmental stimuli. For example, auxin levels decrease on the opposite side of tension wood (section 1.4), which causes the growth

inhibition (Hellgren, et al. 2004). The amount of auxin in cambial tissues is an important player in the regulation of secondary xylem formation. (Björklund et al., 2007). Gibberellin also has a role in xylem formation in *Arabidopsis*, in that it promotes cambial cell division, but needs to work with auxin for the cambium to form properly, and differentiate into xylem (Ragni et al., 2011).

1.4 Wood formation in response to environmental stimuli and the role of ethylene

Wood formation can be altered by environmental stimuli. Reaction wood is one form of environmentally adjusted wood development. Reaction wood forms when a tree responds to gravitational stimuli, for example because of displacement of the stem by wind, snow or growth on a slope. Reaction wood puts a strain on the stem that will correct the tree's position back towards upright. In angiosperms, the reaction wood is referred to as tension wood (TW). TW is characterized by enhanced growth on the upper side of the leaning stem and the formation of a different xylem with proportionally fewer vessel elements, and fibers presenting a thickened secondary cell wall with a gelatinous layer (G-layer). The G-layer is highly enriched in cellulose, contains some hemicellulose and only trace amount of lignin-like compounds (Mellerowicz and Gorshkova, 2012). Because of the high content of cellulose, tension wood is a promising source of information on cellulose biosynthesis, through comparison of gene expression of a tree producing tension wood to a straight grown control tree (Andersson-Gunnerås et al., 2006). Mechanically bending or leaning a tree induces tension wood. Once the cellulose synthesis pathway is figured out, transgenic trees could be created that may over-produce cellulose in the same way as tension wood, but possibly in all of the wood, not just one side of it, and without the gravitational influence. Such material would be highly valuable for second-generation biofuel production.

TW formation in trees is accompanied by enhanced ethylene production on the upper side of the leaning stem (Andersson-Gunnerås, 2003). Also, by treating both the whole plant, as well as only one internode of

the *Populus* plants, Love et al. found that ethylene treatment in poplar can increase cambial growth and therefore xylem formation, simultaneously decreasing internode length (Love et al, 2009). By using ethylene insensitive trees it was further demonstrated that ethylene induced during TW formation partially explains the unilateral enhancement of growth (Love et al, 2009). Even though the effects of ethylene on xylem in poplar are well described (Love et al., 2009), no literature is available on the effect of ACC on *Arabidopsis* xylem development. Investigating those effects was one aim of my Masters project.

Ethylene is the simplest plant hormone, with only two carbons (Figure 1.1) and is responsible for, or shares a role in, regulating germination, organ growth, senescence, and fruit ripening, among many other functions. In times of stress, ethylene helps manage attacks from pathogens, growing around obstructions, and hypoxia (Morgan and Drew, 1997). Many of the details of biosynthetic pathways and signaling cascades for ethylene have been unraveled in the last 20 years, but some pieces of the puzzle are still missing (Wang et al., 2002). Ethylene is synthesized from Methionin, and the reaction taking it from S-adenosyl-Met to 1-aminocyclopropane-1-carboxylic acid (ACC), is one of the key regulatory steps in ethylene biosynthesis (Wang et al., 2002).

Ethylene signals in the cell are perceived by ethylene receptors at the ER membrane. When ethylene binds to its receptors it derepresses the downstream signaling cascade. Ethylene binding to the receptor stabilizes another ER-transmembrane protein, *EIN2*, which then activates the transcription factor *EIN3* (Alonso and Stepanova, 2004). In order to function properly, the ethylene response pathway requires both *EIN3* and EIN3-LIKE protein, EIL1, both of which are downstream of *EIN2*. EIN3 is broken down through proteolysis by *EBF1*/*EBF2*, though this action is repressed by the presence of ethylene (An et al., 2010).

Compared to poplar, there are many more ethylene pathway mutants in *Arabidopsis*, making this an attractive model to investigate the effect of ethylene on wood formation.

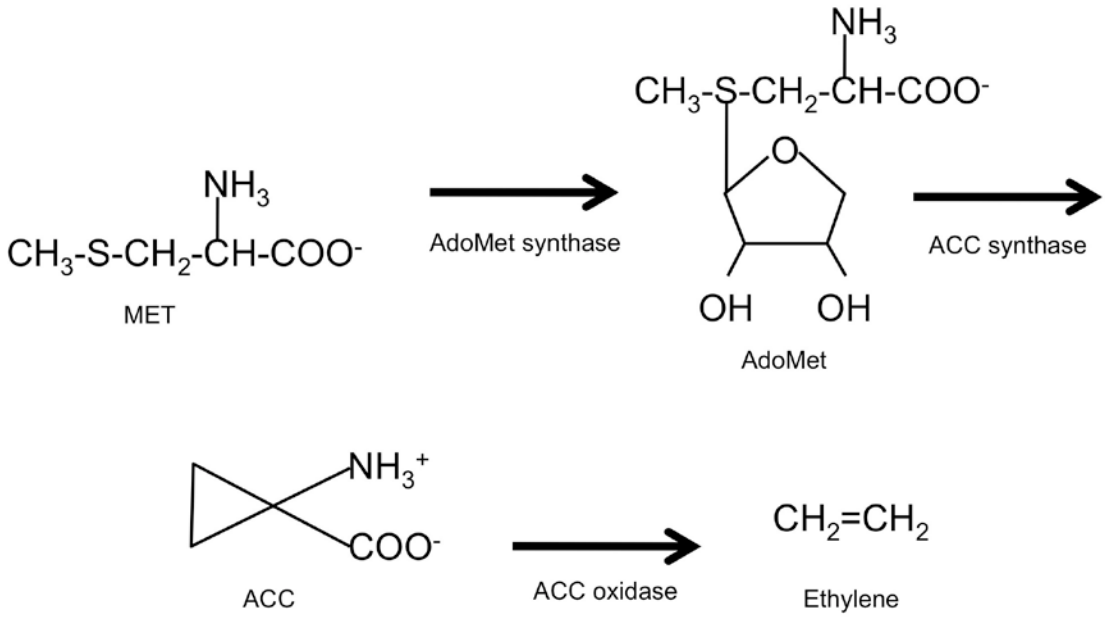

Figure 1.1: The ethylene biosynthesis pathway.

1.5 The possible role of *ARGOS* genes in wood formation

One of the ongoing projects in Björn Sundberg's group, where this Master project was carried out, is to study the effects of ethylene on wood development and to investigate how ethylene signaling and wood formation are connected. Transcript profiling studies in ACC-treated hybrid aspen stems and in tension wood forming trees in the Sundberg group have revealed the

induction of Auxin-Regulated Gene involved in Organ Size (*ARGOS*) and its homolog *ARGOS-LIKE* (*ARL*). Given this inducibility of *ARGOS* and homologs by ACC/ethylene, their expression in the xylem and their known potential to alter cell proliferation and expansion in *Arabidopsis* (Hu et al., 2003 and 2006), we considered these genes as an interesting candidate for investigating their potential to function in wood development.

ARGOS was found when researchers were identifying genes that are responsible for lateral root formation, which is auxin-regulated. *ARGOS*, together with *ARL* and *ORGAN SIZE RELATED1* (*OSR1*), is part of a gene family called Organ Size Related (OSR) genes. OSR genes have the potential to increase plant organ size when overexpressed (Hu et al., 2003 and 2006, Feng et al., 2011). It was found that the *OSR* genes all share a conserved domain in their translated proteins, now referred to as the OSR domain. This domain alone is capable of increasing organ size, even without the rest of the protein (Feng et al., 2011). *ARGOS* increases the number of cells when overexpressed by lengthening cell proliferation, whereas *ARL* positively influences cell-size and *OSR1* incorporates both function. Feng et al. also determined that the OSR family is essentially redundant, for when *ARGOS* and *OSR1* are both overexpressed, there is no additional increase in organ size (Feng et al., 2011). Analysis of the regulation of *ARGOS* and *ARL* in 10-day old seedlings by hormones have shown that *ARGOS* and *ARL* are induced by auxin and cytokinin, (though expression is only slightly elevated in *ARL*), and *ARL* was found to be BR-inducible (Hu et al., 2006). *OSR1* expression is induced by ethylene and repressed by abscisic acid and epi-BL.

When the OSR genes' promoters were further analyzed for hormone-responsive elements using the Plant *Cis*-acting Regulatory DNA Elements database (PLACE) (Higo et al., 1999, Feng et al., 2011), Feng et al. found that there were ethylene, ABA-, and GA-responsive elements in the promoter of *OSR1*, and *ARGOS* and as well as the expected auxin-responsive elements, and ABA- and GA-responsive elements in the *ARL* promoter. This support the observed upregulation of *ARGOS* when *Arabidopsis* is treated with ethylene. Furthermore when co-expression is analyzed for OSR genes in

Arabidopsis, all three genes are found in a network with ethylene related genes, however *ARGOS* is indirectly connected to those genes via *OSR1* and *ARL* (Figure 1.2).

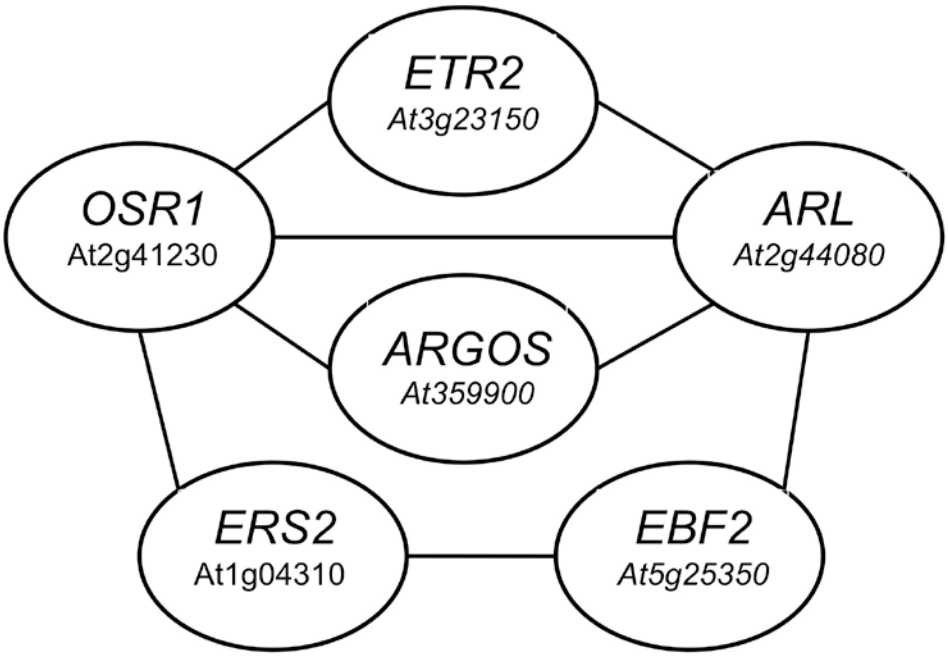

Figure 1.2: Gene cluster created with the help of atted.jp and modified to include the AGI codes. EIN3-binding F box protein (*EBF2*) has a direct correlation to *ARL*, which interacts with both *ARGOS* and *OSR1*. Ethylene response 2 (*ETR2*), which is involved in ethylene perception, is coexpressed with *OSR1* and *ARL*. Ethylene response sensor 2 (*ERS2*) is also involved in ethylene perception and is associated with *OSR1*.

1.6 Project objectives

The objectives of my project were to understand the effect of ACC-treatment on xylem formation in *Arabidopsis* wild-type, *argos*, ethylene-insensitive (*ein3-1*) mutants, using ACC-treatment of *in vitro*, flowering *Arabidopsis* plants, sectioning of woody tissues (hypocotyls and inflorescence stems), combined with histochemical stains for lignin and cellulose, as well as Fournier-transform infrared spectroscopy for chemical wood fingerprinting. A second object consisted of analyzing *ARGOS* and *ARL* expression in hypocotyls of ACC or water-treated *Arabidopsis* plants. This analysis was carried out by qPCR. The overall aim is to fill the gap in knowledge where

ethylene’s influence on *Arabidopsis* xylem formation is concerned, and find out whether *ARGOS* and *ARL* are involved in this process.

2 Materials and Methods

2.1 Plant Material

Arabidopsis thaliana (*A. thaliana*) ecotype 'Columbia' (Col0) was used as a wild-type control. Homozygous seeds of the T-DNA insertion line GK-627B07 (NASC N759439), in which the T-DNA is inserted into the 5'UTR of *ARGOS* (AT3G59900), resulting in *ARGOS* expression 2-3x less than wild-type, and the ethylene insensitive EMS-mutated line *ein3-1* (NASC N8052, Ecker et al., 1995) were used as experimental lines. The transgenic lines used were from a Col0 background.

2.2 Growth Conditions

The seeds were surface sterilized by rinsing them in 70% ethanol, 0.1% Tween, washing them 8 min with Bayrochlore in 70% ethanol, and rinsing them twice with 95% ethanol before letting them dry. The seeds were plated on ½ Murashige & Skoog media (MS), 0.5% sucrose and 0.8% plant agar, pH 5.8, and allowed to stratify in the dark for two days at 4° C. Seeds were germinated in long day growth chambers. On day 4 after germination, the seedlings were transferred to clear 10cm high polypropylene containers with a filter on the lid to allow for gas exchange while remaining sterile. One seedling was placed into each tub with 100 ml of 1 x MS and 0.8% plant agar, pH 5.8. The plants were grown in a controlled growth room with 18-hour daylight, and 6 hours of dark, a light intensity of approximately 200 µmol/m^{-2}/s^{-1}, and 22° C during the day, 18° C at night.

