The role of pectin methylesterase activity in explosive seed dispersal in *Cardamine hirsuta*

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Farnaz Faezi

aus SHIRAZ (IRAN)

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Berichterstatter: ***Prof. Dr. George Coupland***

Prof. Dr. Maria Albani

Prof. Dr. Martin Hülskamp

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Didicated to my Parents

Zusammenfasung

Cardamine hirsuta und *Arabidopsis thaliana* sind nahe Verwandte Pflanzenspezies, die sich dramatisch in ihrer Strategie der Samenverteilung unterscheiden. *C. hirsuta* hat explodierende Samenhülsen, welche die Samen weit in die Umgebung schleudern, wohingegen die Samenhülsen von *A. thaliana* nicht explodieren. Das Ziel meines Projektes ist ein Verständnis dafür zu entwickeln, ob die speziesspezifische Expression von Zellwandmodellierungsgenen im Zusammenhang mit den unterschiedlichen Strategien der Samenverteilung von *C. hirsuta* und *A. thaliana* steht. Gene, die die Aktivität von Pektin-Methyleserase in der Zellwand steuern, wurden mit unterschiedlich hohen Expressionen während der Furchtentwicklung in *C. hirsuta* beobachtet, jedoch nicht in *A. thaliana*. In diesem Projekt charakterisierte ich die Dynamik der Expression dieser Gene. Dabei fand ich 8 Gene von *C. hirsuta*, welche keine eindeutigen Gegenstücke in *A. thaliana* haben. Diese Expression dieser Gene war zeitlich und örtlich beschränkt auf die sich entwickelnden Samen in der Stufe 16 der Fruchtentwicklung. Ich entwarf künstliche microRNAs, um die Expression einzelner oder Gruppen dieser Pektin-Methylesterase Inhibitor Gene zu kontrollieren. Damit erzeugte ich transgenetische Pflanzen welche genutzt werden können, um die Funktion der Zellwandmodellierungsgene in Pflanzen mit explodierenden Schoten zu finden.

Abstract

Cardamine hirsuta and *Arabidopsis thaliana* are close relatives that differ dramatically in seed dispersal. *C. hirsuta* uses explosive pod shatter for ballistic seed dispersal, whereas pod shatter in *A. thaliana* is non-explosive. The aim of my project is to understand whether the species-specific expression of cell wall-remodeling genes is associated with the dramatically different seed dispersal strategies of *C. hirsuta* and *A. thaliana*. Genes that control pectin methylesterase activity in the cell wall were previously identified as differentially expressed during fruit development specifically in *C. hirsuta*, but not *A. thaliana.* In this project, I characterized the expression dynamics of these genes. I found that eight genes unique to *C. hirsuta*, with no clear orthologues in *A. thaliana*, were spatially and temporally restricted in expression. All eight of these genes were expressed only in seeds during stage 16 of fruit development. I designed artificial microRNAs to target individual or groups of these pectin methylesterase inhibitor genes in *C. hirsuta*. Using this approach, I generated transgenic plants that can be used to characterize the function of cell wall-remodeling genes in explosive seed dispersal.

Contents

Chapter 1

Introduction

1.1- Pod shatter in *Arabidopsis thaliana* and *Cardamine hirsuta*

1.1.1- Seed dispersal via pod shatter

Fruit and seed collaborate in many various and ingenious ways to disperse seeds. Most mechanisms of dispersal require biotic or abiotic agents to disperse the seeds e.g. animals, wind, rain (Van der Pijl, 1972). Explosive seed dispersal, on the other hand, depends entirely on the fruit and requires no external agents to disperse the seeds (van der Pijl, 1972). Different fruits employ different mechanisms to explosively eject their seeds including squirting cucumber, touch-me-not etc. (van der Pijl, 1972).

Although adaptations for dispersal are found in both fruits and seeds, fruit represent the dominant means of dispersal in higher angiosperms (Esau, Anatomy of seed plants). Fruit develop from the fertilized gynoecium of the flower and are either fleshy or dry depending on the histology of the fruit wall (Esau, Anatomy of seed plants).

A. thaliana and *C. hirsuta* belong to the Brassicaceae family and have dry, dehiscent fruit called siliques. Each silique consists of two carpels, called valves, joined along their margins to a replum, with a septum that partitions the locule in two (Fig. 1). At maturity, the valves separate from the replum along their margins, leaving the seeds attached to the replum (Fig. 1). This separation process is called dehiscence and requires that valve margin cells differentiate to form a specialized dehiscence zone.

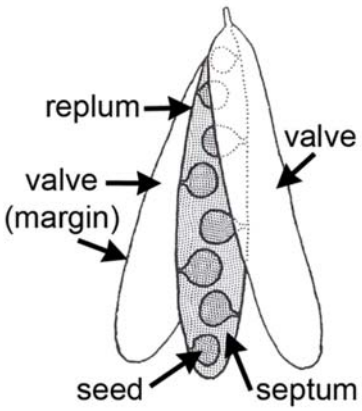

Figure 1: Dry, dehiscent Brassicaceae silique. Figure reproduced from Esau. Anatomy of seed plants. 2nd edition.

Seeds are dispersed by the dehiscent fruit of *A. thaliana* and *C. hirsuta* in a process called pod shatter. During pod shatter, the fruit structure falls to pieces, allowing seed dispersal. This process is explosive in *C. hirsuta* and non-explosive in *A. thaliana*. In *A. thaliana*, pod shatter occurs when the fruit is dry and the seeds are released when the pod is shattered by touch or wind etc. In *C. hirsuta*, the pod shatters when the fruit is still turgid. The valves separate from the replum and coil rapidly, launching the seeds in an explosive manner.

Pod shatter also occurs in oilseed crops that belong to the Brassicaceae family. Crops such as oilseed rape (*Brassica napus*) and canola (specific cultivars of *B. napus*, *B. rapa*, *B. campestris*, and *B. juncea*) have dehiscent siliques (Spence, 1995), similar to *A. thaliana* and *C. hirsuta*. Pre-harvest pod shatter can cause considerable seed loss and is a significant economic factor in oilseed crop production. For example, pre-harvest pod shatter can cause up to 50% reduction in yield in Canola (MacLeod, 1981).

1.1.2- Genetic patterning of Arabidopsis fruit

A dehiscence zone forms in mature Arabidopsis fruit by the differentiation of two cell layers: separation and lignified layers, along each valve margin. The separation layer is adjacent to the replum and the lignified layer is adjacent to the valve. Cells in the separation layer secrete hydrolytic enzymes that break down the middle lamella between adjacent cells, causing the valve to separate from the replum (Roeder *et al.,* 2006). Cells in the lignified layer contain a phenolic cell wall polymer called lignin that confers mechanical strength and hydophobicity (Lijegren *et al.,* 2004). This lignified layer forms a seal along the valve margin following dehiscence.

Fruit dehiscence depends on the precise patterning of these different tissues that enable the fruit to open at maturity. Elegant genetic studies in Arabidopsis have identified a network of transcription factors that control the patterning of fruit tissues such that dehiscence occurs at the correct place and the correct time, to ensure successful seed dispersal (Dinneny & Yanofsky Bioessays, 2004).

Genes that control the development of fruit tissues can be divided into two categories: those that promote valve margin identity and those that repress it. *FRUITFULL (FUL)* is a MADS-box transcription factor that represses valve margin identity (Gu *et al.,* 1998). FUL is expressed in the valve tissue of the fruit and negatively regulates the expression of genes required for valve margin identity, restricting their expression to the valve margin (Ferrandiz *et al.,* 2000). These genes

include the redundant MADS-box genes *SHATTERPROOF1* and *2* (*SHP1,2*), and the basic-helix-loop-helix transcription factor genes *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*). These margin identity genes are ectopically expressed in the valve of *ful* mutants, and the short fruit length of *ful* mutants is rescued by loss of *IND, SHP1,2* and *ALC* activity (Ferrandiz *et al.,* 2000; Lijegren *et al.,* 2004).

REPLUMLESS (RPL) is a BEL1-like homeobox transcription factor that also represses valve margin identity (Roeder *et al.,* 2003). *RPL* is expressed in the replum tissue of the fruit and negatively regulates the expression of *SHP1,2*, restricting their expression to the valve margin (Roeder *et al.,* 2003). These margin identity genes are ectopically expressed in the replum of *rpl* mutants, and the small replum of *rpl* mutants is rescued by loss of *SHP1,2* activity (Roeder *et al.,* 2003). Therefore, repression of valve margin identity genes by *FUL* in the valves, and by RPL in the replum, ensures that the dehiscence zone is specified in a narrow stripe of cells at the valve/replum border.

The other category of genes that control fruit development is those that promote valve margin identity. These include the four genes described above: *SHP1,2*, *IND* and *ALC.* Of these genes, *IND* has the most important function in specifying the identity of valve margin cells. *IND* is expressed in both cell layers of the valve margin and these cell layers fail to differentiate as a dehiscence zone in *ind* mutants (Lijegren *et al.,* 2004). For this reason, *ind* mutants are indehiscent and prevent seed dispersal. Mutations in the *ALC* gene also cause indehiscent fruit (Rajani *et al.,* 2001). *ALC* expression is restricted to only the separation layer of the valve margin, and this cell layer fails to differentiate in *alc* mutants (Rajani *et al.,* 2001). The lignified cell layer of the valve margin forms normally in *alc* mutants, indicating that the two cells layers that comprise the dehiscence zone are differentially regulated. The expression of both *IND* and *ALC* requires the redundant activity of *SHP1,2*, as *shp1,2* double mutants are indehiscent and lack both the lignified and separation layers at the valve margin (Lijegren, 2000). Ectopic expression of *SHP* or *IND* genes causes valve cells to adopt valve margin identity such as ectopic lignification (Lijegren, 2000; Lijegren, 2004). Therefore, the regulatory network controlling fruit development in Arabidopsis works to limit valve margin identity to a narrow stripe of cells at the valve/replum border. These cells differentiate to form a dehiscence zone in the mature fruit that allows subsequent seed dispersal by pod shatter.

1.1.3- Explosive seed dispersal in *C. hirsuta*

Cardamine hirsuta is a relative of the model plant *A. thaliana* that uses explosive pod shatter to forcibly eject its seeds (Fig. 2). This seed dispersal mechanism transfers stored mechanical energy from fruit tissues to the seeds to launch the seeds on ballistic trajectories. This results in the dispersal of seeds over a radius of several meters around a single plant (Hofhuis *et al.,* 2016).