Figure 2.1 shows the in vitro set up. Seedlings were placed in the tubs 4 days after germination. The first picture on the top row shows the seedling 4 days after transfer to the pot. After about 18 days, the plants began to bolt. When the plants reached 8.5 cm (or in some cases a little more), around three-four weeks after transfer, they were treated with 10 or 100 µM ACC, or sterilized water. After treatment, a second pot was placed on top of the first to allow the plant freedom to continue growing, unimpeded. The bottom row of pictures shows the xylem development at different growth times, stained with phloroglucinol to show lignification progress. At 24 days, the xylem I is well developed and the xylem II starts to grow soon after, when the plant is around

8.5 cm tall. In this figure, which is from previous work, the 8.5 cm tall plant had already begun to form xylem II, but in the samples that were harvested before treatment for my experiment, most of the plants were treated before xylem II development had begun, or just after the start, but earlier in development than shown in Figure 2.1.

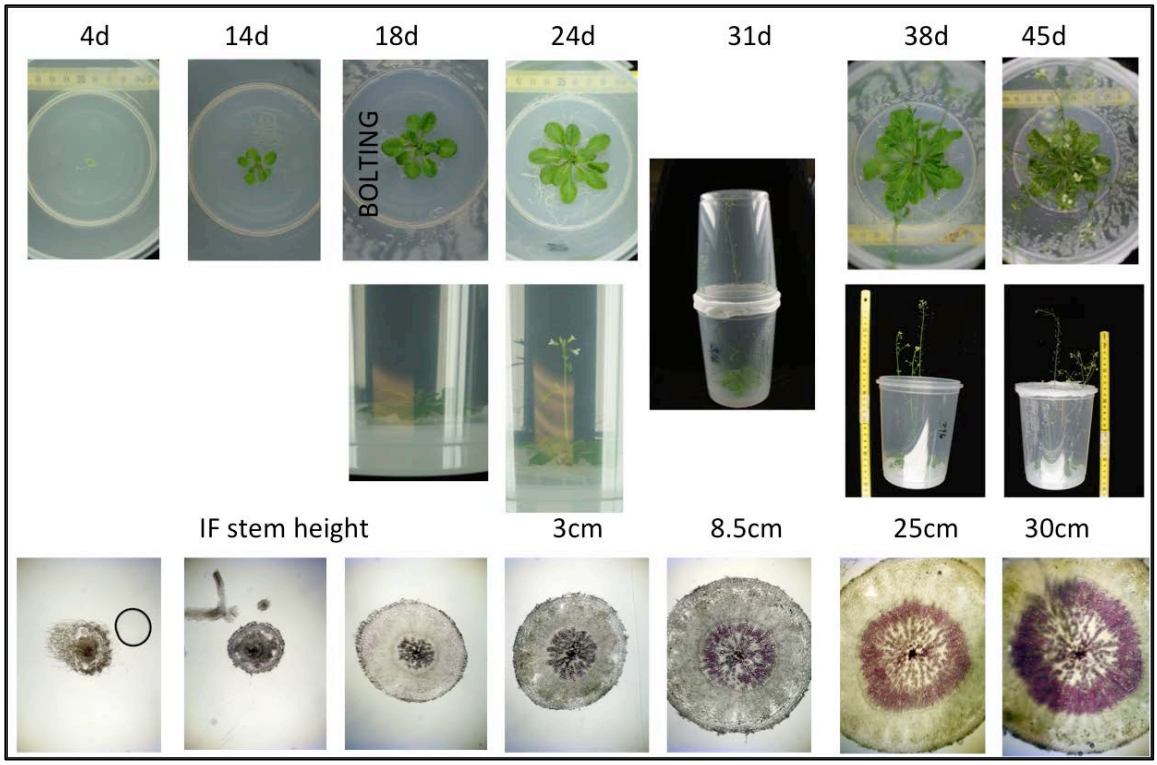

Figure 2.1: This shows the *in vitro* culture system for ACC-treatments if flowering *Arabidopsis* plants. First row: days after transfer to pots, first row of pictures: rosettes, second row of pictures: IF height (note: picture at 31 day time point is not the correct height of IF stem, it is just to indicate that plants were treated at this point and that the lid was replaces by a second pot allow room for free growth). Third row of pictures: shows the development of the xylem at each time point. However, in the 31 day picture, xylem II development has already begun, and that was not the case for the majority of my samples. (Figure credit: Judith Felten)

2.3 ACC-treatment

After the inflorescence stem reached the lid of the *in vitro* pot, or between 8 and 12 cm, the plants were treated. The plants that were treated with 10 µM ACC, were given 2 ml of 0.5 mM ACC dissolved in sterilized distilled water. This resulted in a total concentration of 10 µM ACC in 100 ml of MS medium. The 100 µM treated plants were given 2 ml of 5 mM ACC. The H_2O control plants were given 2 ml of sterilized distilled water. The ACC or H_2O solution for all treatments was prepared fresh before treatment and

distributed around the base of the plant with a pipette, avoiding the rosette leaves to ensure that the roots took up the ACC, instead of being absorbed through the leaves. After treatment the plants were allowed to grow for 2 weeks with a second pot from over the first from the time of treatment on, to allow the inflorescence stem to continue growing freely.

2.4 Harvesting

Two weeks after treatment, the hypocotyls and first 3 cm of the inflorescent stems were harvested. The plant was removed from the growth media (height measurements were taken at this time) and the hypocotyls were collected from the rest of the plant by cutting just below the rosette leaves and above the roots. The hypocotyls of *in vitro* grown Arabidopsis are often twisted and shorter than in soil grown plants, and sometimes difficult to distinguish from roots. Immediately after each sample was harvested, it was put into a labeled Eppendorf tube and flash frozen in liquid nitrogen to preserve the structural integrity, as well as to prevent the RNA from being degraded before extraction, and then stored in a -80° C freezer until used.

2.4 Height measurements

Inflorescence stem height was measured at the time of treatment by making a mark on the side of the pot, with the lid closed to maintain the sterile environment. When the plants were harvested, the length of the inflorescent stem was measured with a ruler and average height growth during treatment was calculated and plotted in Excel. A Student t-test was used to determine the significance of the results and to determine if the resulting height differences were due to the treatment or if it was just natural variation.

2.5 RNA Extraction

Four extraction methods were tested to determine the most effective process for RNA isolation from in vitro grown hypocotyls of flowering Arabidopsis plants. The hypocotyl was ground in 2 mL Eppendorf tubes with a

3mm steel bead using a Retsch bead mill (3x 30sec, 25Hz). The first extraction procedure was the Bio-Rad Aurum™ Total RNA Mini kit. The second and third were QIAGEN RNeasy® Plant Mini Kit with either RLT or RLC extraction buffer supplemented with 20 mg PEG8000 per 0.5 mL RLC extraction buffer. PEG8000 helps to scavange polyphenols from plant tissues that may negatively interfere with the extraction. The fourth method tested was the CTAB (hexadecyltrimethylammoniumbromide) extraction (Chang, 1993), which proved to have the greatest yield of RNA from the hypocotyls (results of extraction test shown in Table 2.1). 750 uL of warm CTAB extraction buffer (10 g CTAB, 50 ml 1 M Tris-HCl, 25 ml 0.5 M EDTA, 200 ml 5 M NaCl, 205 ml dH_2O, 10 g PVP and 20 µl β-mercaptoethanol per 1 ml extraction buffer) was added to the frozen, ground material, and incubated for 1-3 minutes on a 65° C heating block. The phases were separated with 750 µl of chloroform:isoamylalcohol (24:1), centrifuged at full speed (13,000 rpm) for 15 minutes at room temperature. The upper layer was transferred to a new tube, separated again with another 750 µl chloroform:IAA, and centrifuged the same as before. The RNA was precipitated out of the upper phase with ¼ volume of 10 M lithium chloride by freezing for two hours at -20° C. After that, the samples were centrifuged at full speed again, the supernatant was discarded and the pellet resuspended in 500 µl RNAse free water (DEPC H_2O), and the RNA allowed to precipitate overnight at -20° C with 1/10 volume of sodium acetate and 2 volumes of absolute ethanol. The next day, it was centrifuged at full speed for 15 minutes and 4° C, then washed with 1 ml of 70% ethanol and allowed to dry. Finally, it was resuspended in 50 µl DEPC H_2O. The concentration of RNA was measured on a Nanodrop spectrophotometer.

After extraction the samples were treated with DNase I to remove the genomic DNA that was still in the sample. To each sample tube, 5 µl of DNase I buffer (10X DNase I Buffer, 100 mM Tris pH 7.5, 25 mM MgCl2, 5 mM CaCl2) and 1 µl of DNase I enzyme were added and incubated at 37° C. Nucleotide concentration of the CTAB extraction after DNase treatment is given in Table 2.1.

RNA was extracted from Col0, *argos*, and *ein3-1* plants that had been treated with either water or 10 µM ACC.

Table 2.1: RNA yield for each extraction method. CTAB was the only method used which yielded sufficient amounts of RNA to proceed with DNase treatment to remove DNA.

Extraction method	RNA yield before DNase treatment	Yield after DNase treatment
CTAB	105.4 ng/µl	81.3 ng/µl
Aurum	3.7 ng/µl	N/A
Qiagen-RLT	1.7 ng/µl	N/A
Qiagen-RLC	5.3 ng/µl	N/A

2.5.1 cDNA production

Reverse transcriptase was used to synthesize cDNA from the extracted mRNA. Approximately 150 ng (volumes were rounded to the nearest 1/10 of a µl) of mRNA from each sample was added to 4 µl of 5x iScript™Reverse Transcription Supermix (reverse transcriptase, RNase inhibitor, dNTPs, $MgCl_2$), 1 µl of enzyme mix, brought to a total volume of 20 µl with DEPC water, and were incubated in a thermocycler for 30 minutes at 42° C, 5 min at 85° C, and kept at 4° C until they could be put into the freezer.

2.6 Gene Expression

Quantitative-PCR (qPCR, also called real-time PCR) was accomplished with a Bio-Rad C1000 Thermal Cycler® with CFX96 Real-Time System. Each 15 µl PCR reaction mixture consisted of 7.5 µl iQ™ SYBR® Green Supermix (SYBR® Green I dye, 50 U/ml iTaq™ DNA polymerase, 0.4 mM each dNTP, 6 mM $MgCl_2$, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, 20 nM fluorescein), 2.75 µl of 1.6 µM forward and reverse primers, and 2 µl diluted cDNA (a dilution series was used for the primer efficiency test, see section 2.6.2 for dilutions). The thermocycler settings for these reactions were: 95° C 5 min, (95° C 30 seconds, 55° C 30 seconds, 72° C 30 seconds) x 30 cycles, 72° C 5 min, then hold at 4° C. Melting curves, with one-half-degree

increases, were recorded to test for specific amplification, and ensure the absence of primer dimers. The measurements made by the Real-Time System were recorded and calculated by the Bio-Rad CFX Manager™. All solutions were thoroughly mixed at each step of setting up the plates before each run to ensure that the reagent and cDNA concentrations were uniform throughout the solutions.

2.6.1 Selection of Reference Genes

Gutierrez et al. validated the stability of several commonly used reference genes (Gutierrez et al., 2008). Based upon the findings of the paper, *EF1*α (elongation factor 1-α) and *APT1* (adenine phosphoribosyltransferase 1) were two of the most stably expressed genes in *Arabidopsis* and were used in this study. In addition, *UBQ10* (Polyubiquitin) and Clathrin, commonly used in the host-lab, were included as reference genes. The four reference genes used, their primers, and fragment sizes are given in a table below (Table 2.2)

2.6.2 Primer Efficiencies

The efficiencies of the primers were determined by making a plate which used a mix of 1 µl from each cDNA sample, and a dilution series of 1/5, 1/25, 1/125, 1/625, and 1/3125 from this mix. A technical replicate was made for each reaction. All of the primers were included on this plate, and each with the full dilution series, (as well as a duplicated set of water wells as negative controls,) so they could be compared to one another. Primer efficiencies are given in Table 2.3.

2.6.3 Expression of *ARGOS* and *ARL*

Each qPCR plate was designed to run technical replicates of all samples for two different primers per plate.

Table 2.2: Primer sequences for the four reference genes used in qPCR gene expression analysis, with fragment size of PCR product.

Gene	Primer Sequence	Fragment size (bp)
UBQ10	ATCACCCTTGAAGTGGA GAAACCACCACGAAGAC	194
APT1	GAGACATTTTGCGTGGGATT CGGGGATTTTAAGTGGAACA	348
Clathrin	GTTTGGGAGAAGAGCGGTTA CTGATGTCACTGAACCTGAACTG	200
EF1α	TGGTGACGCTGGTATGGTT TCCTTCTTGTCCACGCTCTT	264

Table 2.3: Primer efficiencies as calculated by Bio-Rad CFX Manager^{TM} as well as R^2 value.

Primer	Efficiency	R^2
CLATHRIN	83.4%	0.954
ARGOS1	114.5%	0.979
UBQ10	92.9%	0.999
ARL1	109%	0.996
EF1α	98.2%	0.991
APT1	122.2%	0.932
ERF1	184.8%	0.992

2.7 Sectioning

The collected hypocotyls and inflorescence stems that would be stained were sectioned with a Microm cryotome at a thickness of 50 µm, while being kept at -19° C. The samples designated for Fourier transform infrared spectroscopy (FTIR) analysis were sectioned at 18 µm thickness. The samples were attached to the sample holder using freezing mounting

medium, and then sectioned at the desired thickness. The mounting medium was washed away with water before staining/drying the sections.

2.8 Histochemical Staining

The sections were stained with two different stains that differentiate different cell wall components. Phloroglucinol HCl (1.0 g Phloroglucinol, 100 ml ethanol, 16 ml concentrated HCl) is used to stain lignin. When the section is exposed to phloroglucinol it will turn pink to red, depending on the lignin content, with the darker the red color indicating more lignin. The Safranin/ Alcian Blue mix (1 g Alcian Blue, 2.5 ml glacial acetic acid, 0.2 ml formalin 40%, then bring volume to 100 ml with dH_2O) stains the lignified cells red, while the cells with polysaccharide rich primary cell walls blue to green. Phloroglucinol was used to highlight the presence of lignin and was used considerably. While the safranin/alcian blue could have been used to determine cambial changes in cell size or number, this did not get analyzed for lack of time.

2.9 Xylem and lignification measurements

The stained sections were used for microscopic observations and subsequent image analysis to characterize lignification pattern induced by ACC-treatments. ImageJ was used to measure the ratio of xylem II to total xylem, first by measuring from the center of the xylem I to the outer edge of the xylem II, then the inside point of the measuring line was moved to the inner edge of the xylem II (figure 2.2). The width of xylem II was then divided by the total xylem to get the percentage that was made up by the xylem II. The percentages were compared between treatments and between genotypes.

To measure how much of the xylem II that did not have gaps in lignification, I measured the xylem II all the way out to the furthest lignified cells, and then measured to where the bottom edge of the gaps began (figure 2.3). The percentage of xylem II without gaps was calculated by dividing the non-gapped xylem II by the total xylem. Water treated plants from all genotypes had no gaps, so they were designated as 100% lignified, in order

to be comparable to the ACC-treated plants.

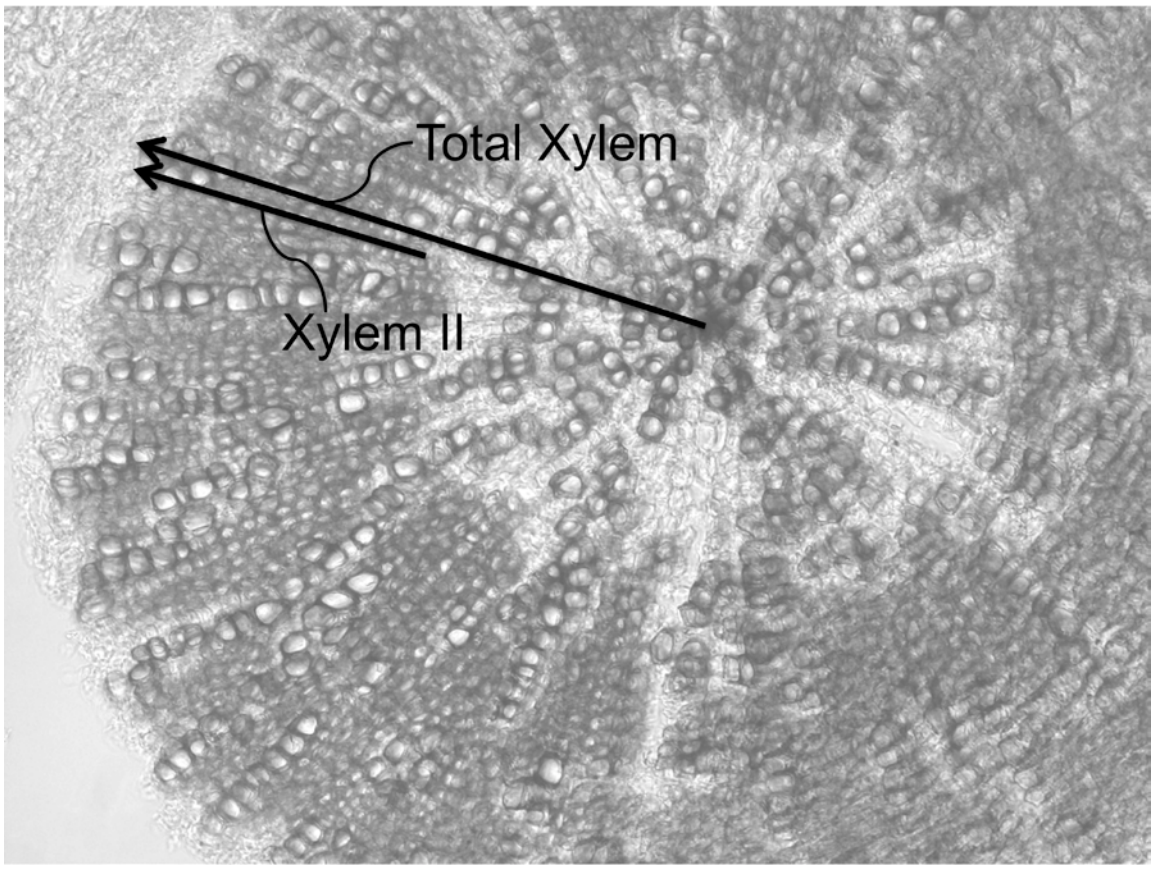

Figure 2.2 Measurement of the proportion of xylem II to total xylem.

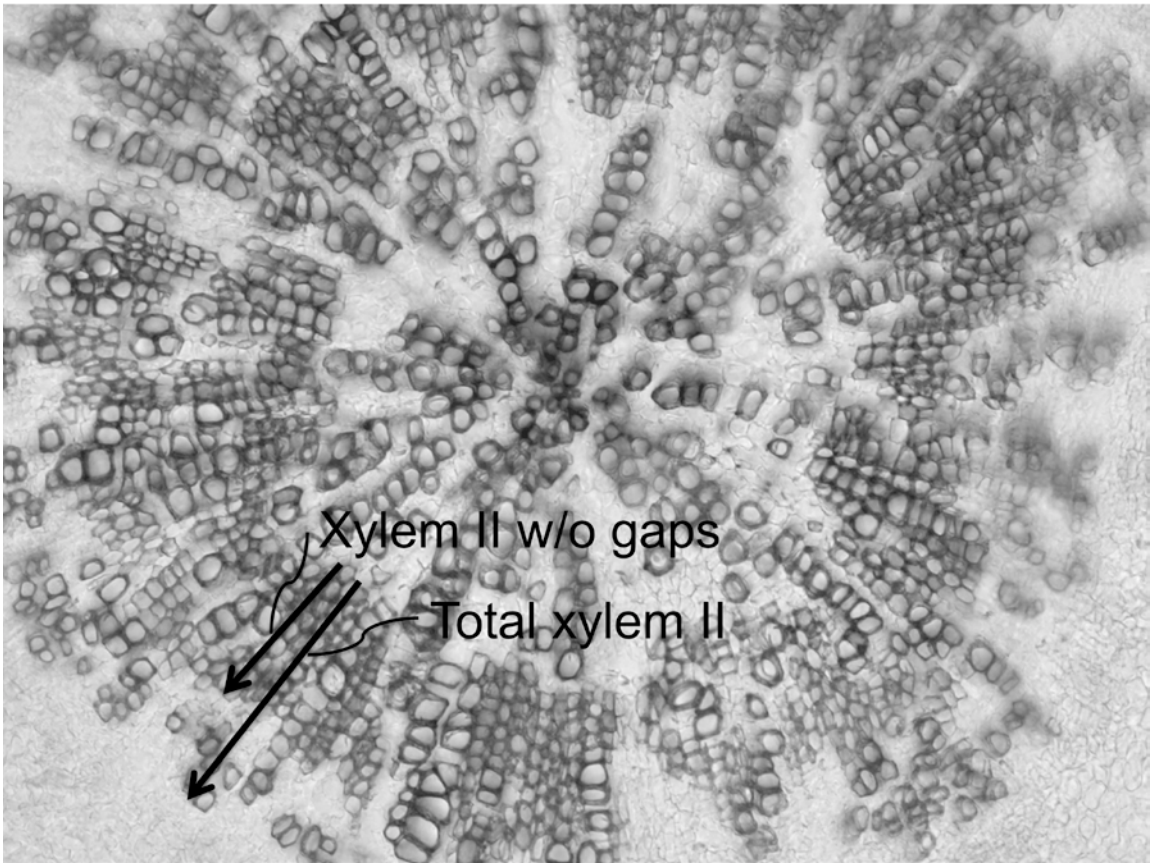

Figure 2.3 Measurement of the percentage of xylem II without gaps in lignification

2.9 Fourier transform infrared microspectroscopy

The 18 µm thick hypocotyl sections were dried between two microscope slides. Slides were cracked open and sections were carefully scraped off the glass slides and transferred to an FTIR-transparent BaF_2 crystal. Spectra from two 0.17 x 0.17mm (64x64 pixel) sized positions (see Figure 2.4) for each section (i.e. 2 images per genotype/treatment and 3-4 biological replicates) were recorded on a Bruker Tensor 27 spectrometer equipped with a microscopy accessory (Hyperion 3000) including a 64x64 focal plane array (FPA) detector (Bruker Optics), providing a maximum spatial resolution of approximately 5 µm at 4000cm^{-1}. Visual photographs of the samples for assigning pixels for data analysis within the recorded area were taken by a Sony Exwave HAD colour digital video camera (http://www.sony.com/) mounted on the top of the microscope. The sample

tray was boxed, and the chamber was continuously purged with dry air. Spectra were recorded in transmission mode over the range of 900–3900 cm^{-1} with a spectral resolution of $4cm^{-1}$. For each image, 32 interferograms were co-added to obtain high signal-to-noise ratios. Prior to sample measurements, background spectra were recorded for each sample at a nearby empty spot on the BaF_2 crystal with the same number of scans.

2.10 Data Analysis

2.10.1 Interpreting qPCR Data

The primer efficiency data obtained from the Bio-Rad CFX Manager program and Normfinder (Andersen et al., 2004) was used to find the best reference gene to use to normalize the results from the *ARGOS* and *ARGOS-LIKE* (*ARL*) reactions. Once the expression values were normalized, the ACC-treated sample expression was compared to the H_2O treated samples to determine if the change in expression was due to the treatment.

The Cq values (the cycle number at which the fluorescence increased beyond the threshold of background fluorescence) for each target gene, and reference gene were determined by the CFX Manager. The Cq values were averaged between the technical replicates to get the average Cq of each biological replicate. The RQ (relative quantification) was obtained by subtracting the Cq of the target gene from the Cq of the chosen reference gene, and raising 2 by that number. The equation used is this: $RQ=2\text{^}(Cq_{reference} - Cq_{target})$. The RQ values were then rescaled by dividing each RQ by the lowest value for each target gene. Because the resulting values did not have normal distribution, they were then transformed by $\log_2$ in order to perform the Student t-tests to compare the results.

2.10.2 Image analysis

ImageJ was used to measure changes in wood formation as observed from stained sections. The radius of the xylem II, as well as the depth of unlignified gaps in the xylem, was measured in three to six places on each section, two sections per sample, on three or four samples for each genotype and treatment. The radius of the total xylem and the radius of only the xylem II

were also measured, in three places with the same number of sections as the other measurements. Those numbers were recorded in Microsoft Excel to determine whether changes were due to treatment. The size of the image was obtained from Axiovision software when the microscopy pictures were taken, and that information was used to set the scale in ImageJ.

2.10.3 FTIR-microspectroscopy data processing

For data analysis fiber spectra were selected from pixels that either showed strong or very weak intensity in the 1510 cm^{-1} band (aromatic –C=C– vibrations, indicative of lignification) (Figure 2.2). Fibers were visually identified on the sections (Figure 2.2). Spectra with high or low intensity at 1510 cm^{-1} are denominated as originating from “lignified fibers” or “non-lignified fibers”, respectively. Spectra from 3 lignified and 3 non-lignified fibers per sections were exported and processed. Spectral treatments included 13-point smoothing, a two-point linear baseline correction between 906 and 1801 cm^{-1} and total sum (area) normalization over the same spectral range. All spectra were processed together in the previously described way.

2.10.4 Multivariate analysis

Multivariate analysis (unsupervised such as PCA,(Trygg et al., 2006); or supervised with pre-defined classes such as Orthogonal Projections of Latent Structures Discriminant Analysis, OPLS-DA (Trygg and Wold, 2002; Stenlund et al., 2008)) were employed for analyzing FTIR microspectroscopy data as described before (Gorzsás et al. (2011). All analyses were carried out in SIMCA-P+, version 12.0 (Umetrics AB, Umeå, Sweden).