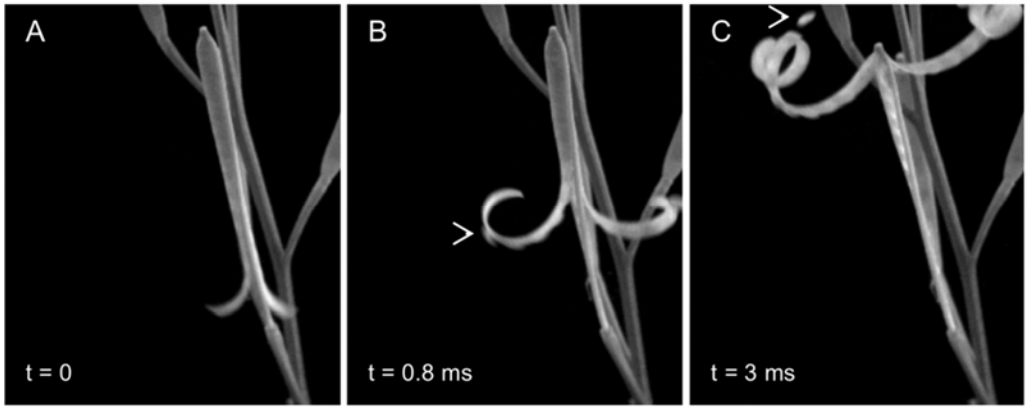

Figure 2: Explosive seed dispersal in *C. hirsuta*. Explosive seed dispersal recorded at 15,000 fps. (A) The two valves detach from the fruit, (B) curl back with seeds adhered to the inner valve surface, and (C) launch seeds while coiling; t: time between frames, arrows indicate seeds. Figure reproduced from Hofhuis *et al*. 2016.

C. hirsuta is an annual, weedy plant with a short generation time, small size, inbreeding habit, and abundant progeny (Hay & Tsiantis COGD, 2016). Like *A. thaliana*, these features allow for large scale cultivation and make *C. hirsuta* a good laboratory subject. Importantly, *C. hirsuta* is a diploid species with a small genome and eight chromosomes, which together with simple, high frequency genetic transformation, makes *C. hirsuta* a good model species for molecular genetics research (Hay *et al.,* Plant J., 2014).

Regarding fruit development in *C. hirsuta*, plants produce up to 147 fruit within a month after germination (Vaughn *et al.,* 2011). Approximately ten days after fruit start to elongate, the fruit pod is competent to explode when manually triggered (Vaughn *et al.,* 2011). This stage of fruit development occurs at the transition between stages 17a and 17b (Fig. 1 in chapter 3). Fruit development in *C. hirsuta* can be staged according to similar landmarks in *A. thaliana* fruit (Roeder & Yanofsky, 2006). Fruit development starts at stage 14 of flower development, after fertilization of the egg and central cells occurs within the gynoecium (Roeder & Yanofsky, 2006). At stage 15,

the fruit elongates above the other floral organs, and by stage 16, these floral organs abscise as the fruit continues to elongate (Fig. 1 in chapter 3) (Roeder & Yanofsky, 2006). Stage 17 is a long stage during which the fruit attains its full length (stage 17a) and width (stage 17b) (Fig. 1 in chapter 3) (Roeder & Yanofsky, 2006). In stage 17b, the valve margins differentiate into dehiscence zones. The middle lamella between cells in the separation layer breaks down and lignification of both the lignified layer of the valve margin and the endocarp *b* layer of the valve occurs (Hofhuis *et al.,* 2016; Roeder & Yanofsky, 2006). At this stage, the *C. hirsuta* fruit valve is competent to disperse seeds by explosive pod shatter (Hofhuis *et al.,* 2016). In contrast to this, *A. thaliana* fruit go through additional stages of development (stages 18-20) where the fruits dehisce, dry, and the seeds fall from the replum when the pod shatters (Roeder & Yanofsky, 2006).

The mechanism of explosive seed dispersal in *C. hirsuta* requires biomechanical features of the exocarp and endocarp *b* cell layers in the valves (Hofhuis *et al.,* 2016). In the outer exocarp layer, the cells contract in length, producing tissue tension. During fruit development, cortical microtubules in the exocarp cells reorient from a transverse to a longitudinal orientation. Cortical microtubules guide the deposition of cellulose microfibrils which increase cell wall stiffness in the longitudinal direction, thereby restricting growth in this direction (Paredez *et al.,* 2006; Cosgrove, 2005). This dictates that cells expand in the transverse direction, causing cell geometry to change from elongated to square. Computational modeling predicts that this geometry and anisotropy of exocarp cells causes them to contract in the longitudinal direction in response to turgor pressure (Hofhuis *et al.,* 2016).

In the inner endocarp *b* layer of the valve, cells are asymmetrically lignified. This lignin confers stiffness to the endocarp *b* layer, such that it resists the contraction of the outer exocarp layer. This differential contraction of the two layers causes the valves to coil when separated from the rest of the fruit. However, the valves have a bowed geometry in cross section while attached to the fruit, and this geometry prevents them from coiling. Similar to a toy slap bracelet, the valves need to flatten in cross section in order to release tension by coiling. The geometry of the lignified secondary cell walls of endocarp *b* cells is critical for the valve to transition from a bowed to flat cross section. The lignified wall in each endocarp *b* cell is shaped like a hinge. Tension in the valves drives the opening of these hinges, allowing the valves to flatten when separated from the rest of the fruit, and release the tension by coiling (Hofhuis *et al.,* 2016). Genetic evidence for the importance of the endocarp *b* layer came from characterizing the *less lignin 2* mutant in *C. hirsuta*.

Fruit valves of this mutant are missing the entire endocarp *b* layer at maturity and pod shatter is non-explosive, indicating that the endocarp *b* layer is necessary for explosive seed dispersal (Hofhuis *et al.,* 2016).

1.2- Comparative transcriptome analysis of *C. hirsuta* and *A. thaliana* fruit development

The recent publication of a high-quality reference genome for *C. hirsuta* enabled comparative genome and transcriptome analyses between *C. hirsuta* and *A. thaliana* (Gan *et al.,* 2016). In this paper, transcriptomes of immature and mature fruit were compared using the DESeq algorithm to identify genes that were differentially expressed during fruit development. This experiment was performed in both *C. hirsuta* and *A. thaliana* and orthologous genes were compared between the two datasets using the following criteria: adjusted $P < 0.05$ in *C. hirsuta*, adjusted $P > 0.3$ in *A. thaliana*, to identify genes that were differentially expressed only in *C. hirsuta* (Gan *et al.,* 2016). From this analysis, 319 genes were identified as differentially expressed during development of explosive fruit in *C. hirsuta*, but not during the development of non-explosive fruit in *A. thaliana*.

Gene Ontology (GO) analysis was used to determine which biological processes, functions, and/or locations were significantly over-represented in these 319 differentially expressed genes (DEG) (Gan *et al.,* 2016). GO terms describe gene products in terms of their associated biological processes, cellular components and molecular functions, and terms that are represented in a gene set more often than expected by chance, provide a functional profile for this gene set (Rhee *et al.,* Nature reviews genetics, 2008). Six highly enriched GO terms related to cell wall and pectinesterase activity were identified in these 319 DEG (Fig. 3). The differential expression of 10 pectin methylesterase (PME) genes and their inhibitors (PMEI) were largely responsible for the over-representation of these six GO terms amongst this gene set (Fig. 6). This was an exciting result given the importance of the cell wall in the mechanism of explosive seed dispersal in *C. hirsuta*.

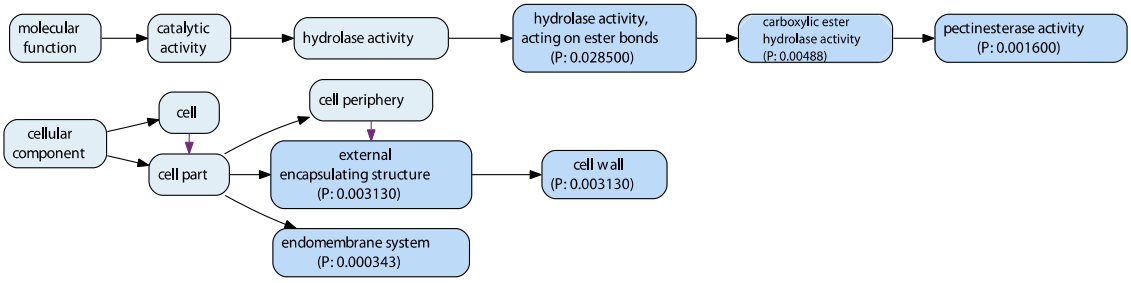

Figure 3: Gene Ontology (GO) analysis of differentially expressed genes (DEG) specific to *C. hirsuta* fruit development. Enriched GO terms (dark blue) in the set of 319 DEG specific to *C. hirsuta* and not *A. thaliana* fruit development, and their parental terms (light blue). *P* values were

obtained from exact Fisher tests after correcting for multiple hypothesis testing. Black arrows indicate an 'is a' and orange arrows indicate a 'part of' relationship between respective terms in the GO graph.

Comparative genome analyses were used to identify gene families that had expanded in *C. hirsuta*, relative to other Brassicaceae species such as *A. thaliana* (Gan *et al.,* 2016). Two expanded *PME/I* gene families were identified by this approach (Fig. 4). One family contained eight genes unique to *C. hirsuta* with no clear orthologues in *A. thaliana*, while the other family contained five genes in *C. hirsuta* and one gene in *A. thaliana* (Fig. 4). Therefore, the *C. hirsuta* genome contains unique *PME/I* genes that have no clear orthologues in *A. thaliana*.

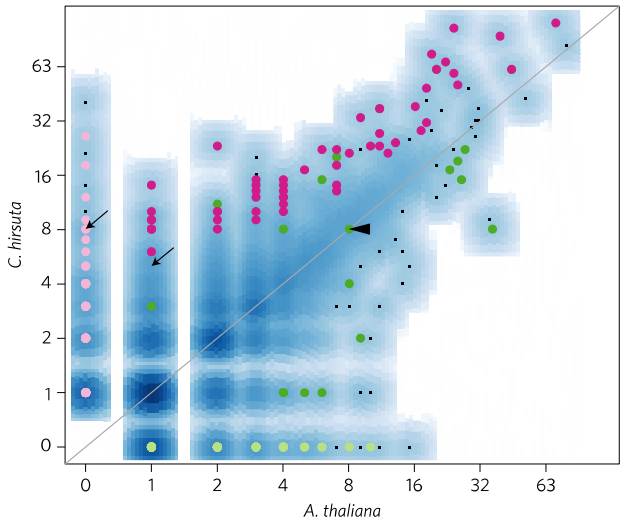

Figure 4: Logarithmically scaled smooth scatterplot of gene families showing the number of species-specific members in *A. thaliana* (x-axis) and *C. hirsuta* (y-axis). Dots above the grey line represent gene families that are significantly expanded in *C. hirsuta* (pink) or contracted in *A. thaliana* (green), and dots below the grey line represent gene families that are expanded in *A. thaliana* (green), based on Hahn's test with eight species; pale pink dots represent families that are unique to *C. hirsuta* and pale green dots represent families that are unique to *A. thaliana*. The arrows indicate two families containing pectin methylesterase (PME) and PME inhibitor genes; one gene family has no members in *A. thaliana*. Figure reproduced from Gan *et al.,* 2016.