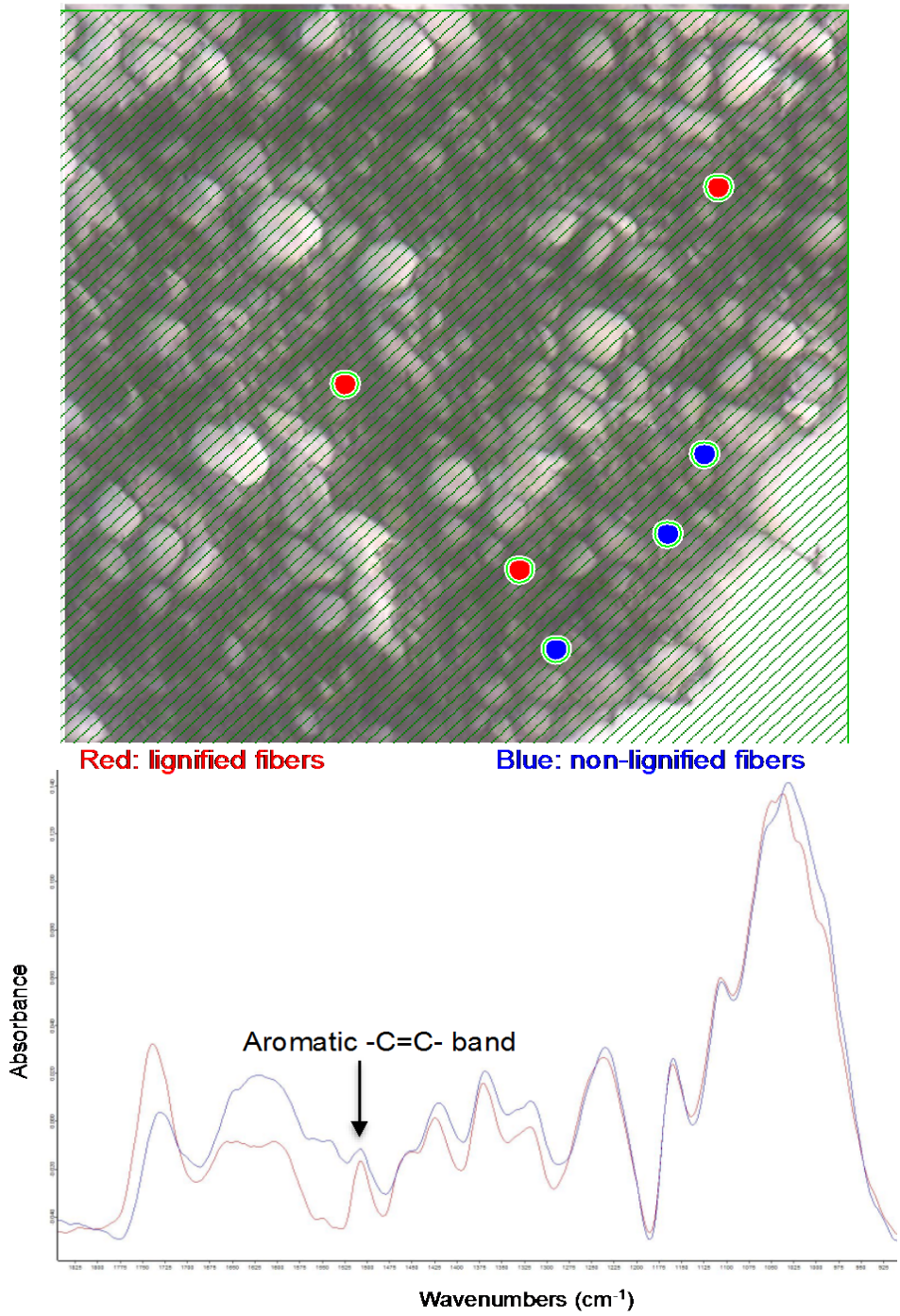

Figure 2.4: Example of lignified and non-lignified fibers chosen from the images obtained by FTIR spectroscopy. Note the intensity of the band at 1510 cm^{-1} in the red- and blue-labeled spectra (corresponding to red and blue labels on the sections, respectively). Section: *argos* mutant, similar sections and classification were obtained for Col0 and *ein3-1* independently of treatments.

3 Results

3.1 Influence of ACC on overall plant growth

Ethylene is known to influence elongation growth in plants. We measured the height of inflorescence stems in ACC and water-treated plants to investigate the impact of the different applied ACC concentrations on overall plant growth.

In the wild-type, the 100 µM ACC-treatment led to a significant reduction of inflorescence growth compared to water-treated plants (Figure 3.1). However there was no significant impact on inflorescence growth in the 10 µM ACC-treatment. This points to an ACC-dose response of inflorescence growth in Col0. Although there was a trend towards height growth decrease at 10 and 100 µM ACC-treatment in the *argos* mutant, this change was not significant. In the *ein3-1* genotype that is supposed to be ethylene insensitive, height growth was decreased with 10 µM ACC-treatment, and even more so at 100 µM ACC-treatment (by 41% compared to water-treated). This result was unexpected.

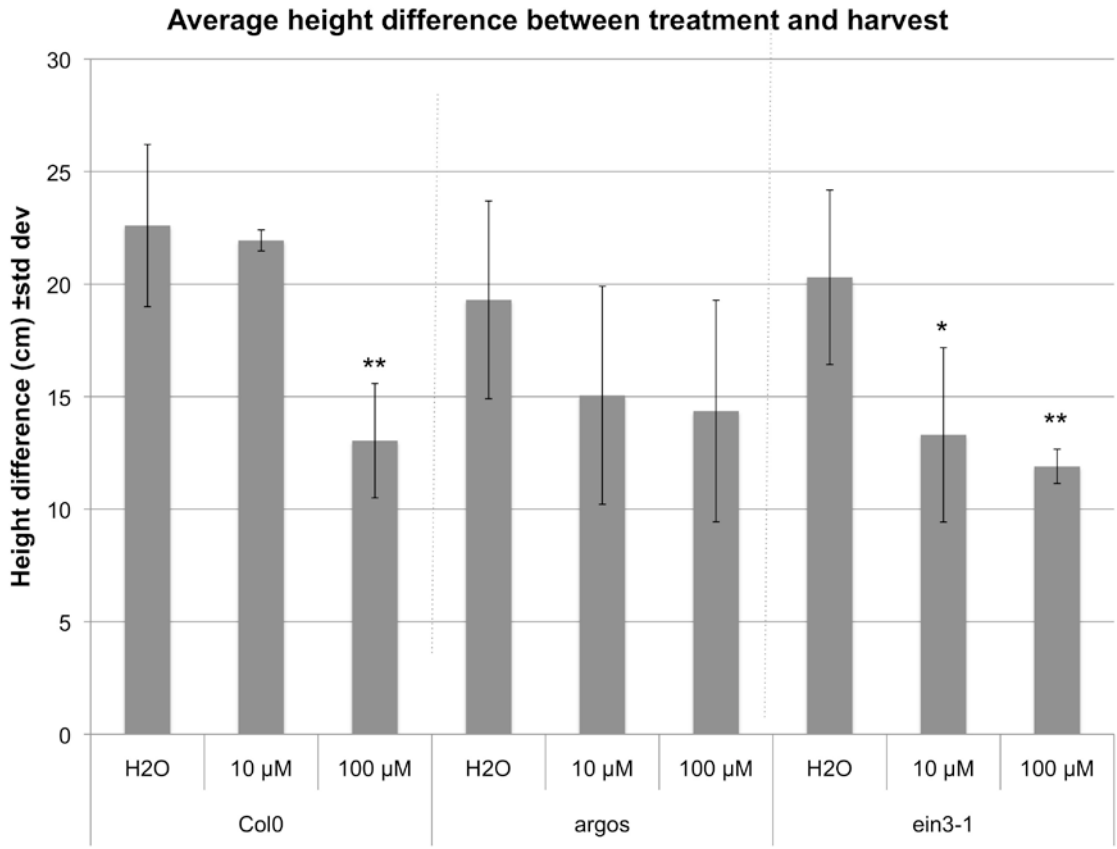

Figure 3.1: Average height growth of the inflorescence stem after treatment of Col0, *argos,* and *ein3-1* with different concentration of ACC. The height of inflorescence stems (4-5 plants per treatment) was measured before and after treatment and the differences between those two heights averaged for plants of the same genotype and treatment. The resulting p-values of two-tailed t-tests between treatments are marked with "*" when less than 0.05, and "" when less than 0.01. An ACC-treatment of 10 µM was enough to impair inflorescence stem growth in *ein3-1*, and a 100 µM treatment affected both Col0 and *ein3-1*. Error bars on this and all following charts are derived from the standard deviation for each average.**

3.2 Influence of ACC on ethylene related gene expression in the hypocotyl

ARGOS has been found to be greatly upregulated by ACC in poplar stems. We wanted to investigate whether *ARGOS* and its homolog *ARGOS-LIKE* (*ARL*) are also induced by ACC in the hypocotyl of *Arabidopsis*. In addition we wanted to know whether the *argos* mutant reacts the same way to ACC as wild-type plants. Ethylene Response Factor 1 (*ERF1*) in Arabidopsis is rapidly induced by ACC-treatment (An et al., 2001). We therefore used this gene as a reporter for ethylene responsiveness in our qPCR study. The results represented the relative expression of each target gene, to compare the expression of water and ACC-treated plants.

ARGOS showed a significant increase of 62-fold at 10 µM ACC-treatment in Col0 plants compared to the water treated controls (p-value 0.0003). ACC did not significantly enhance *ARGOS* expression in the *argos* mutants. The *ein3-1* mutants though, also had a significant increase in *ARGOS* expression with ACC-treatment over the water treatment. The increase from water treated to the ACC-treated *ein3-1* samples was 17-fold (p-value 0.014). *ARGOS* expression was increased by ACC-treatment in Col0 and *ein3-1*, but not in the *argos* (Figure 3.2, black).

ARL transcript levels in wild-type plants were also induced by the ACC-treatment, with a 9.7-fold increase over water treated plants (p-value 0.001). In *argos* mutants, the increase was 27-fold (p-value 0.018). In *ein3-1* ACC failed to increase *ARL* levels significantly. The ACC-treatment caused an increase in expression of *ARL1* in the wild-type and in *argos*, but not in the *ein3-1* mutant (Figure 3.2, white).

ERF1 was used as a positive control to be sure that ACC was in fact recognized, and causing an ethylene response, and indeed, the levels of *ERF1* were greatly increased in both the wild-type and *argos* mutant (Figure 3.2, grey). In Col0, *ERF1* expression increased by 7.6-fold (p-value 0.007). In *argos*, the expression increased by 68-fold (p-value 0.018). In *ein3-1* an apparent reduction of *ERF1* transcript in response to ACC was observed, but this change was not significant.

Taken together, ACC-treatment enhanced expression of all three target genes in the wild-type, of *ARL* and *ERF* in the *argos* mutants, and of *ARGOS* in *ein3-1*. *ARGOS* and *ARL* seem to be ACC-inducible genes but at least for *ARGOS*, the absences of *EIN3* is not enough to inhibit this response.

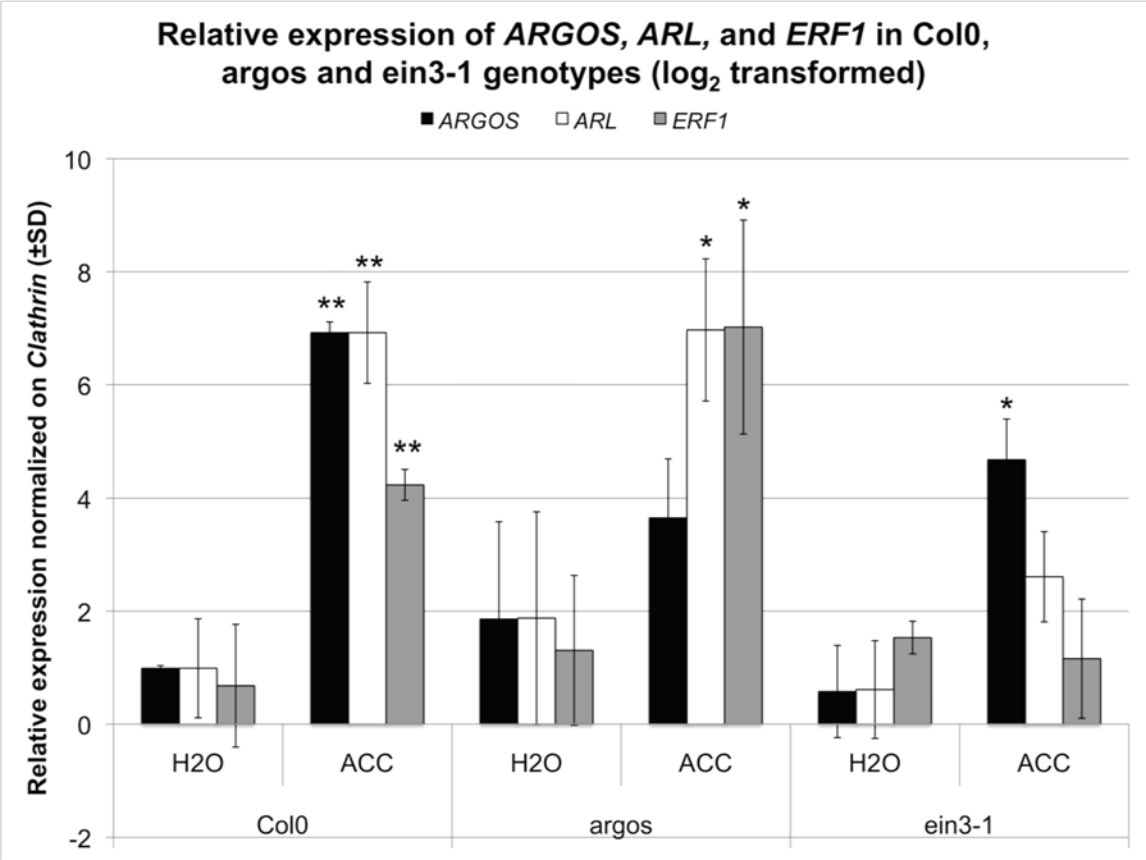

Figure 3.2: Relative gene expression for *ARGOS (*black*), ARL* (white*), and ERF1* (grey) in Col0, *argos* and *ein3-1* hypocotyls. The expression was normalized on *Clathrin*, and rescaled to the lowest expression before averaging expression for each genotype and treatment.