Interestingly, 8 of the 13 genes from expanded *PME/I* gene families in *C. hirsuta* were differentially expressed during *C. hirsuta* fruit development (Fig. 5) (Gan *et al.,* 2016). These 8

genes were all significantly up-regulated in mature fruit compared to immature fruit (Fig. 5). Furthermore, 5 of these differentially expressed *PME/I* genes arose by tandem duplication in *C. hirsuta* (Fig. 5) (Gan *et al.,* 2016). Previous studies have identified regulatory changes in tandemly duplicated genes as causes of morphological diversity in plants (Vlad *et al.,* 2014; Hanikenne *et al.,* 2008). Therefore, it is interesting to find differential expression of tandemly duplicated *PME/I* genes associated with the derived trait of explosive seed dispersal in *C. hirsuta*.

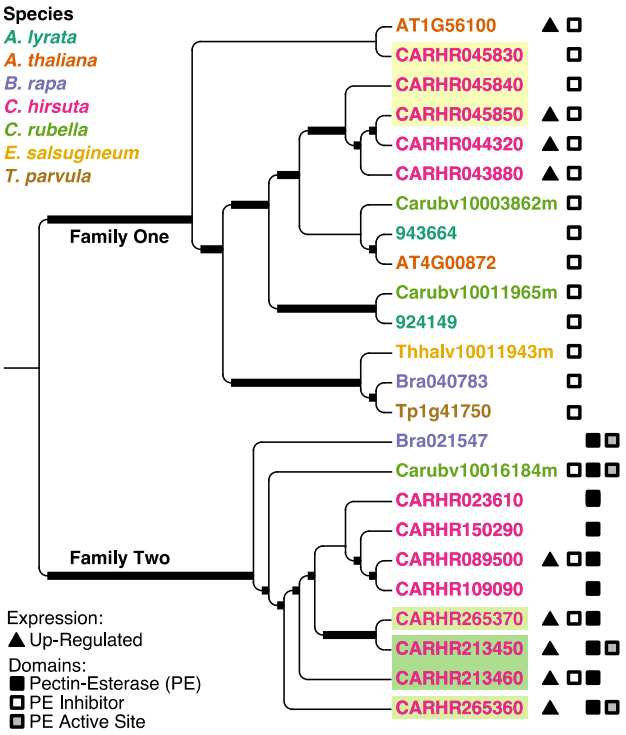

Figure 5: Maximum likelihood tree of expanded PME(I) gene families in *C. hirsuta*. Gene identifiers are color-coded to indicate species; tandem gene duplicates are highlighted in yellow, light green and dark green; triangles indicate significant up-regulation during fruit development; boxes indicate which conserved protein domains are found in each gene: white, PMEI (IPR006501); black, PME catalytic (IPR000070) and pectin lyase (IPR011050, IPR012334); grey, PME active site (IPR018040). Bold branches have maximum confidence. Figure reproduced from Gan *et al.,* 2016.

An initial characterization of three differentially expressed *PME/I* genes (CARHR043880, CARHR044320 and CARHR045850, Fig. 6) showed that they were highly expressed in *C. hirsuta* seeds (Gan *et al.,* 2016). Furthermore, another DEG from this analysis (CARHR143060, Fig. 6) is

orthologous to the *PMEI6* gene in *A. thaliana,* which is required in the seed to promote mucilage release upon imbibition of the seed coat (Saez *et al.,* 2013).

chi.gene	chi.stage9	chi.stage16	chi.foldChange	chi.padj	ath.gene	ath.stage9	ath.stage16	ath.foldChange	ath.padj
CARHR143060	4.90	143.76	29.32	4.13E-04	AT2G47670	1.60	5.29	3.31	0.90
CARHR085300	229.62	1730.60	7.54	2.49E-02	AT3G10720	280.87	255.63	0.91	1.00
CARHR118350	13.51	265.95	19.68	7.01E-04	AT2G26440	640.40	702.79	1.10	1.00
CARHR173850	682.48	48.41	0.07	1.98E-03	AT5G47500	1621.01	1831.29	1.13	1.00
CARHR043880	0.00	9058.78	Inf	1.55E-18	AT4G00872	0.00	0.59	Inf	1.00
CARHR214060	0.00	34.50	Inf	1.45E-03	AT5G38610	4.01	9.38	2.34	0.90
CARHR089480	700.32	6075.53	8.68	1.22E-02	AT3G14310	950.92	1070.50	1.13	1.00
CARHR276140	115.84	806.03	6.96	3.49E-02	AT5G62360	258.72	446.94	1.73	0.80
CARHR004800	0.00	36.02	Inf	1.37E-03	AT1G05310	0.40	0.58	1.46	1.00
CARHR156040	5.70	66.06	11.58	2.63E-02	AT3G47400	23.32	43.98	1.89	0.82
CARHR045850	1.33	19915.54	15022.14	1.05E-18					
CARHR044320	0.00	22229.74	Inf	1.10E-20					
CARHR089500	0.00	197.29	Inf	2.83E-08					
CARHR213450	0.00	42.74	Inf	3.36E-04					
CARHR213460	0.00	17.56	Inf	2.74E-02					
CARHR265360	0.00	24.21	Inf	6.81E-03					
CARHR265370	0.00	16.05	Inf	4.01E-02					

Figure 6: DEseq results for differentially expressed *PME/I* genes in *C. hirsuta.* Note that the adjusted *P* values are significant for *C. hirsuta* genes but not for *A. thaliana* genes. Figure reproduced from Gan *et al*. 2016.

Based on these findings, the authors hypothesized that up-regulation of *PMEI* gene expression during *C. hirsuta* seed development may inhibit pectin methylesterase (PME) activity (Gan *et al*. 2016). To test this hypothesis, they measured PME activity and found that wild-type *C. hirsuta* seeds had lower PME enzymatic activity per unit protein than wild-type *A. thaliana* seeds (Fig. 7) (Gan *et al*. 2016). Furthermore, this reduction in PME activity was comparable to the reduction found in seeds of the *35S::PMEI6* genotype relative to wild-type *A. thaliana* seeds (Fig. 7). The authors went on to show that *C. hirsuta* but not *A. thaliana* seeds accumulated pectin with a high degree of methyl-esterification in thickened cell walls of the seed coat (Gan *et al*. 2016). Taken together, these findings suggest that some of the differentially expressed *PME/I* genes that are specific to *C. hirsuta* may inhibit PME activity in the seed coat. However, such a function remains to be tested. In addition, it is unknown whether the other differentially expressed *PME/I* genes identified by Gan *et al.* function in fruit or seed development in *C. hirsuta*.

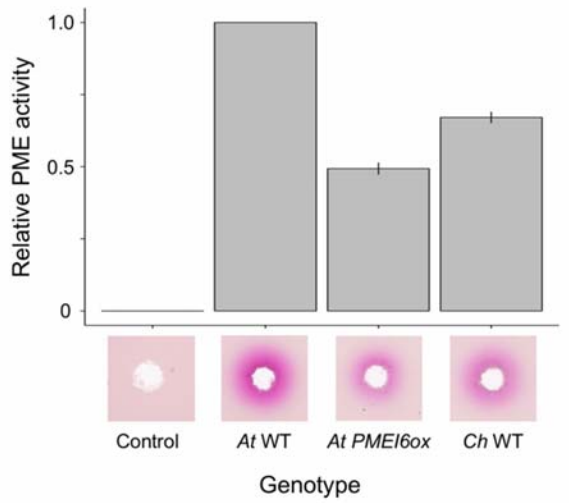

Figure 7: Relative PME activity in 15 µg protein extracts from seeds of *A. thaliana* wild type, *A. thaliana* PMEI6ox, and *C. hirsuta* wild type, quantified from ruthenium red-stained gel assays (representative assays shown below graph); control assay contains no protein. Figure reproduced from Gan *et al.,* 2016.

1.3- Pectin methylesterase activity in the cell wall

1.3.1- Plant cell wall

In growing cells, the primary cell wall is composed of cellulose microfibrils embedded in a hydrated matrix of complex polysaccharides (Fig. 8). Each cellulose microfibril is a linear chain of β-D-1,4-glucan molecules that crystallize into strong, thin rods. These are the load-bearing elements of the cell wall. They are synthesized by cellulose synthase complexes in the plasma membrane, which are encoded by *CESA* genes (Fig. 8). Matrix polysaccharides include pectins and cellulose-binding glycans, called hemicelluloses. These polysaccharides are synthesized in the Golgi apparatus and secreted into the cell wall (Fig. 8). Hemicelluloses interact with cellulose microfibrils, either by coating the microfibrils and tethering them together to form a load-bearing network (Scheller and Ulvskov, 2010), or by attaching microfibrils together at a limited number of junctions, which act as biomechanical hotspots for wall loosening (Park and Cosgrove, 2012).

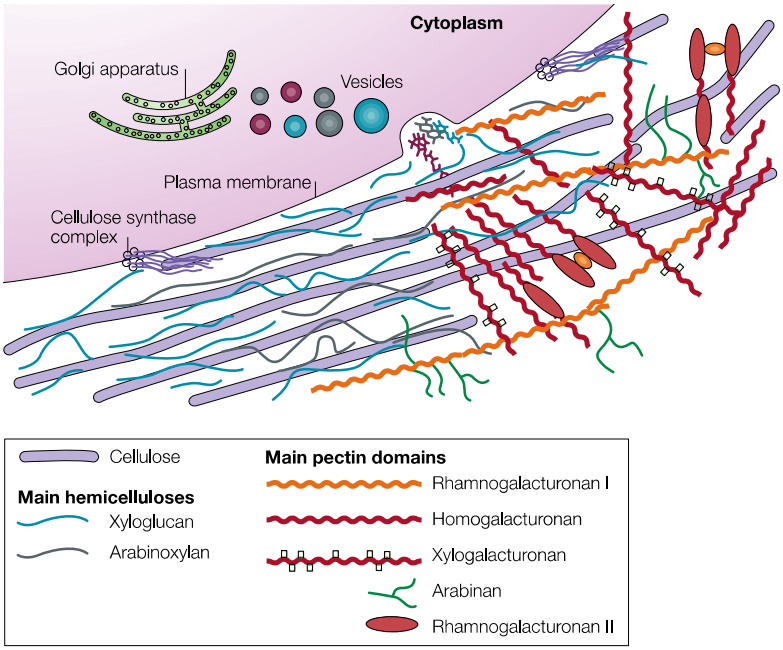

Figure 8: Structure of the primary cell wall. Cellulose microfibrils (purple rods) are synthesized in the plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles. The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I and rhamnogalacturonan II. Image reproduced from Cosgrove 2005.

Pectins are a complex and heterogeneous group of acid polysaccharides. They are characterized by chains of galacturonic acid molecules linked at their 1 and 4 positions. The major types of pectins include homogalacturonan (HGA), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II), and xylogalacturonan (XGA) (Fig. 7). The carboxyl groups of HGA and XGA are often methylesterified (Fig. 9). Methylesterification occurs in the Golgi (Zhang and Staehelin, 1992). This modification blocks the acidic group and protects pectins from the action of lyases. It also reduces the ability of these pectins to form gels by Ca^{2+} crosslinking (Fig. 10).

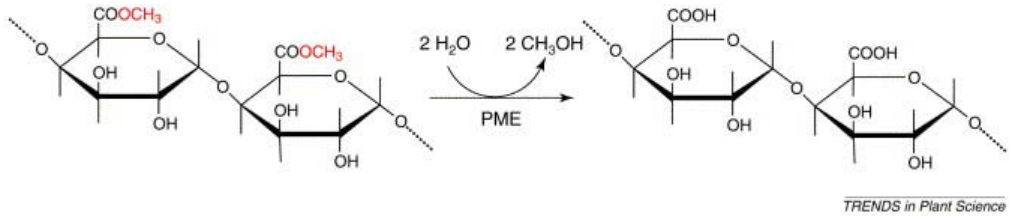

Figure 9: Demethylesterification of pectins by pectin methylesterases (PME). Figure reproduced from Micheli. F. (2001) TRENDS in Plant Science.

1.3.2- Pectin methylesterase activity

When pectins are secreted into the cell wall, they integrate into the wall network by binding to cellulose, hemicellulose, and other pectins. This allows the formation of structures with hydrogel characteristics. In particular, HGA forms stiff gels through Ca^{2+}-mediated crosslinking of its carboxyl groups (Fig. 10). Prior to crosslinking, the methylester group must first be removed by pectin methylesterases enzymes (PMEs), which are secreted by plant cells into their wall space (Micheli, 2001). PMEs hydrolyse the methylesters and free the carboxyl group for Ca^{2+} crosslinking and gel formation (Fig. 9).

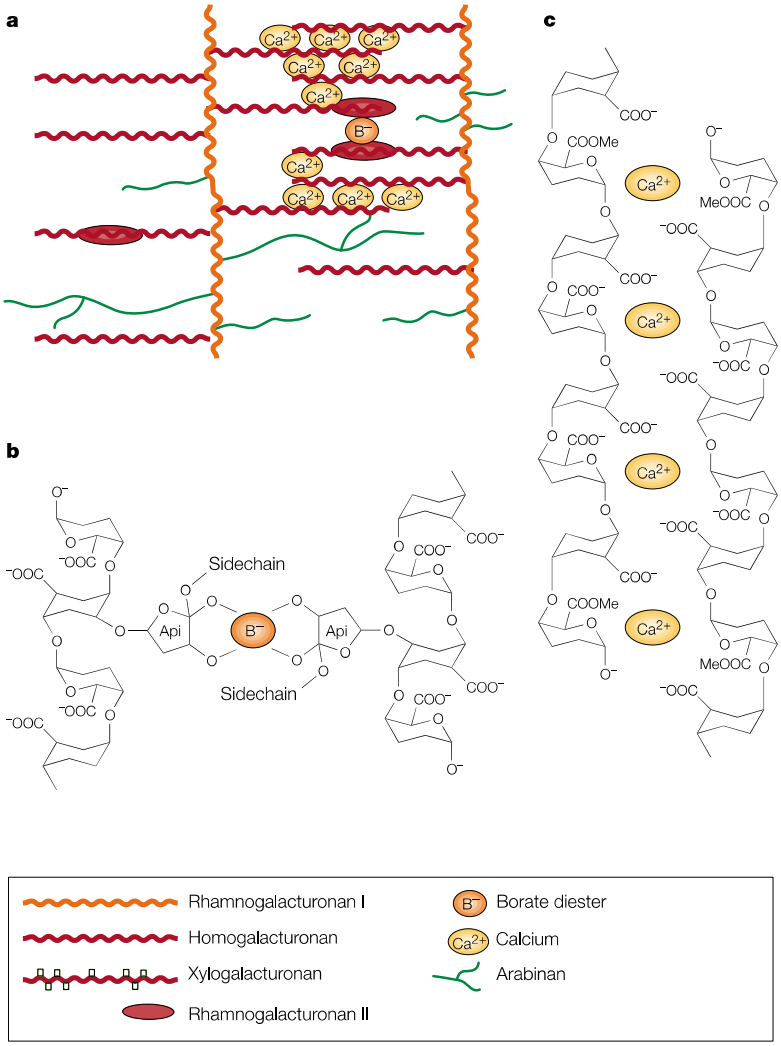

Figure 10: Formation of pectin networks. (a) Pectin domains covalently crosslink each other, they also form linkages involving boron and calcium. (b) Homogalacturonans form stiff gels through Ca2+-mediated crosslinking of its carboxyl groups. Image reproduced from Cosgrove 2005.

De-methylesterified HGA can have two general fates that differentially affect the mechanical properties of the cell wall: (1) stable gel formation as described above (Fig. 10), which causes cell wall stiffening, or (2) degradation by polygalacturonases, which causes cell wall loosening (Levesque-Tremblay *et al.,* 2015). Whether PMEs act randomly (like in fungi) or linearly (like in plants) along a pectin chain may influence the fate of de-methylesterified HGA (Micheli, 2001) (Fig. 11).

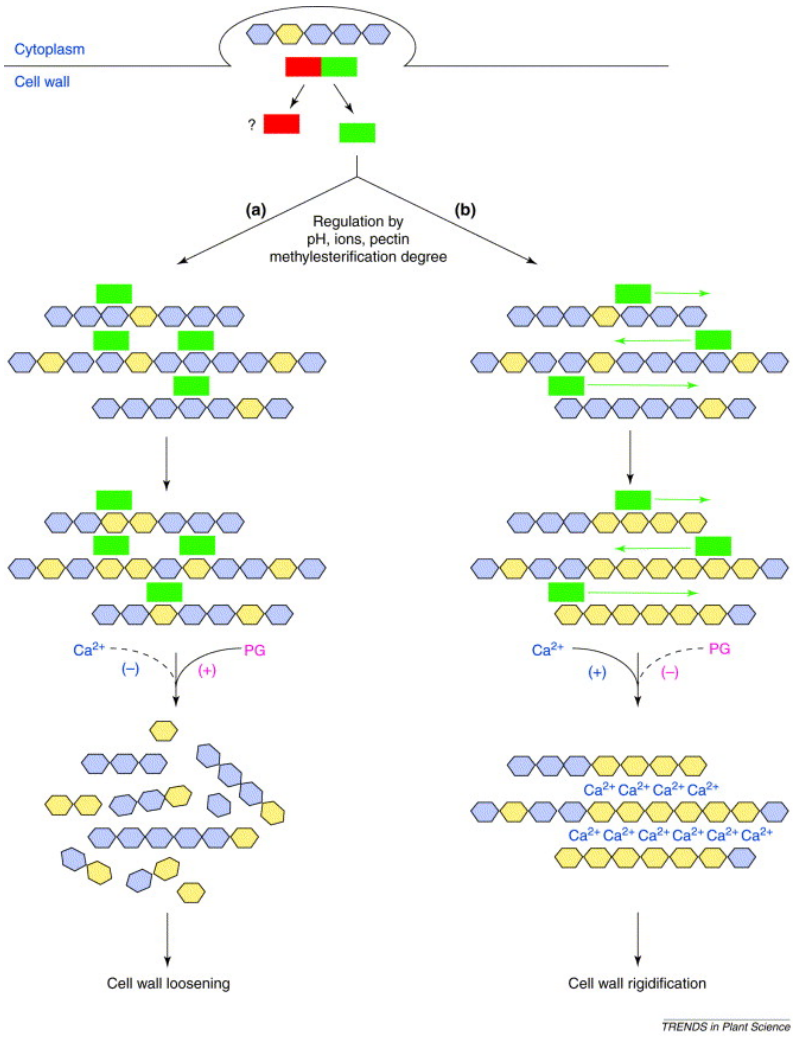

Figure 11: Modes of action of pectin methylesterases (PMEs). Depending on cell wall properties, PMEs (green) can act randomly (a), promoting the action of polygalacturonases (PG) and contributing to cell wall loosening, or can act linearly (b), giving rise to blocks of free carboxyl groups that interact with Ca2+, so rigidifying the cell wall. Methylesterified galacturonic acids are represented in blue and demethylesterified galacturonic acids in yellow. Figure reproduced from (Micheli, 2001) TRENDS in Plant Science.

1.3.3- Pectin methylesterase inhibitors

PME activity in the cell wall is inhibited by proteins called PME inhibitors (PMEI). A recent phylogenetic analysis identified 67 PME and 69 PMEI genes in *A. thaliana* (Scheler *et al.,* 2015).

These 136 genes clustered into three groups: Two groups of PME genes contained a PME domain (Pfam 01095), which harbours motifs important for PME activity. Twenty two genes clustered as one group that contained only this PME domain, while 45 genes clustered as another group that additionally contained a PMEI domain (Pfam 04043). A third group of 69 PMEI genes contained only a PMEI domain (Scheler *et al.,* 2015). The PME/PMEI system is potentially very important, since the degree and pattern of pectin demethyl-esterification might strongly influence biomechanical properties of the cell wall (Ali and Traas, 2016; Peaucelle *et al.,* 2011).

1.3.4- PME and PMEI activities in plant development

In the majority of cases where growth processes have been investigated in plant development, PME activity leads to cell wall stiffening and PMEI activity to cell wall softening (Levesque-Tremblay *et al.,* 2015). However, there are several examples where the opposite was true, which complicates our understanding of the role of PME activity in cell wall mechanics, growth, and development. For example, overexpression of *PME5* in the shoot apical meristem of *A. thaliana* decreased pectin methylesterification, but caused cell wall softening rather than stiffening (Peaucelle *et al.,* Current Biology, 2011; Peaucelle *et al.,* Current biology, 2008). Conversely, overexpression of *PMEI3* increased pectin methylesterification and caused cell wall stiffening rather than softening (Peaucelle *et al.,* Current Biology, 2011; Peaucelle *et al.,* Current biology 2008). Moreover, these modifications of PME activity affected organogenesis at the shoot apical meristem with overexpression of *PME5* and *PMEI3* causing ectopic organ formation and inhibition of organ formation, respectively (Peaucelle *et al.,* Current biology, 2008). However, another study found no obvious differences in the degree of pectin methylation related to organ formation at the shoot apical meristem (Yang *et al.,* Current Biology, 2016). Taken together, these findings imply that organogenesis requires pectin properties to remain constant.