3.3 Wood formation in the hypocotyl under the influence of ACC

The xylem formed in the hypocotyl of *ein3-1* plants was phenotypically quite different from the other two genotypes. Both wild-type and *argos* hypocotyls had the usual organization of xylem; an orderly xylem I and II, with patterns of cells approximately radiating out from the center, similar to spokes in a wheel. The *ein3-1* hyopotyls on the other hand were quite disorganized. The xylem I lacked the almost ray-like arrangement of vessels and instead had what looked like randomly spaced lignified vessels. The xylem II appears to be slightly more organized, but is not quite as round as the other two genotypes, though this could have been due to the growth of the hypocotyls, which were more distorted and twisted than seen in the other two genotypes, and it was more difficult to get a clean cross-section of pure hypocotyl without sectioning into the adjacent root tissues. The insensitivity to ethylene may be

involved in this disarrangement, but the common consensus is that ethylene insensitivity does not affect development in the absence of stress. However, the FT-IR results suggest that ACC-treatment still reduced the amount of lignin in the lignified cells, compared to water treated.

We next studied the effect of 10 and 100 uM ACC on xylem formation in Arabidopsis hypocotyls and investigated whether these responses were altered in *argos* and *ein3-1* mutants. Wood formation in the hypocotyl varied between the genotypes of *Arabidopsis*, and between the different treatments. The proportion of xylem II was measured in transverse sections from three plants within each genotype and treatment. We observed gaps in the lignification at the periphery of xylem II in ACC-treated plants, and measured the proportion of xylem II that was free of these gaps. The Phloroglucinol stain (Figure 3.3) was best for observing lignification patterns, but the unlignified cells were very difficult to distinguish. Safranin/alcian blue staining was useful for visualizing the cells that were not lignified, especially at higher magnification (Figure 3.4).

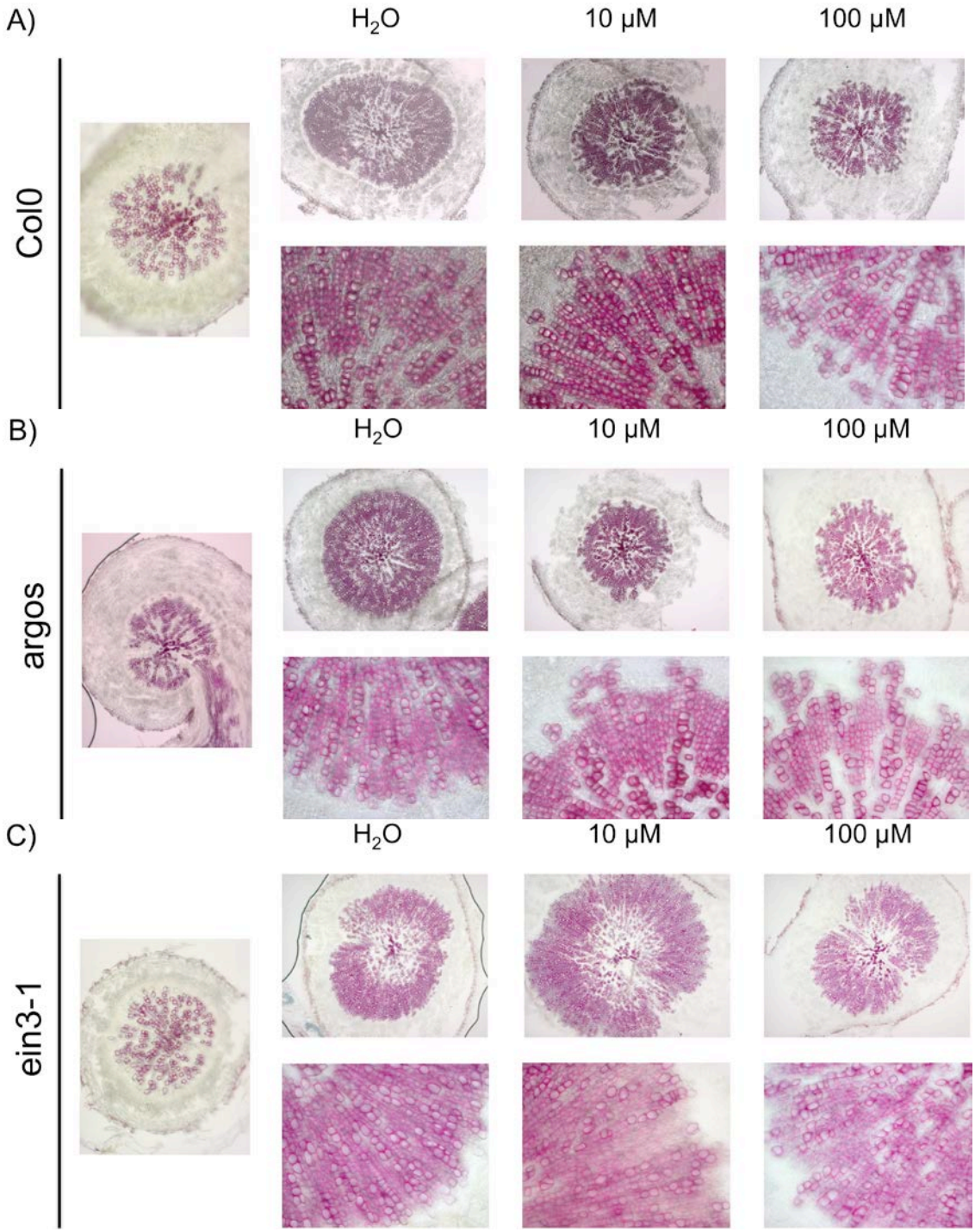

Figure 3.3: *Arabidopsis* hypocotyls stained with phloroglucinol to reveal lignification pattern. A-C show sections from their respective plant genotypes, with (left to right) a representative section from each genotype before treatment; water, 10 µM and 100 µM ACC, 10x magnification in the top row, 20x on the bottom. Sections from Col0 (A) *argos* (B) and *ein3-1* (C).

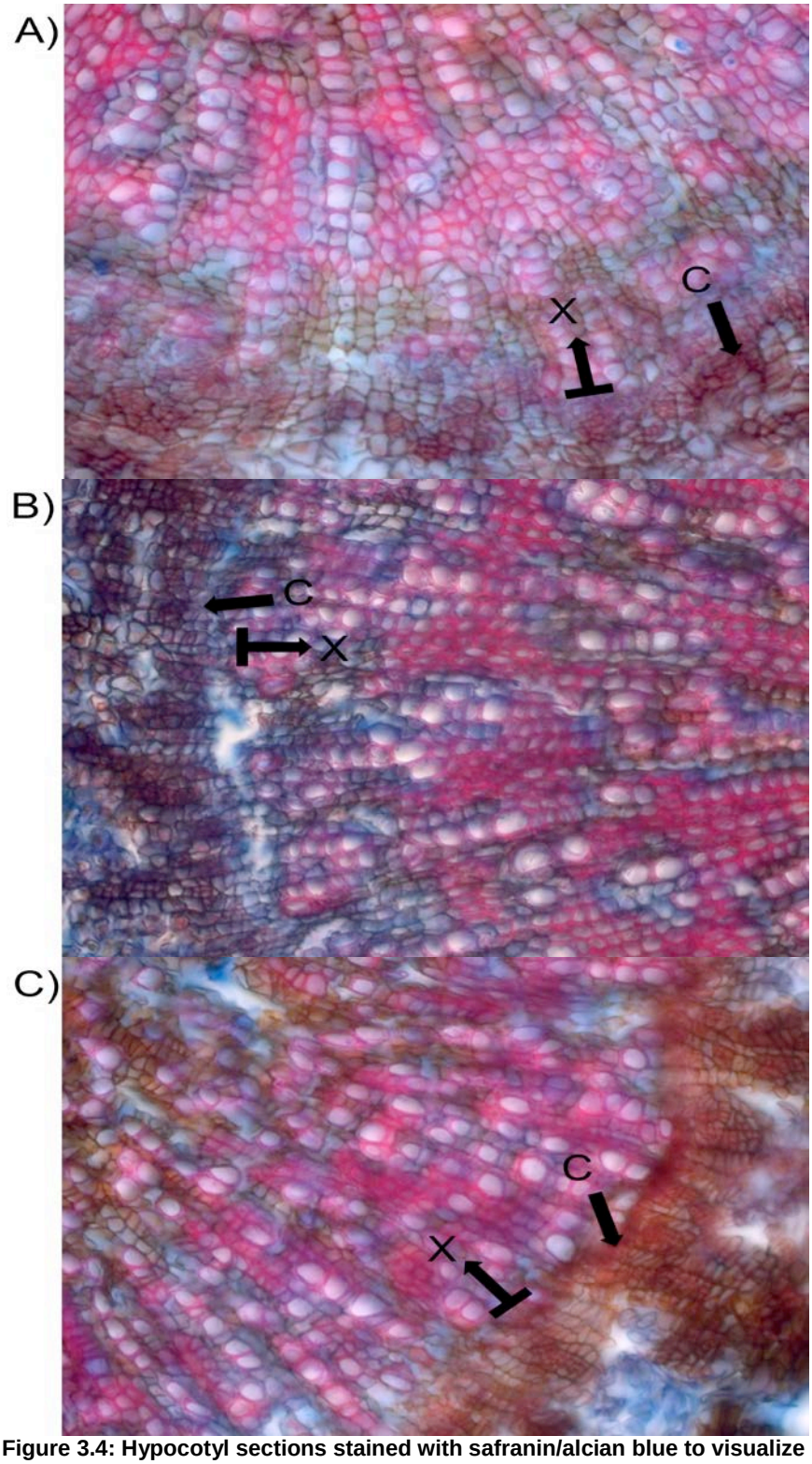

Figure 3.4: Hypocotyl sections stained with safranin/alcian blue to visualize morphology of unlignified cells (which stain blue-brown, lignified cells are red-pink) for 100 µM ACC-treated A) Col0, B) *argos*, and C) *ein3-1* genotypes. The periphery of xylem is shown, with regions phase I and II of the secondary xylem (X), as well as the cambium (C). 40x magnification.

3.3.1 Xylem growth

In control Col0 plants, the xylem II made up an average of 57% of the total xylem area in the hypocotyl. Because ACC-treatments were started at

the initiation of xylem II development, it took place under the influence of the treatment. In Col0, high concentrations of ACC (100 µM) had a significant effect on the amount of xylem II formed in the hypocotyl, with only 51% of the total xylem being made up of xylem II, which is 89% of the proportion in the water treated plants. There was no significant difference in xylem II area in the 10 µM ACC-treated plants compared to water treated ones (Figure 3.5, Col0).

In *argos* plants, the 10 µM treatment led to a reduction of 9% in the xylem II width (50% compared to 59% in the water treated plants). The 100 µM plants had 7% less xylem II than the water treated plants, decreasing to 52% of the total xylem (Figure 3.5, *argos*).

Col0 had significant reductions in proportion of xylem II with 100 µM ACC-treatment, while argos has significant changes with both concentrations. The proportion of xylem II in hypocotyls of *ein3-1* plants were not influenced by either 10 nor 100 µM ACC-treatments (Figure 3.5, *ein3-1*).