A more straightforward role for PMEI activity is in seed mucilage release in *A. thaliana*. Upon contact with water, specialized cells in the seed coat release cellulose fibrils and large quantities of pectins as a gel around the seed (Haughn and Western, 2012). Mucilage is not essential for seed germination in *A. thaliana* but may serve as an adhesive or short-term water reservoir for the seed (Western, 2000). *PMEI6* is expressed in epidermal cells of the seed coat and required for normal mucilage release, as natural and induced alleles of *PMEI6* showed delayed release of seed mucilage (Saez-Aguayo *et al.,* 2013). In summary, the regulation of pectin

methylesterification by PME/I activity has important consequences for cell wall biomechanics and the function of genes such as *PMEI6* in *A. thaliana* seed coat development are clear. However, a general understanding of how PME/I activities relate to cell wall elasticity, plant growth, and development, is not straightforward.

1.4- Aim of study

In this project, I aim to investigate the role of cell wall-remodeling genes in explosive seed dispersal in *C. hirsuta*. As a basis for this project, I will use the 17 PME/I genes that were previously identified as differentially expressed during fruit development specifically in *C. hirsuta*, but not *A. thaliana* (Gan *et al.,* 2016). I aim to understand whether the species-specific expression of these genes is associated with the dramatically different seed dispersal strategies of *C. hirsuta* and *A. thaliana*. To do this, I will characterize the spatial and temporal expression of these 17 PME/I genes during *C. hirsuta* fruit development, and generate transgenic plants expressing artificial miRNAs that target 7 of these genes for silencing. By analyzing endogenous mRNA levels of targeted genes in these transgenic plants, I aim to identify lines that can be used to assess the function of PME/I genes in *C. hirsuta*. These transgenic plants will provide a permanent resource to study how PME/I activities influence explosive seed dispersal and other traits in *C. hirsuta*.

Chapter 2

Materials and methods

2.1- Plant material and growth conditions

2.1.1- Plant growth conditions

Cardamine hirsuta plants of the reference Oxford (Ox) accession: herbarium specimen voucher Hay 1 (OXF) (Hay & Tsiantis, 2006), were sown on soil in 7-cm square pots (1-6 plants per pot), or large trays (for Basta selection). Seeds were then stratified at 4°C in the dark for seven days to break seed dormancy, and grown under long day conditions (16 hr light/8 hr dark) in the greenhouse or in a controlled environment chamber (16 hr light at 21°C, 8 hr dark at 18°C).

2.1.2- Solid media:

Solid growth media containing 0.5x Murashige and Skoog (MS), mineral salts (sigma), no sucrose, 50 mg/L hygromycin and 0.6-0.8 % agar, was prepared in square Petri dishes. Sterilized seeds were sown on the media surface, covered with aluminum foil and stratified at 4°C for seven days, then grown in a horizontal orientation in long day conditions for one day, before being covered again with aluminum foil and germinated in the dark. Petri dishes were then uncovered and transgenic plants that were hygromycin resistant were identified after a few days growth as tall seedlings with green cotyledons.

2.1.3- Surface sterilization of seeds:

Seeds were sterilized by incubating them in 70% ethanol, 0.1% triton X-100 solution for 10 minutes, and then briefly washed with 96% ethanol. This procedure was repeated two times, and then seeds were quickly air-dried under a laminar flow hood.

2.1.4- Seed harvesting and storage:

Mature (yellowing) siliques were bagged before pod shatter. Seeds were harvested, collected in small envelopes and stored in boxes containing silica gel (2-5mmm Carl Roth GmbH) to dry for a minimum of two weeks before planting.

2.2- Bacterial and Plant Transformation

2.2.1- Heat shock Transformation

An amount of 2 µl of plasmid DNA was added into a tube of competent cells (E.*coli* strain *DB3.1* or strain *DH10B*), mixed gently, and incubated for 30 minutes on ice. Cells were heat shocked for 40 seconds at 42°C, then immediately transferred on ice for 2 minutes, after that 500 µl liquid LB or SOC was added, the cells were recovered in the 37°C shaking incubator for 60 minutes, plated on LB-Agar + antibiotic, and incubated at 37°C overnight.

2.2.2- Transformation of Agrobacterium

An amount of 2 µl of plasmid DNA was transformed via electroporation into *Agrobacterium tumefaciens GV3101::pMP90* strain which has gentamycin resistance on the *Ti* plasmid, and selected with antibiotics: Spectinomycin + Gentamycin or Kanamycin + Gentamycin for *pOPIn2-AtRPS5a::LhGR2*-amir and *pMCD32*-amir plasmids, respectively.

2.2.3- Plant transformation

Transformation of *C. hirsuta* wild-type plants with *Agrobacterium* was done using the floral dip method (Hay *et al*., 2014). Transgenic seeds were harvested and selected with the appropriate selection method: Basta spray for *pOPIn2-AtRPS5a::LhGR2*-amir and Hygromycin on solid MS media for *pMCD32*-amir plasmids, respectively. Resistant seedlings from each construct were transferred to 7-cm pots to analyze individual T1 lines. Seeds of the first-generation transgenic plants (T1) were harvested from individual plants. Approximately 40 seeds from individual T1 lines were selected with either Basta spray or on hygromycin solid media and segregation ratios of resistant to sensitive plants were scored in this T2 generation.

2.3- Artificial miRNA construction

2.3.1- Computational tools

To design amiRNAs we used the Web MicroRNA Designer program (WMD), which is available at http://wmd3.weiglword.org (Schwab *et al*., 2006). To design gene-specific qRT-PCR primers,

we used IGV and DNASTAR software. amiRNA sequences are listed in Table 1 and primers used in this thesis are listed in Table 2.

2.3.2- amiRNA sequences

To design amiRNAs, we submitted sequences of our target genes in FASTA format as single or multi targets to the WMD program, and then we selected 21mer amiRNA sequences from the WMD output that had high-confidence predictions to silence our genes (Table 1).

Table 1. amiRNA sequences and target genes

amir	Target gene	amir sequence	Hybridisation energy of amir to perfect complement	Hybridisation energy of amir to target gene	
PMEI6	CARHR143060	TTAACGTATGAGCTGTACCGA	-42.87		-42.08
4a	CARHR043880	TAGTTTTGTAGTTGCCTGCGT	-42.49	CARHR044320	-34.75
	CARHR044320			CARHR045850	-34.67
	CARHR045840			CARHR043880	-36.75
	CARHR045850			CARHR045840	-32.39
3a	CARHR043880	TTTTGACGATAATAAATCCGC	-36.98	CARHR044320	-30.24
	CARHR044320			CARHR045850	-34.18
	CARHR045850			CARHR043880	-30.24
3c	CARHR089500	TAAAACGTTCGATGGTCGCCT	-43.82	CARHR213460	-34.13
	CARHR213460			CARHR089500	-36.21
	CARHR265370			CARHR265370	-34.13
2a	CARHR213460	TATGATGGCATAAGTTAGCGT	-41.32	CARHR213460	-37.60
	CARHR265370			CARHR265370	-37.60

Colour of amiRNA sequence indicates a quality ranking given by WMD (green is best, yellow is intermediate) based on different criteria such as, amiRNA sequence composition (e.g. degree of 5' instability), mismatch positions when paired to intended target(s), hybridization energy when paired to intended target(s), number of other genes which have 5 or less mismatches to the amiRNA (not following the mismatch rules, preferably very few), hybridization energies of other genes with 5 or less mismatches to the amiRNA (not following mismatch rules, preferentially much higher) etc.

2.3.3- amiRNA construction by Overlapping Polymerase Chain Reaction

I used the WMD program to design oligonucleotides specific for each amiRNA, for use in overlapping PCR (four oligonucleotides per amiRNA: I, II, III and IV, Table 2). To construct amiRNA stemloops I used two other oligonucleotides based on the template plasmid (pRS300) sequence (primers A and B, Table 2) (Schwab *et al*., 2006). The plasmid RS300 (miR319a pBSK, kindly provided by Detlef Weigel), which contains the *A. thaliana atb*-miR319a precursor, was used as template in PCR reactions (Fig. 1). With the use of overlapping PCR and six primers (I, II, III, IV, A and B) for each amiRNA, the original miRNA sequences of miR319a were replaced with these amiRNAs. Reactions took place in the Bio-Budget lab cycler Thermal cycler, using the following program: initial denaturation 98°C (30 seconds), 35 cycles of: 98°C (10 seconds), 50°C (30 seconds), 72°C (15 seconds), and a final extension of 72°C (7 minutes). Overlapping PCR reactions were carried out with proof-reading high fidelity DNA polymerase (Thermo Scientific Phusion Hot Start II). For a 50µl reaction, the following were mixed in a 0.2 ml PCR tube: 5X Phusion Buffer, 10 mM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 0.02 U/µl Phusion Hot Start II DNA polymerase, and 1 µl template DNA (pRS300), in water. With this technique, initially amplified products were used as a template mix (0.5 µl each) in a single PCR reaction to amplify a single final product.

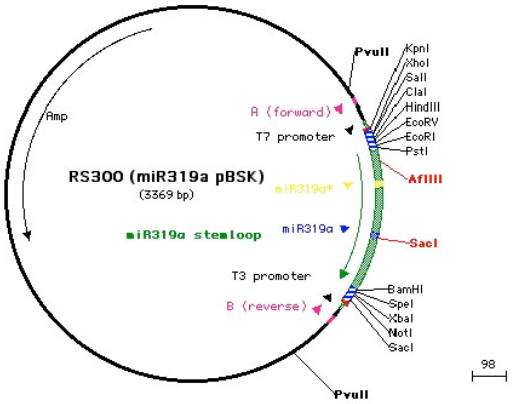

Figure 1: The plasmid RS300 (miR319a pBSK), which contains the *A. thaliana atb*-miR319a precursor.

2.3.4- Gateway Cloning

2.3.4.1- Creation of amiRNA entry vectors

After replacing the original miR319a precursor with the amiRNA sequences for my genes of interest, I subcloned the resulting stemloops into the pCR8 vector by TA cloning using the pCR8®/GW/TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. TOPO TA reactions were transformd into competent *E.coli DH10B* cells and spread on Spectinomycin containing LB agar plates. Plates were incubated at 37°C overnight. To screen for positive clones I used PCR colony screening. Positive colonies were propagated, then single colonies were mini-prepped, purified using a DNA purification kit (Machery-Nagel GmbH & Co.KG), and plasmid DNA was analyzed by *EcoR1* restriction digest. To check for orientation, I used *Afl Π* restriction digest (which is a unique restriction site in the pCR8®/GW/TOPO vector) and *Sal I* (which is a unique restriction site in the amiRNA insert). The fragments were then purified and dissolved in 50 µl AT Buffer. The plasmid DNA concentrations were measured by Nanodrop, diluted to 50 ng/µl according to the Sanger sequencing information of Max-Planck Institute with primer pair A and B (Table 2), and sent for sequencing. Positive entry clones were used for LR reactions to create final amiRNA vectors.

2.3.4.2- Creation of amiRNA destination vectors

I created amiRNA constructs in both constitutive and inducible expression vectors. For inducible expression, I used the transactivated and chemically induced gene expression system provided by (Moore *et al.,* 2002). This system enables you to have inducble expression via a two component system contained on a single T-DNA insert. I used the vector *pOPIn2-AtRPS5a::LhGR2* in order to drive my genes of interest under the *AtRPS5a* costitutive promoter (Fig. 2A). For constitutive expression, I used the *pMDC32* vector (with a pCambia 1300 backbone), which contains a 2X *35S CaMV* promoter upstream of a Gateway cloning cassette. Therefore, I constructed two types of destination vectors via recombination from amiRNA entry vectors: with inducible expression (*pOPIn2-AtRPS5a::LhGR2*) or constitutive expression (*pMDC32*) (Fig. 2B). Before use, the *pMDC32* vector was first transformed into *DB3.1* competent cells, then plated out on LB-Agar + Spectinomycin 100 µg/ml to select colonies for plasmid isolation. The *pOPIn2-AtRPS5a::LhGR2* vector was plated on LB-Agar + Kanamycin 100 µg/ml to select colonies for plasmid isolation.

Plasmid DNA concentration was measured by Nanodrop, diluted to 20 fmol and used as a destination vector in order to recombine with amiRNA entry vectors via LR reactions.

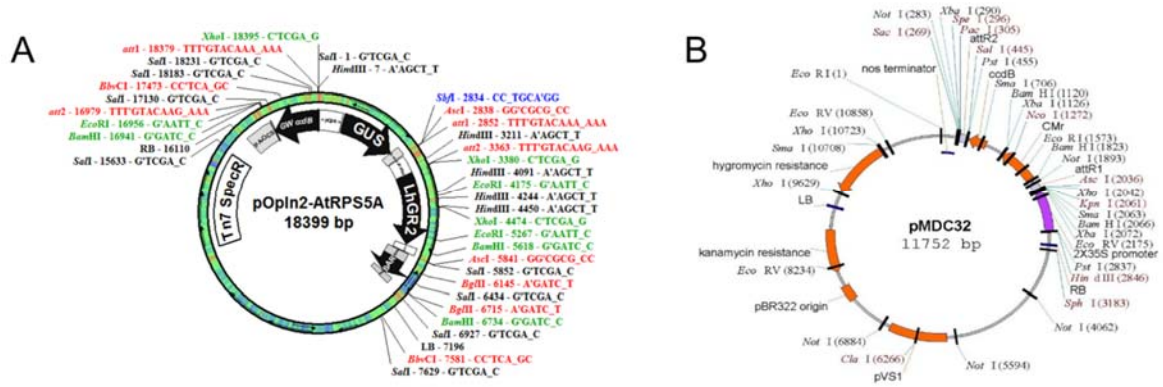

Figure 2: Destination vectors used to create amiRNA constructs with inducible expression: *pOPIn2-AtRPS5a::LhGR2* (A) or constitutive expression: *pMDC32* (B).

2.3.4.3- Creation of final amiRNA constructs

Using the Gateway LR Clonase II Enzyme Mix, I recombined the amiRNA entry vectors, described in section 2.3.4.1, via LR reactions, with both *pOPIn2-AtRPS5a::LhGR2* and *pMCD32* destination vectors described in section 2.3.4.2 (cloning procedure, Fig. 3). For *pOPIn2-AtRPS5a::LhGR2*, I linearized the entry vectors by *BglI* restriction digest because both entry and destination vector have the same selection (Fig. 4). To screen for positive clones, plasmids were purified and digested with *NotIHF* and *AscI* restriction enzymes for *amir-pOPIn2-AtRPS5a::LhGR2* plasmid, and *NcoI /BspHI* digestion for *amir-pMDC32*.

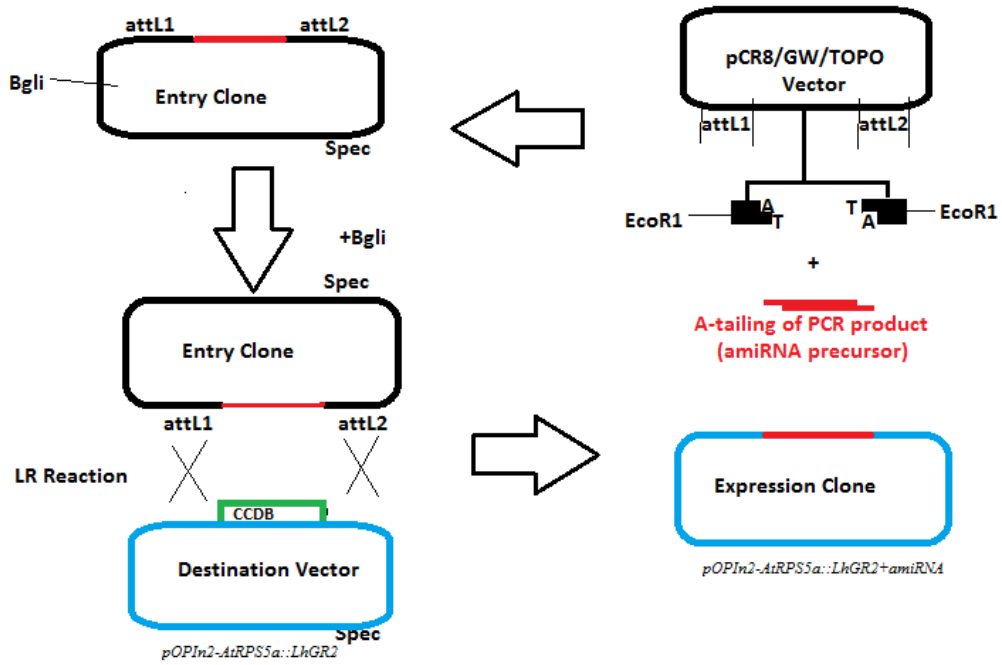

Figure 3: Schematic of LR reaction for creating dexamethasone-inducible amir constructs in the *pOPIn2-AtRPS5a::LhGR2* destination vector.

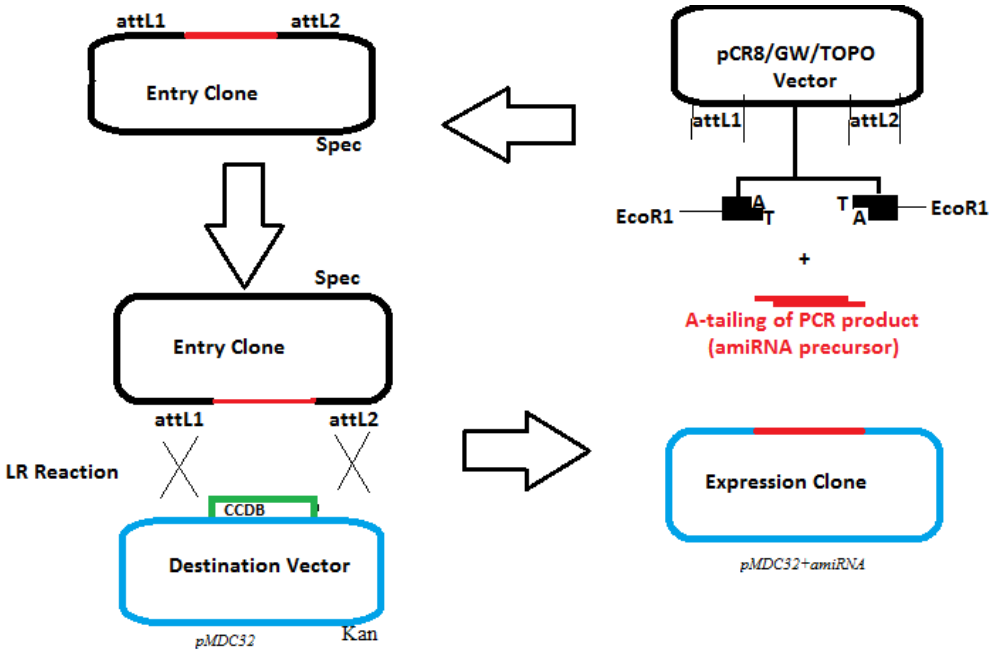

Figure 4: Schematic of LR reaction for creating constitutively expressed amir construts in the *pMCD32* destination vector.

2.4- Molecular methods

2.4.1- Analysis and purification of PCR products

PCR products were run on a 1 % agarose (Biozym Scientific GmbH) gel electrophoresis, then isolated and purified with NuleoSpin® Gel and PCR Clean up gel extraction kit (Machery-Nagel GmbH & Co.KG). Purified PCR products were then sequenced to confirm there are no PCR-generated errors.

2.4.2- Gel electrophoresis

Gel electrophoresis was carried out in 1X TAE buffer in the presence of Midori Green Advanced dye. Gels were run at 110 V for between 60 to 90 minutes. Bands were visualized with Biodoc-It ^{tm} Imaging System.

2.4.3- RNA Isolation

Samples from different stages of C. *hirsuta* wild type fruit (stages 9, 15, 16, 17a, see Fig. 1 chapter 3) as well as three tissue samples (valve, seeds and rest of fruit, see Fig. 6 chapter 3) from stage 17b fruit and also different C. *hirsuta* wild type plant tissues (seedling, root, rosette leaf number five, fruit stage 16 and floral shoot, see Fig. 9 chapter 3) were dissected and flash frozen in liquid nitrogen. I carefully dissected both valves off each fruit with a sharp, thin needle under a stereo microscope, then dissected out the seeds, leaving the remaining fruit tissues. Fruit tissue samples were pooled from 5-6 plants per replicate. Total RNA was isolated from three biological replicates of each sample using a Spectrum plant total RNA kit (Sigma-Aldrich). RNA concentration was calculated using Agilent 2100 Bioanalyzer II.