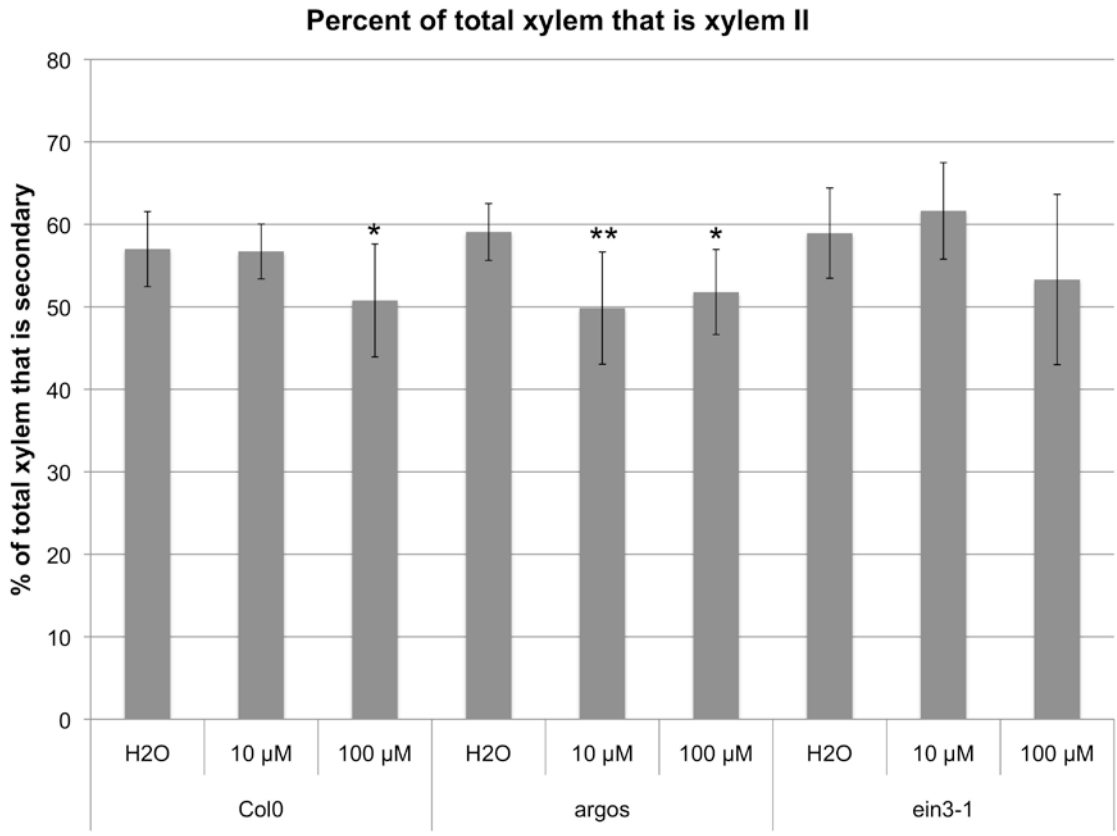

Figure 3.5: The percent of total xylem that was made up of xylem II is shown with the average percentage of each treatment and genotype. Significant changes are marked with "*" for a p-value ≤ 0.05 and "" for p ≤ 0.01.**

3.3.2 Fiber lignification

The ACC-treatment affected the way the xylem cells lignified, meaning that there were gaps where fibers (or very rarely, vessel elements) hadn't lignified, and lignification of fibers derived from the cambium during the treatment generally seemed to be retarded. In all three genotypes, the ACC-treatment caused gaps to form in the xylem II. The total width of xylem II, including the areas with gaps, was compared to the amount of xylem that did not have gaps in the lignification (Figure 3.6).

In Col0 plants, the water treated samples had no gaps in lignification, so the xylem was designated as 100% lignified. With 10 µM ACC-treatment, the Col0 plants developed gaps in lignification in the outer 37% of the xylem, and the plants treated with 100 µM had gaps in the outer 35%.

This trend was also seen in the *argos* mutant plants, with the treated plants having gaps in the lignified fibers of the secondary xylem, where the water treated plants did not. The unlignified regions of 10 and 100 µM occupy 38.7% and 35.8%, respectively.

In *ein3-1* plants, 10 µM ACC-treatment resulted in gaps covering 14.3% of the secondary xylem. With the higher treatment, only 8.1% of the secondary xylem had gaps.

In summary, ACC-treatment caused lignification gaps to appear in secondary xylem of all *Arabidopsis* genotypes, at both concentrations.

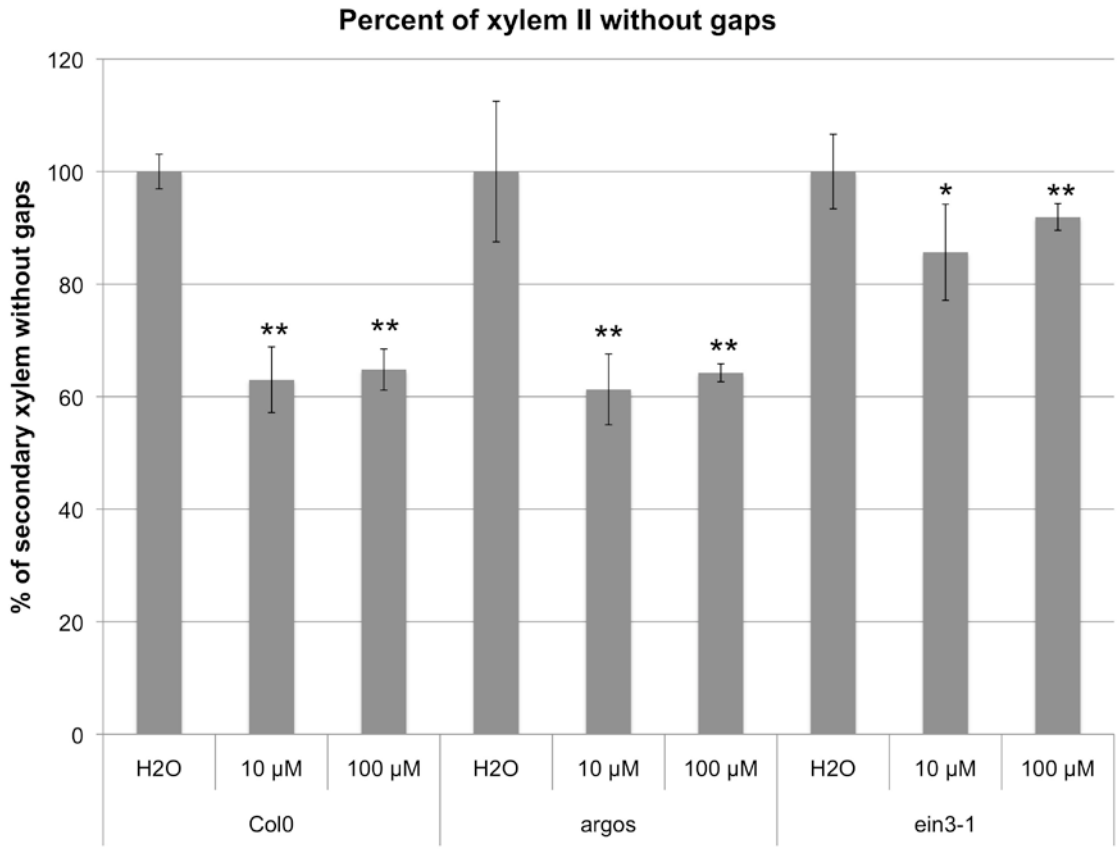

Figure 3.6: The percent of xylem II that was free of gaps in lignification. The t-test results are marked with "*" for p ≤ 0.05, and "" for p ≤ 0.01 to show their significance. The water treated samples had no gaps, and are therefore shown at 100% free of gaps. All ACC-treated genotypes had gaps, while none of the water treated plants had gaps in the xylem.**

3.4 Effect of ACC on secondary cell-wall composition in Arabidopsis hypocotyls

We used Fourier transform infrared (FTIR) microspectroscopy to investigate whether the ACC-treatment induced any chemical changes in fibers of *Arabidopsis* hypocotyls. FTIR spectroscopy, which yields a chemical fingerprint of the analyzed material, in combination with microscopy and a focal plane array detector, which provides spatial information, enables characterization of the chemical composition of different cell types (Gorzsás et al. 2011). Spectral information is extracted from distinct pixels in the recorded area. Because our anatomical analysis suggested a different lignification pattern in Arabidopsis hypocotyls after ACC-treatment compared to controls, we interpreted spectral information with regard to lignification pattern of the fibers and treated spectra from lignified and non-lignified fibers separately (see material and methods, section 2.9 and 2.10.3-4).

Multivariate analyses such as Principal Component Analysis (PCA) or Orthogonal Projections to Latent Structures Discriminate Analysis (OPLS-DA) are well adapted to identify and characterize cell wall chemotypes in different genotypes and/or treatments by using information from the full spectral range. We carried out a PCA on all 357 extracted spectra from all genotypes and treatments and colored the data either according to genotypes (results not shown), treatments (results not shown) or sampling position (lignified or non-lignified) (Figure 3.7) to investigate which of these parameters gave the largest variation between the samples. The result showed that the largest variation was obtained between lignified (red triangles) and non-lignified (blue triangles) fibers. There is a certain degree of overlap between both groups, which is explained by the fact that spectra from each group contain a varying amount of lignin, and also non-lignified fibers are not completely devoid of lignin (see Figure 2.2 in M&M) or spectra are influenced by lignified cells in the proximity due to spatial resolution constrains. The largest variation within those groups was seen in the non-lignified class. The separation of lignified and non-lignified spectra in the PCA supported our choice to treat spectral information from those respective fibers separately.

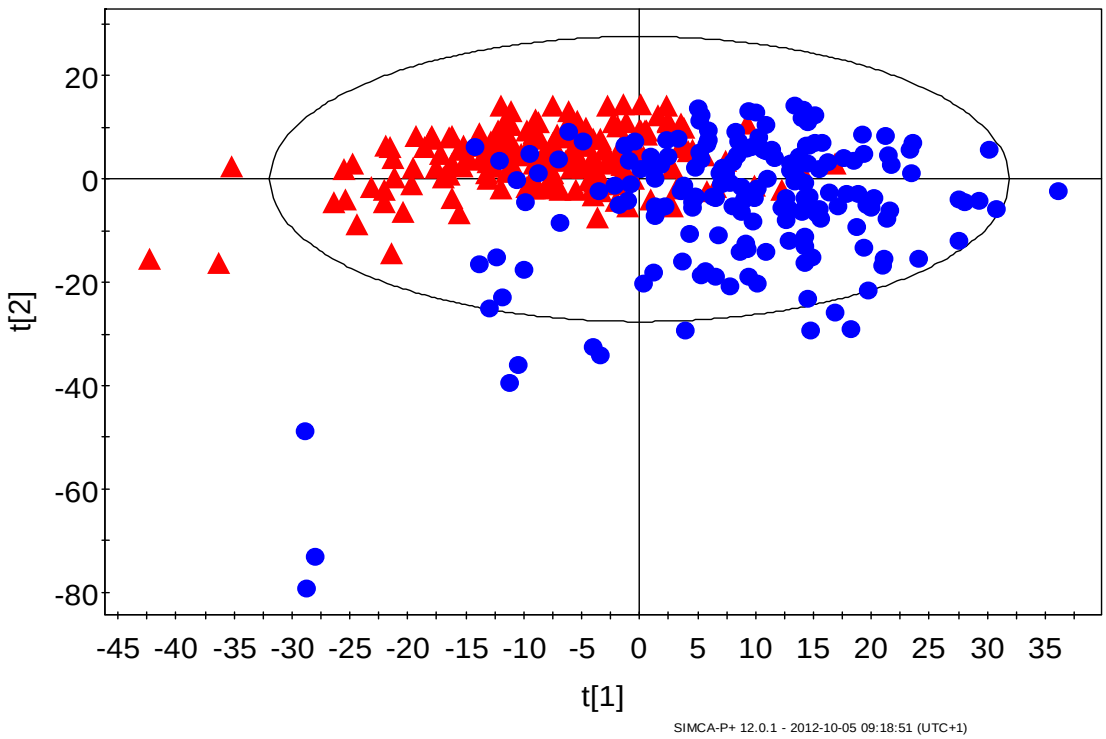

Fig 3.7: Principal component analysis (PCA) on the 357 obtained spectra from FTIR microscopy. The PCA has 17 components, R2X(cum)=0.977, Q2(cum)=0.964. Red triangles (lignified), blue dots (non-lignified)

For all further analysis OPLS-DA was used. This type of multivariate analysis is supervised and therefore based on pre-defined classes (here ACC or H_2O treatments) and separates the systematic variation in the FT-IR spectroscopic data into two parts: predictive and orthogonal components, which can be visualized in a Scores plot. Predictive components are correlated to the predefined classes and can originate e.g. from cell wall chemotypes. Orthogonal components are not correlated to the predefined classes and are caused by experimental (biological or technical) variation. The predictive variation between the classes is shown in a Loadings plot, from which the importance of different FT-IR bands for the separation of the classes can be identified. FT-IR bands can be diagnostic for cell-wall polymers (either as single bands, or as band combinations). Taken together, Scores plots can be used to understand whether two (or more) sample types are significantly different in their chemical fingerprints and Loadings plots are used to identify the origin of the chemical differences. The predictive ability of the classes in the Scores plot is given by the Q2-value, the higher the Q2 value the better defined and the more distinct are the chemotypes in the classes.

In order to check for ACC-induced wood chemotypes we have created Scores plots with fiber spectra from ACC versus water treated plants for the genotypes Col0, *ein3-1* and *argos*. If a dosage effect in the ACC response between 10 and 100 µM ACC-treatment exists (increasing difference in chemotypes with increasing hormone concentrations), we would expect stronger models (higher Q2 values) for "H_2O versus 100 µM" than for "H_2O versus 10 and 100 µM combined" cases. Details of the models obtained are listed in Table 3.1, Q2 values are additionally shown as histogram in Figure 3.8 and Scores Plots in Figure 3.9. Generally we found that ACC-treatment induced a difference in chemotypes in lignified and non-lignified fibers in both Col0 and *argos* mutants and to a much lesser extent in the ethylene insensitive mutant *ein3-1* (Table 3.1, Figure 3.8). Interestingly there was a

dosage effect of ACC in Col0 non-lignified but not in lignified ones. As for *ein3-1,* a dosage effect was seen in both lignified and non-lignified fibers with a bigger increase in Q2 values from 10 µM to 100 µM than in the wild-type. Finally, ACC-treatments on the *argos* mutant did not show any dosage effect, not even in non-lignified cells.

Table 3.1: OPLS-DA models for the various comparisons, all made with 1+2 (predictive + orthogonal) components. N = number of observations (=spectra), R2X = Cumulative sum of squares of the entire X matrix (wavenumbers) explained by all extracted components, R2Y = Cumulative sum of squares of all the y-variables (classes) explained by the extracted components, Q2 = Cumulative "predictive ability" (i.e. the fraction of the total variation that can be predicted) for all the x-variables and y-variables for the extracted components

Genotype	Comparison	Lignified cells				Non-lignified cells			
		N	R2X	R2Y	**Q2**	N	R2X	R2Y	**Q2**
Col0	H_2O vs 10/100µM ACC	54	0.709	0.793	**0.645**	54	0.757	0.639	**0.419**
	H_2O vs 100µM ACC	42	0.580	0.849	**0.649**	42	0.773	0.736	**0.611**
argos	H_2O vs 10/100µM ACC	63	0.653	0.774	**0.687**	63	0.543	0.862	**0.787**
	H_2O vs 100µM ACC	45	0.643	0.805	**0.707**	45	0.496	0.872	**0.786**
ein3-1	H_2O vs 10/100µM ACC	63	0.644	0.393	**0.135**	60	0.629	0.398	**0.067**
	H_2O vs 100µM ACC	40	0.686	0.546	**0.342**	36	0.639	0.614	**0.417**

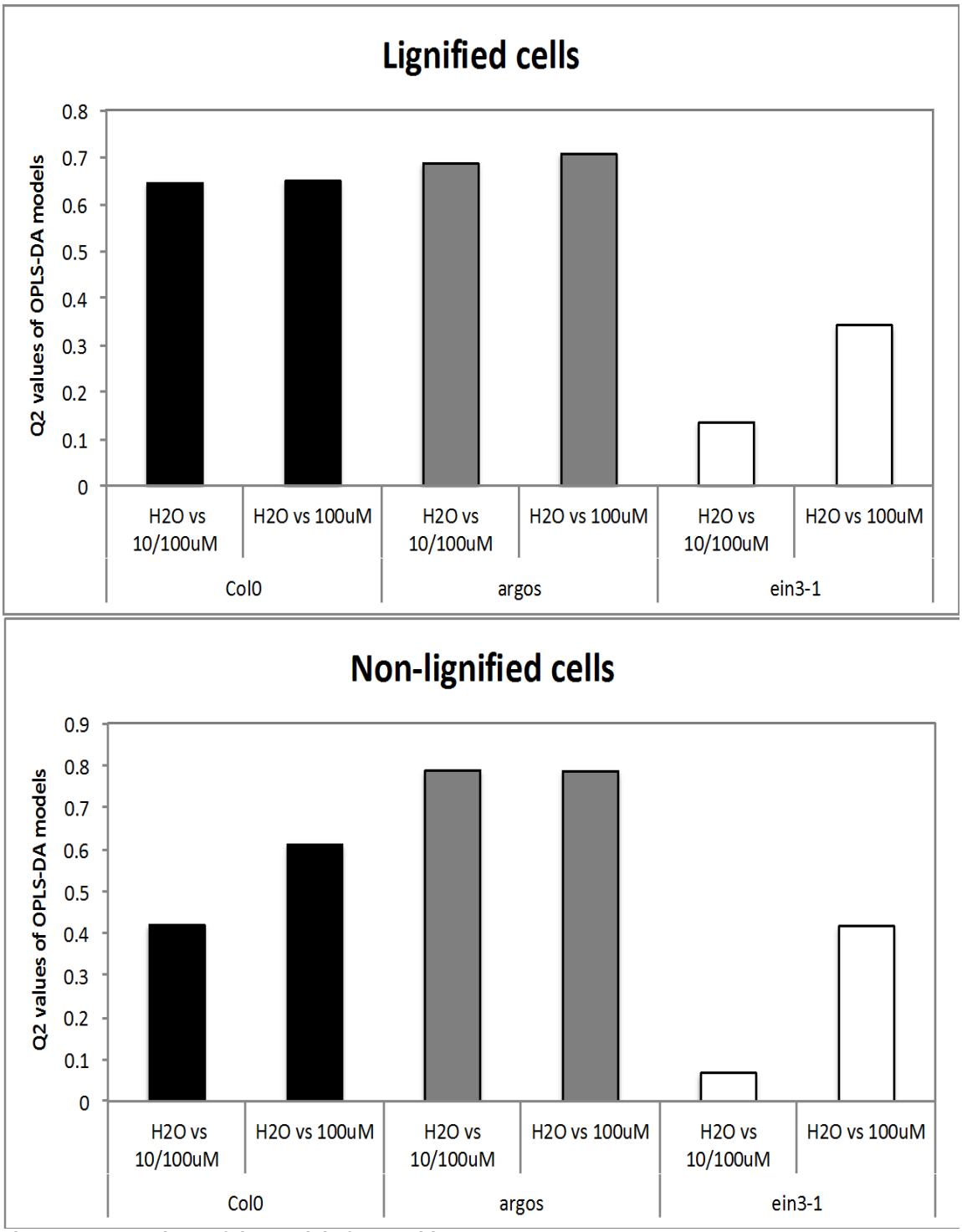

Figure 3.8: Q2 values of the models from Table 3.1

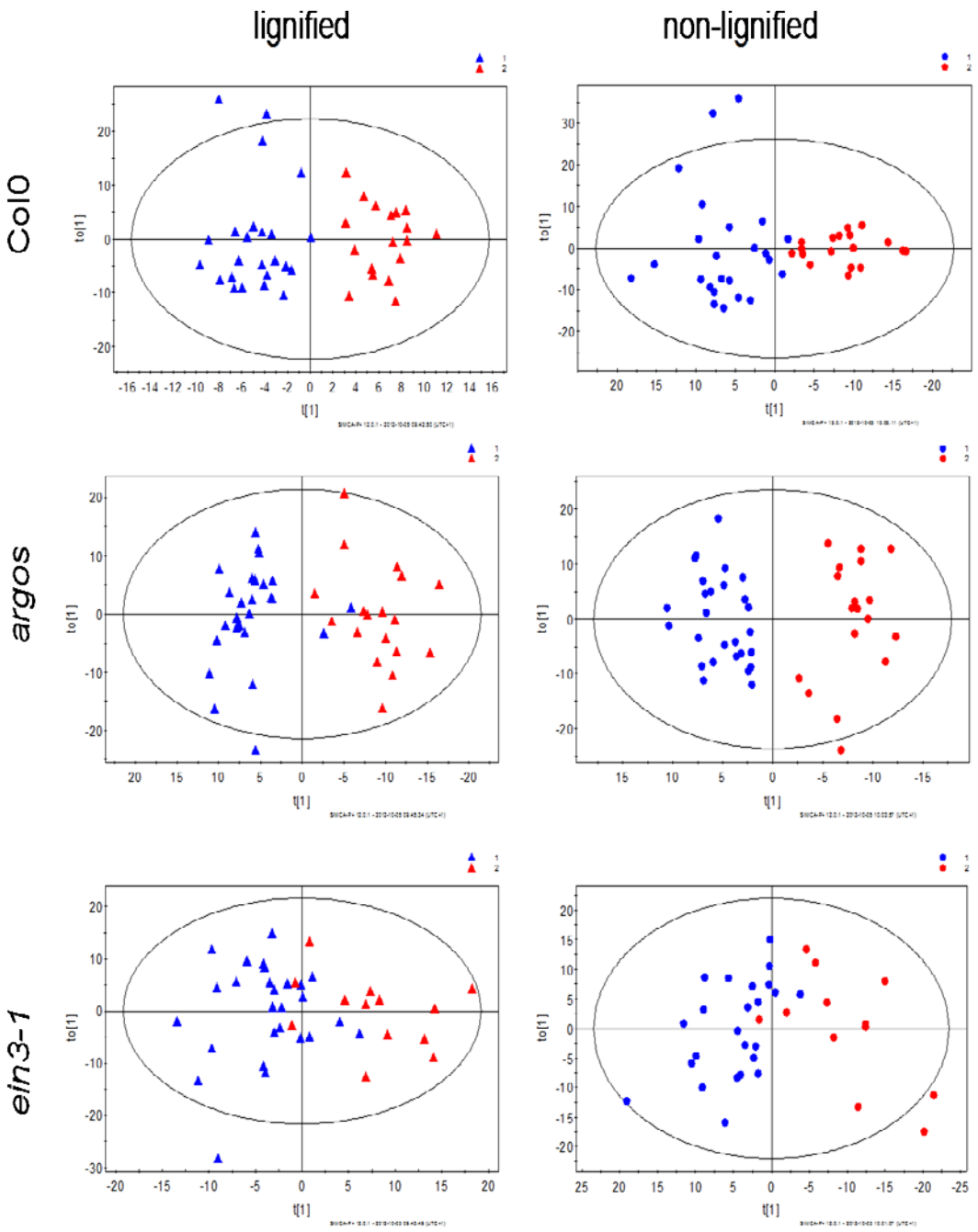

Figure 3.9: Scores plots between water treated (blue) and ACC-treated samples (red) for each genotype. The predictive component is shown on the horizontal axis, whereas the first orthogonal component is shown on the vertical axis.

In the next step we investigated the chemical differences that were induced by ACC through interpretation of the Loadings plots. For easier visualization we highlighted in the spectrum the bands that were induced or reduced by 100µM ACC-treatment (Figure 3.10). FT-IR spectral bands are generally not diagnostic for any particular cell wall polymer, but rather reflect chemical bonds that may be present in several wall components (Gorzsás et al., 2011). Interpretation of Loadings plots should therefore be done with care and preferentially be based on the co-variation of several bands

In lignified Col0 fibers ACC application induced an increase in C-O vibrations (1200-1220 cm^{-1}), an increase in carbohydrate vibrations of non-identified nature (900-1003 cm^{-1}) and in absorbed water/proteins vibrations (around 1650 cm^{-1}), whereas a decrease in C-H vibrations (1240-1480 cm^{-1}) and a shift of C=O vibrations towards lower wavenumbers (1700-1740 cm^{-1}), indicative for de-esterification/ deprotonation of carboxyl groups, were observed. ACC-induced chemical differences in *argos* were very similar to changes induced in Col0, except that aromatic –C=C– vibrations (1510 cm^{-1} and 1595 cm^{-1} indicative of lignins) increased in *argos* whereas they did not show any change in Col0, and vibrations associated to asymmetric –C-O-C– stretches (glycosidic linkages, 1165cm^{-1}) decreased. Strikingly, ACC induced the opposite changes in *ein3-1* compared to wild-type and *argos*: The band associated to glycosidic linkages (1165 cm^{-1}) and C-H vibration (1240-1380cm^{-1}) intensities increased and the aromatic –C=C– band at 1510cm^{-1} and water/proteins associated bands (at around 1650 cm^{-1}) decreased. In addition, the Loadings plots indicated a structural change in carbohydrates (around 1003 cm^{-1}). The only similarity between ACC-treated *ein3-1* and wild-type was the shift of the C=O band (1740 cm^{-1}) indicating de-esterification/deprotonation.

Concerning non-lignified fibers, we found that ACC-treatment in wild-type plants increased the intensity of carbohydrate vibrations (around 1003 cm^{-1}), absorbed water/protein vibrations (1650 cm^{-1}) as well as that of the aromatic –C=C– vibration at 1595 cm^{-1} (which can be indicative of S-lignin (Faix, 1991). On the other hand, bands associated to C-O and C-H vibrations (1250-1450cm^{-1}) were negatively impacted by the treatment. Generally, many

(but not all) changes in the non-lignified fibers were similar to those in the lignified fibers. Again, ACC had very similar impacts on *argos* compared to wild-type, except that similarly to lignified fibers, the asymmetric –C-O-C–(at 1165 cm^{-1} glycosidic linkage vibrations) were decreased and vibrations associated to aromatic –C=C– vibrations (at 1510 and 1595 cm^{-1}, associated to G and S-type lignins, respectively (Faix, 1991)) were increased in *argos*. Again, the chemotype of ACC-treated non-lignified fibers in *ein3-1* was very different compared to the same fibers from wild-type and *argos.* In *ein3-1*, C-O and C-H-vibrations increased and vibrations associated to the asymmetric –C-O-C– (at 1165 cm^{-1} glycosidic linkage vibrations), water/proteins/lignin (1600-1720cm^{-1}) in response to ACC-treatment.