2.4.4- Checking for DNA contamination

Isolated RNA was checked for DNA contamination by PCR. The PCR reaction contained as follows: 5u/µl Ampliqon *Taq* DNA polymerase, 10X standard Buffer, 10 mM dNTPs, using housekeeping gene primers: 0.5 µM NB80 forward primer, 0.5 µM SH843 reverse primer (table 1). PCR reaction was done using the following programme: Initial denaturation 95$^{°C}$ (1 minute and 30 seconds), 40 cycles of: 95$^{°C}$ (30 seconds), 58$^{°C}$ (30 seconds), 72$^{°C}$ (1 minute), and a final extension of 72$^{°C}$ (5 minutes). PCR products together with genomic DNA (which used as a positive

control) were then ran on 1.5% agarose gel electrophoresis to identify the DNA contamination. DNase I digestion was done after PCR check using Turbo DNA-free kit (Ambion) for those PCR Products which have the same size of bands as our positive control (*C. hirsuta* genomic DNA).

2.4.5- Semi-quantitative RT-PCR

Superscript III reverse transcription kit (Thermo Fisher Scientific) was used for cDNA synthesis according to the manufacturer's instructions. Synthesized cDNA was then quality checked via PCR using the housekeeping gene primers NB80 and SH843 (Table 2) with conditions described above in section 2.4.4, with two different cycle numbers: 23X and 35X. Amplified PCR products, and also genomic DNA as a control, were loaded on a 1% agarose gel, electrophoresed and the intensity and size of bands were analyzed. The size of bands for our cDNA were smaller than the size of band for gDNA. PME/I genes were then amplified by PCR using this cDNA template with gene specific primers (Table 2), using the conditions described above in section 2.4.4 with 35 cycles. PCR products were run on 1.5% agarose gel electrophoresis and the intensity and size of bands were analyzed.

2.4.6- Quantitative RT-PCR

Total RNA was extracted from three biological replicates of each tissue sample as described in section 2.4.3. RNA was converted into cDNA using SuperScript III Reverse Transcriptase and an oligo-dT primer as described in section 2.4.4. Quantitative PCR was performed in triplicate using Power SYBR Green Master Mix (Fisher Scientific) and the ViiA 7 Real-Time PCR System (Fisher Scientific). Primer efficiency and expression level were determined as previously described (Pfaffl, 2001). Expression levels of each PME/I gene was normalized to the reference gene *CLATHRIN/AP2M.* All primers used are described in Table 2.

2.4.7- Determining transgene copy number and zygosity

Transgene copy number and zygosity was determined using *g-Count* technology by idnaGenetics, Norwich, UK. In this service, the company extracts DNA from leaf tissue, provided by the customer, and uses the hygromycin marker gene present in my construct to measure the number of transgene copies relative to an invariant gene in the *C. hirsuta* genome. Young leaf tissue (1cm x 1cm) was collected from 85 T3 plants including five *35S::amir-4a* lines, two *35S::amir-2a* and

two *35s::amir-3c* lines. The leaf samples were collected in capped Qiagen sample collection tubes, freeze dried and send to iDNA Genetics, Norwich, UK for multiplex PCR analysis.

2.5- Protein assays

2.5.1- PME activity assay

PME activity was determined by ruthenium red-stained gel assays as previously described (Gan *et al.,* 2016). In brief, dry seeds (50-100 mg) of *A. thaliana* Col-0, *35S::PMEI6* and *pmei6-1* (Saez-Aguayo *et al.,*2013), and *C. hirsuta* Ox and *35S::amir-4a-1*, were ground in 250 µl of cold extraction buffer (4°C, 1M NaCl, 12.5 mM citric acid, 50mM Na2HPO4, pH 6.5 in dH2O) with a motorized tissue grinder and left at 4°C for 4h. Samples were centrifuged at 14.2 k rpm for 15 minutes and supernatants were collected. Protein concentrations were determined by Bradford assay and 80 µl of extracts containing 15, 10, 5 and 2.5 µg of protein were loaded into 0.5 cm wells in a 1% agar plate supplemented with 0.1% of ≥85% esterified citrus fruit pectin (Sigma, cat. Nr. P9561), 50mM Na2HPO4 pH 6.5, and 12.5 mM citric acid. Plates were incubated overnight at room temperature and subsequently stained with 500 µg/ml Ruthenium Red for 45 minutes. Background stain was reduced by destaining with dH2O for 8 h and 48 h at room temperature and 4°C, respectively. Plates were then imaged with a scanner.

2.5.2- Bradford protein assay

Protein concentration of seed extracts was determined by Bradford assay (Bradford, 1976). In brief, Bradford reagent (Bio RAD, cat. Nr. 500-0205) was added to each tube containing water blanks, BSA protein standards, or seed extract dilutions, mixed and incubated at room temperature for 5 min. Absorbance was measured at 595 nm with a spectrophotomer (Eppendorf). A standard curve was calculated using the BSA standards and used to determine the concentration of protein in seed extracts.

Table 2. Primer sequences used in this thesis

primer	sequence (5'...3')	length (bp)	orientation	target	template
A	CTGCAAGGCGATTAAGTTGGGTAAC	25	forward	pRS300	overlapping PCR
B	GCGGATAACAATTTCACACAGGAAACAG	28	reverse	pRS300	overlapping PCR
UniL	CGTTGTAAAACGACGGCCAGT	21	forward	pCR8®/GW/TOPO vector	Gateway Cloning
revL	CAGGAAACAGCTATGACCATG	21	reverse	pCR8®/GW/TOPO vector	Gateway Cloning
f103	gaTAGTTTTGTAGTTGCCTGCGTtctctctttgtattcc	40	forward	4a_I_miR-s	PMEI amiRNA oligos
f104	gaACGCAGGCAACTACAAAACTAtcaaagagaatcaatga	40	reverse	4a_II_miR-a	PMEI amiRNA oligos
f105	gaACACAGGCAACTAGAAAACTTtcacaggtcgtgatatg	40	forward	4a_III_miR*s	PMEI amiRNA oligos
f106	gaAAGTTTTCTAGTTGCCTGTGTtctacatatatattcct	40	reverse	4a_IV_miR*a	PMEI amiRNA oligos
f107	gaTTTTGACGATAATAAATCCGCtctctctttgtattcc	40	forward	3a_I_miR-s	PMEI amiRNA oligos
f108	gaGCGGATTTATTATCGTCAAAAtcaaagagaatcaatga	40	reverse	3a_II_miR-a	PMEI amiRNA oligos
f109	gaGCAGATTTATTATGGTCAAATtcacaggtcgtgatatg	40	forward	3a_III_miR*s	PMEI amiRNA oligos
f110	gaATTTGACCATAATAAATCTGCtctacatatatattcct	40	reverse	3a_IV_miR*a	PMEI amiRNA oligos
f111	gaTAAAACGTTCGATGGTCGCCTtctctctttgtattcc	40	forward	3c_I_miR-s	PMEI amiRNA oligos
f112	gaAGGCGACCATCGAACGTTTTAtcaaagagaatcaatga	40	reverse	3c_II_miR-a	PMEI amiRNA oligos
f113	gaAGACGACCATCGATCGTTTTTtcacaggtcgtgatatg	40	forward	3c_III_miR*s	PMEI amiRNA oligos
f114	gaAAAAACGATCGATGGTCGTCTtctacatatatattcct	40	reverse	3c_IV_miR*a	PMEI amiRNA oligos
f115	gaTATGATGGCATAAGTTAGCGTtctctctttgtattcc	40	forward	2a I miR-s	PMEI amiRNA oligos
f116	gaACGCTAACTTATGCCATCATAtcaaagagaatcaatga	40	reverse	2a II miR-a	PMEI amiRNA oligos
f117	gaACACTAACTTATGGCATCATTtcacaggtcgtgatatg	40	forward	2a III miR*s	PMEI amiRNA oligos
f118	gaAATGATGCCATAAGTTAGTGTtctacatatatattcct	40	reverse	2a IV miR*a	PMEI amiRNA oligos
n119	gaTTAACGTATGAGCTGTACCGAtctctctttgtattcc	40	forward	PMEI6_I miR-s	PMEI amiRNA oligos
n120	gaTCGGTACAGCTCATACGTTAAtcaaagagaatcaatga	40	reverse	PMEI6_II miR-a	PMEI amiRNA oligos
n121	gaTCAGTACAGCTCAAACGTTATtcacaggtcgtgatatg	40	forward	PMEI6_III miR*s	PMEI amiRNA oligos
n122	gaATAACGTTTGAGCTGTACTGAtctacatatatattcct	40	reverse	PMEI6_IV miR*a	PMEI amiRNA oligos
NB80	AGCTCCGTATTGCTCCTGAA	20	forward	Binds to ChACT8	House keeping gene
SH 843	CAGTGAGGTCACGACCAGCA	20	reverse	Binds to ChACT8	House keeping gene
22	TCGATTGCTTGGTTTGGAAGATAAGA	26	forward	MKp1-p1-Clatherin	endogenous control q-PCR
23	TTCTCTCCCATTGTTGAGATCAACTC	26	reverse	MKp2-p2-Clatherin	endogenous control q-PCR
n147f	CCGTTTGCAATCGTGGCTAA	20	forward	CARHR143060-PMEI6	qPCR
n148r	AATCCTGGTCCAGTTTCCGT	20	reverse	CARHR143060-PMEI6	qPCR
n149f	CGCGCTTAACCGTAACTTGA	20	forward	CARHR085300	qPCR
n150r	ATTCACCCAAGTTCCGGCTA	20	reverse	CARHR085300	qPCR
n151f	AAGCGTTGGAGATGGATGGA	20	forward	CARHR118350	qPCR
n152r	AGGCTACAAAATCCGCGTTC	20	reverse	CARHR118350	qPCR
n153f	TCTTCCTCTTCCGACCGATC	20	forward	CARHR214060	qPCR
n154r	CCCTCACAGCCTCTTCCAAT	20	reverse	CARHR214060	qPCR
n155f	GCCTCACTCTCAACACAAGC	20	forward	CARHR276140	qPCR
n156r	CTCCTCCAACGTGTCACCTA	20	reverse	CARHR276140	qPCR
n159f	CTGGGACGATTGGGACCATA	20	forward	CARHR173850 PME5	qPCR
n160r	AGGAGCGATCCAATGTCTCC	20	reverse	CARHR173850 PME5	qPCR
n163f	TTATGGGAGATGGTCGGACG	20	forward	CARHR089480	qPCR
n164r	AGGCGGAGAAATCAGAACCA	20	reverse	CARHR089481	qPCR
n165f	CGGAGATGAGTCAGCGTTTG	20	forward	CARHR004800	qPCR
n166r	TGACCCTGGGCTCAATTGAT	20	reverse	CARHR004800	qPCR
n169f	AACCAACATTGAGACGTGCC	20	forward	CARHR156040	qPCR
n170r	CTCGTGTCTTGAAACCCAGC	20	reverse	CARHR156040	qPCR
f120	AGGCAACTACAAAACTGTCG	20	forward	CARHR043880	qPCR
f121	TTTGACTAAATAATGATTCAGATT	24	reverse	CARHR043880	qPCR
f132	AAGCAGCGGCAGAGAGTTGGAG	22	forward	CARHR044320	qPCR
f134	GATTGCGTTTGGCGTTACTGTGAGA	25	reverse	CARHR044320	qPCR
f126	CCTCCATTAGTGCCAACGCTCT	22	forward	CARHR045850	qPCR
f127	AATTCATCATACGGTGCCTTGCT	23	reverse	CARHR045850	qPCR
n 129 F	TAGTGGAAGATGGGTCGTGG	20	forward	CARHR089500	qPCR
n 130 R	ATCCCTGGAAAGCACATCTG	20	reverse	CARHR089500	qPCR
n124f	CTT AAG AGT CTC AGA GAT TAT ATG A	25	forward	CARHR213460	qPCR
n180r	GTA AAT TCT TAT CAA CTT GTT TGG C	25	reverse	CARHR213460	qPCR
n181f	GGATACGGCGGTGCTGGACA	20	forward	CARHR265370	qPCR
n182r	GAA ACC CGA GAA TCT CCA CAT GTT C	25	reverse	CARHR265370	qPCR
n173 f	CAAGGACTGCGACATTGTTGGC	22	forward	CARHR213450	qPCR
n176r	CTT GTT GTC CCA CGA AAC C	19	reverse	CARHR213450	qPCR
n187r	GGA CTC GTC GTG CTG TTC CAC TG	23	reverse	CARHR265360	qPCR
n188f	CTGGCGGCGATGATCCAAACATG	23	forward	CARHR265360	qPCR