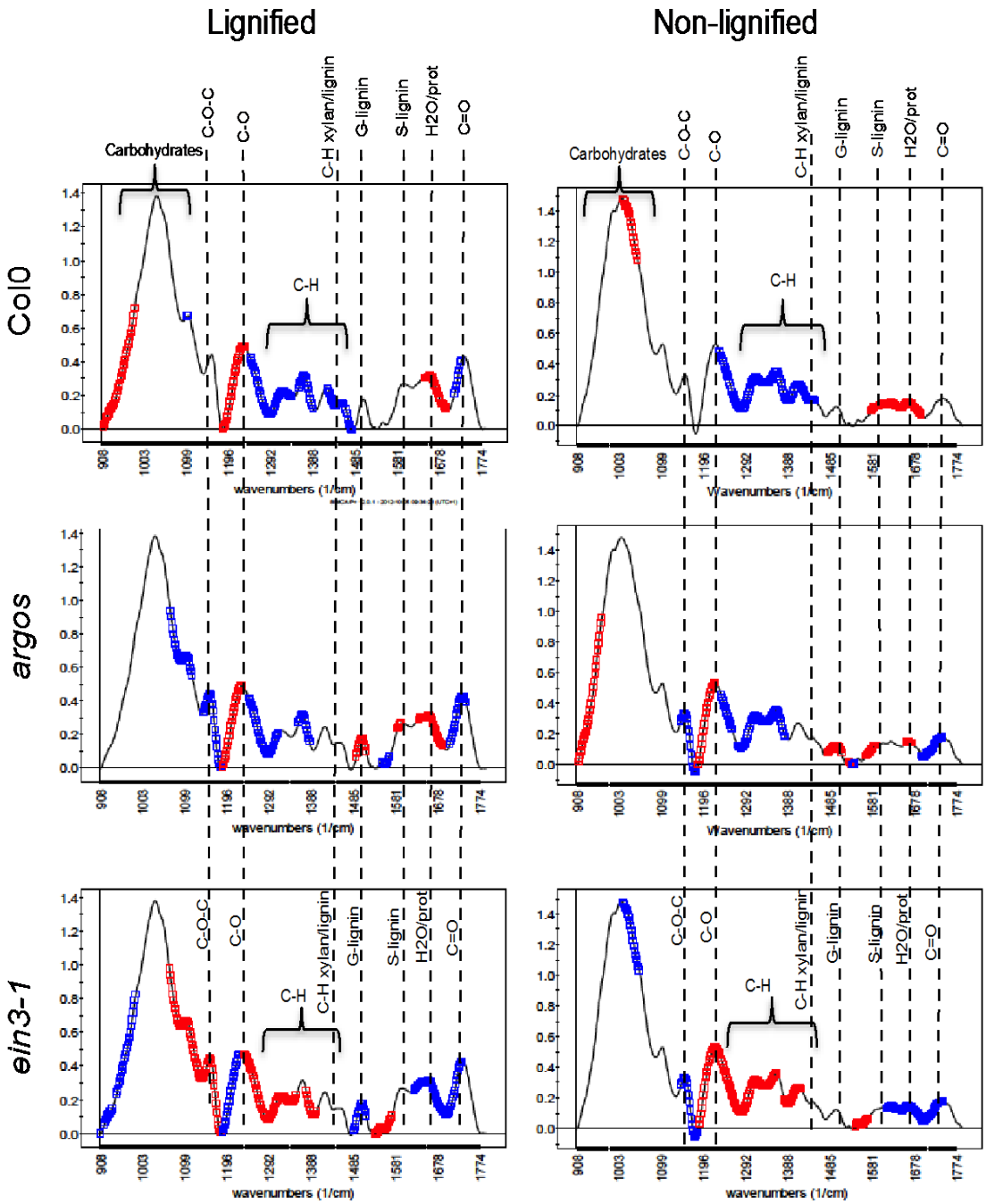

Figure 3.10: Example FTIR spectra, with highlighted bands that were more intense in controls (blue) or in ACC-treated samples (red). Differences are based on the comparison between controls and 100 µM ACC-treated samples.

4. Discussion

In *Arabidopsis*, ACC-treatment has an inhibiting effect on the growth of the inflorescence stem. Inflorescence stems from plants that were treated with ACC were shorter than on the water treated plants. This was true for each genotype, and the phenotype was intensified with the greater ACC concentration. Both *argos* and *ein3-1* had greater reactions to 10 µM ACC than wild-type (though not statistically verified for *argos*).

This was expected, as the literature has shown that ethylene has an inhibitory effect on some forms of growth, such as internode elongation (e.g. Love et al., 2009) and hypocotyl elongation (e.g. Pierik et al., 2006). An unexpected result was the change in height for *ein3-1* plants. The reaction to 10 µM ACC was greater in *ein3-1* than in the other two genotypes, and was the only genotype with a significant height change for that concentration. It appears that *EIN3* isn't responsible for all ethylene responses, and that *ein3-1* can still react to ACC.

Assessing *ERF1* expression was used to verify that the applied ACC does indeed penetrate into the growth medium and is taken up by the roots and transported to woody tissues to generate an ethylene response. *ERF1 is* induced in wild-type and the fact that *ERF1* was not significantly induced in *ein3-1* confirms that this genotype is indeed ethylene insensitive, at least in the hypocotyl. From this assay alone, we cannot confirm that *ein3-1* is ethylene insensitive in all tissues, though it has been verified by others (Roman et al., 1995, who tested for root, hypocotyl, and seedling length, as well as apical hook angle). However, *ARGOS* is still induced 17-fold in the *ein3-1* plants with ACC-treatment.

That *ARGOS* is induced by ACC-treatment in wild-type, and to a lesser extent in the *ein3-1* mutant, makes *ARGOS* an interesting candidate to investigate for its role in xylem formation. Since *ARGOS* was still induced by ACC in *ein3-1*, its interaction with ethylene is worth investigating further. Ethylene-induced *ARGOS* induction does not appear to be completely through *EIN3*. ACC may also induce *ARGOS* independent of the ethylene-signaling pathway.

ACC-treatment in *Arabidopsis* resulted in decreased amounts of mature xylem II, while in poplar, caused an increase in stem width, through increased secondary xylem production. I measured the width of the lignified xylem, whereas in most studies in poplar, measurements are made out to the cambium. However, the cambium is not easily recognized in the phloroglucinol stained sections, hence my reasoning for measuring only to the edge of the lignified xylem. This could account for some of the difference in results between *Arabidopsis* and poplar. But regardless of differences in measuring techniques, the amount of lignified xylem was changed by ACC-treatment.

The 10 µM ACC-treatment had no significant effect on the proportion of secondary xylem in wild-type, but the 100 µM treatment did. ACC-treatment of 10 µM was either not sufficient to make a difference, or it was just enough to delay lignification and make it appear that there was a smaller percentage of secondary xylem that had lignified. With 100 µM treatment, there was a much larger decrease in how much of the total xylem was made up by lignified secondary xylem.

The *argos* mutants were the most affected, both in 10 µM and 100 µM ACC-treatments, of all the genotypes tested. With 10 µM treatment, the amount of lignified xylem II is a decrease of 15.6% compared to the water treated plants. In wild-type, there was only a significant decrease with the 100 µM ACC-treatment, whereas in *argos* the 10 µM treatment already caused a significant change and there was no further decrease. This points to a possible oversensitivity to the ACC-treatment in *argos*.

A different reaction was seen in *ein3-1* on the addition of 10 µM ACC. In *ein3-1* there is no significant change to the proportion of xylem II to the total xylem. Since this genotype is ethylene-insensitive, it was expected that there would be no change.

All ACC-treated genotypes contained gaps within the lignification of the secondary xylem. The fact that they were only found towards the periphery, close to the cambium suggests that lignification was merely delayed and not altogether halted, since treatment began at the beginning of xylem II formation, and there are no gaps in the older cells, closer to the xylem I.

In wild-type plants and in *argos*, the depth to which the gaps existed in was nearly the same. The p-values were extremely low, which shows, with very high confidence, that the results were the outcome of ACC-treatment, and not an artifact. In both genotypes, the 100 µM treated plants exhibited less of the xylem being inundated with gaps in lignification, while in the measurements pertaining to the proportion of xylem II, it was found that the higher treatment results in a smaller proportion than with the 10 µM.

Taken together, the FT-IR analysis revealed that ACC induced very similar chemotypes in lignified and non-lignified cells in Col0 and *argos*, except that ACC-treated *argos* mutants showed increased proportions of lignin and decreased proportions of asymmetric –C-O-C– vibrations (glycosidic linkages) which were not observed in wild-type. The differences in chemotypes were stronger in *argos* compared to wild-type, even at lower ACC concentrations and especially in non-lignified fibers (as judged by the Q2 values of OPLS-DA models). The *ein3-1* mutants had a weaker chemotype and ACC induced very different changes than in wild-type.

The OPLS-DA models for the comparisons between water and ACC-treated plants show that ACC-treatment can change the composition of the cell walls, in both lignified and non-lignified fibers. In Col0 and *ein3-1*, the dosage effect was seen most clearly in the non-lignified fibers. In Col0 the dosage effect for lignified cells was very small (0.645 vs. 0.649; Q2 H_2O vs 10/100 µM ACC and Q2 H_2O vs 100 µM, respectively), while in non-lignified cells the difference was much larger (0.419 vs. 0.611). The greatest dosage effect was observed in *ein3-1*, with a lignified Q2 H_2O vs 10/100 µM of 0.135 and Q2 H_2O vs 100 µM of 0.342, and non-lignified Q2 values of 0.067 and 0.417.

4.1 Future Work

For further insight into the role of *ARGOS,* it would be useful to look at gene expression in other parts of the plants. Gene expression analysis on inflorescence stems was planned, but there was not enough material. Testing

for expression of *ARGOS* and *ARL* in the inflorescence stem would give a broader picture of their interaction with ethylene and wood development.

Another interesting facet to examine would be to look at cambial activity in the hypocotyl. While the safranin/alcian blue could have been used to determine changes in the number of cambial cells, it did not get analyzed for lack of time. Since *ARGOS* overexpression instigates increased cell division (Hu et al, 2003), and is induced by ethylene application, the cambial activity could have increased in the ACC-treated plants, but the xylem cells were delayed in their lignification, and therefore were not observed with the phloroglucinol staining.

It is hard to know whether cells were alive or dead at the time of harvest as phloroglucinol and safranin/alcian blue stain based on the compounds found in the cell walls. Consequently, we only know if a cell is lignified or not with the two stains used in this project. Nitroblue tetrazolium would be useful for determining if cells are alive or dead, by interacting with reactive oxygen species that are indicative of living cells. We tried to make use of this stain, but early attempts did not work, and in the interest of time, continued with what did work. Another alternative would be a use a marker to indicate that processes necessary to living cells were occurring, for example, on housekeeping genes.

As explained in the introduction, there is redundancy in the *OSR* genes, and *OSR1* could have been able to take over the role that *ARGOS* plays, especially since expression in *argos* was only reduced by 2-3x from wild-type. (Overexpression of both *OSR1* and *ARGOS* does not result in larger organs than if only one of them is overexpressed (Feng et al., 2011)). It would be interesting to find out whether the *argos* plants actually have different cambial activity or if the other OSR genes successfully compensated for the decrease in *ARGOS*.

The *argos* mutant appears to be hypersensitive to ACC, as the reactions to 10 µM ACC-treatment is more severe than the wild-type, and sometimes the treatments of 10 µM ACC resulted in a stronger phenotype than for 100 µM ACC (for example, in the formation of xylem II). The decrease in inflorescence stem growth is much more pronounced in the *argos* mutant,

than wild-type, *ERF1* is more heavily induced, and the decrease in the amount of xylem II formed is also much less than the decrease seen in 10 µM ACC-treated wild-type plants. The *argos* genotype used in this experiment was not a knock-out mutant, and only had 2-3x reduced *ARGOS* level from wild-type. If a less leaky mutant had been used, the phenotype of hypersentivity and xylem changes probably would have been more pronounced. The OPLS-DA data shows that *argos* reacts to both concentrations of ACC, with no dosage effect, where Col0 and *ein3-1* do show a dosage effect. The Q2 H_2O vs 10/100 µM for non-lignified cells is nearly the same as Q2 H_2O vs 100 µM, and if there was a dosage effect (meaning that 100 µM ACC had a greater effect on the cell wall composition than 10 µM) then the Q2 H_2O vs 100 µM would be larger. This is more evidence of a hypersensitivity to ACC.

4.2 Conclusions

The results of this project show that there are discrepancies in the response of *Arabidopsis* and poplar wood development to applied ACC. The findings of Love et al 2009 on the effect of ethylene on poplar growth are likely because we used two different species. In Hu et al. 2006, they did not find *ARGOS* and *ARL* to be induced by ACC as I did. However, they were testing expression in seedlings and not specifically in the hypocotyl. On the other hand, an earlier experiment from this group by Yuan Ma found that *ARGOS* was induced in seedlings treated with ACC.

Love et al., 2009 also saw a decrease in internode length, and my plants also had significant decreases in inflorescence stem length in wild-type and *ein3-1* genotypes. The effect of ethylene and ACC seem to be the same between poplar and *Arabidopsis* in terms of elongation, but different for cambial activity.

In Hu, et al., 2006, figure 6 indicates that *ARL* and *ARGOS* are not induced by ACC-treatment in 10-day old *Arabidopsis* seedlings. At ten days old, *Arabidopsis* has not yet begun to form secondary xylem in the hypocotyl, which was the only tissue assayed for gene expression in my project. It is

possible that ACC affects *ARGOS* and *ARL* expression differently in different organs, and it may change over time with development of the plant.

That the *ein3-1* genotype does not show a significant increase in *ERF1* mRNAs means that *ERF1* is downstream of *EIN3*. However, *ein3-1* does show a response to both low and high concentrations of ACC. There was a significant change in the lignification of the secondary xylem, though the gaps comprised less of the xylem of the ACC-treated wild-type and *argos* plants. In the end, I conclude that ACC reduces inflorescence stem elongation, leads to reduced xylem growth and delayed lignification, with changes in wood chemistry. These effects were enhanced in *argos*, especially the effect on xylem development. *ARGOS* is an ACC-inducible gene, but its response seems to be only partially mediated by the ethylene signaling pathway.

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