Chapter 3

Results

Analysis of PME/I gene expression in *C. hirsuta*

3. Analysis of PME/I gene expression in *C. hirsuta*

3.1- Introduction

In order to identify genes that were differentially expressed during explosive seed dispersal in *C. hirsuta*, an RNAseq experiment was previously performed in the lab (Gan *et al*., 2016). The results of this experiment, together with an analysis of expanded gene families in the *C. hirsuta* genome, identified 17 PME/I genes that were differentially expressed during *C. hirsuta* fruit development (Gan *et al*., 2016). For the results presented in this chapter, I used quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to validate the previous RNAseq results and analyse the spatiotemporal dynamics of PME/I gene expression during *C. hirsuta* fruit development. For all qRT-PCR experiments, I employed the expression of the housekeeping gene Clatherin to normalize the expression of PME/I genes. For all experiments, I present the mean and standard error of three biological replicates for each sample and use the Student's t-test to test for differential gene expression.

3.2- Results

3.2.1- Validation of RNAseq results

To validate the results of the previous RNAseq experiment (Gan *et al*., 2016), I performed qRT-PCR to analyse PME/I gene expression during early and late stages of *C. hirsuta* fruit development. RNAseq and qRT-PCR are very different techniques to quantify gene expression; cDNA sequence reads are directly counted in RNAseq, whereas the cDNA is amplified in qRT-PCR and quantified via the incorporation of fluorescent dye. Despite these differences, it is expected that the results from both techniques should agree.

In the RNAseq experiment, gene expression was compared between immature *C. hirsuta* fruit at stage 9 and mature *C. hirsuta* fruit at stage 16 (Fig. 1). Therefore, I compared gene expression by qRT-PCR between these same fruit stages. I was able to validate the differential expression of all 17 PME/I genes that were identified by RNAseq. All genes, with the exception of CARHR173850/PME5, were expressed significantly higher in stage 16 fruit than in stage 9 fruit (Table 1, Fig. 2, Fig. 3). CARHR173850/PME5 was expressed significantly lower in stage 16 fruit than in stage 9 fruit (Table 1, Fig. 2).

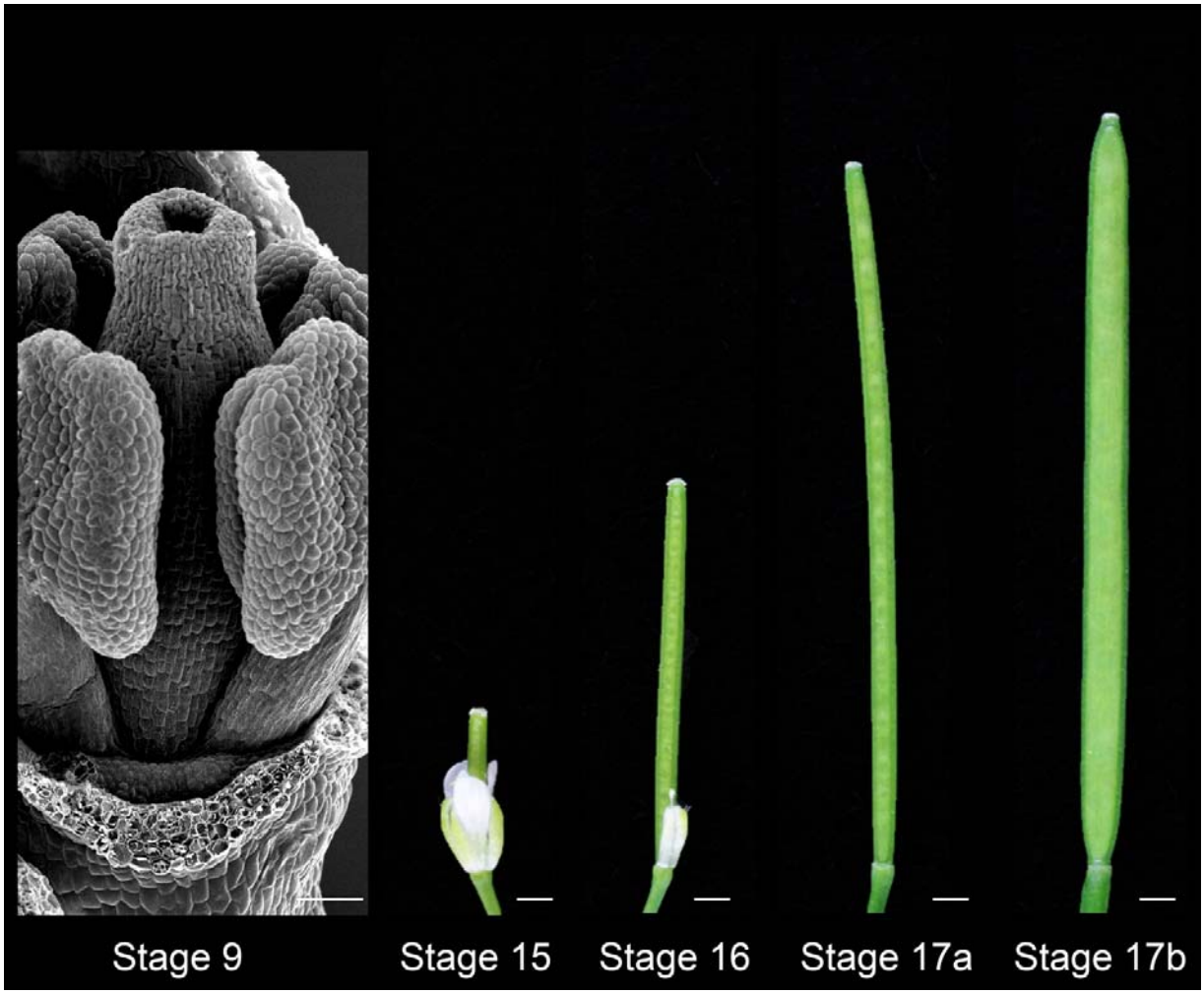

Figure 1: Stages of *C. hirsuta* fruit development. Stage 9 carpel is shown in a scanning electron micrograph of an unopened flower bud with an obscuring sepal dissected away and fours stamens surrounding the central carpel. Stages 15 through 17b fruit are shown in photographs. Note that floral organs are present in stage 15 flowers and abscise during stage 16. Scale bars: stage 9 (50 μm), stages 15, 16, 17b (1 mm).

Table 1. Validation of differential PME/I gene expression during *C. hirsuta* fruit development by qRT-PCR quantification.

Gene Name	*C. hirsuta* fruit stage 9	*C. hirsuta* fruit stage 16	
	mean	mean	P-Value
CARHR143060 PMEI6	2,66E-03 ± 9,40E-05	1,94E-02 ± 5,30E-03	3,44E-02
CARHR085300	6,04E-03 ± 2,34E-04	5,56E-02 ± 3,90E-03	2,22E-04
CARHR118350	2,47E-04 ± 3,23E-05	9,93E-04 ±2 ,59E-05	5,55E-05
CARHR214060	6,12E-04 ± 1,53E-04	2,42E-02 ± 6,03E-03	1,75E-02
CARHR276140	2,61E-01 ± 5,29E-02	5,84E-01 ± 6,13E-02	1,62E-02
CARHR173850 PME5	7,84E-02 ± 8,33E-03	3,72E-03 ± 4,89E-04	8,61E-04
CARHR089480 PME3	5,84E-01 ± 1,00E-01	2,55E+00 ± 1,54E-01	4,36E-04
CARHR004800	2,41E-03 ± 1,51E-03	5,31E-01 ± 1,43E-02	3,27E-06
CARHR156040	2,04E-02 ± 2,38E-03	1,44E-01 ± 1,98E-02	3,46E-03
CARHR043880	0	1,20E+00 ± 2,48E-01	6,04E-04
CARHR044320	0	5,78E+00 ± 2,48E-01	6,04E-04
CARHR045850	1,50E-01 ± 1,36E-02	2,58E+01 ± 1,77E+00	1,30E-04
CARHR089500	3,30E-05 ± 8,34E-06	2,44E-02 ± 1,54E-03	9,34E-05
CARHR213460	1,10E-05 ± 5,30E-06	2,62E-03 ± 2,05E-04	2,19E-04
CARHR265370	2,51E-05 ± 2,65E-06	4,02E-03 ± 3,27E-04	2,58E-04
CARHR213450	8,65E-04 ± 4,87E-04	1,26E-01 ± 1,04E-02	2,80E-04
CARHR265360	8,34E-05 ± 6,80E-06	1,10E-02 ± 3,85E-04	9,20E-06

C. hirsuta fruit stages are described in Fig. 1. Gene expression is compared pairwise between stage 9 and stage 16 fruit using Student's t-test. Values are shown as means of 3 biological replicates ± standard error of the mean.

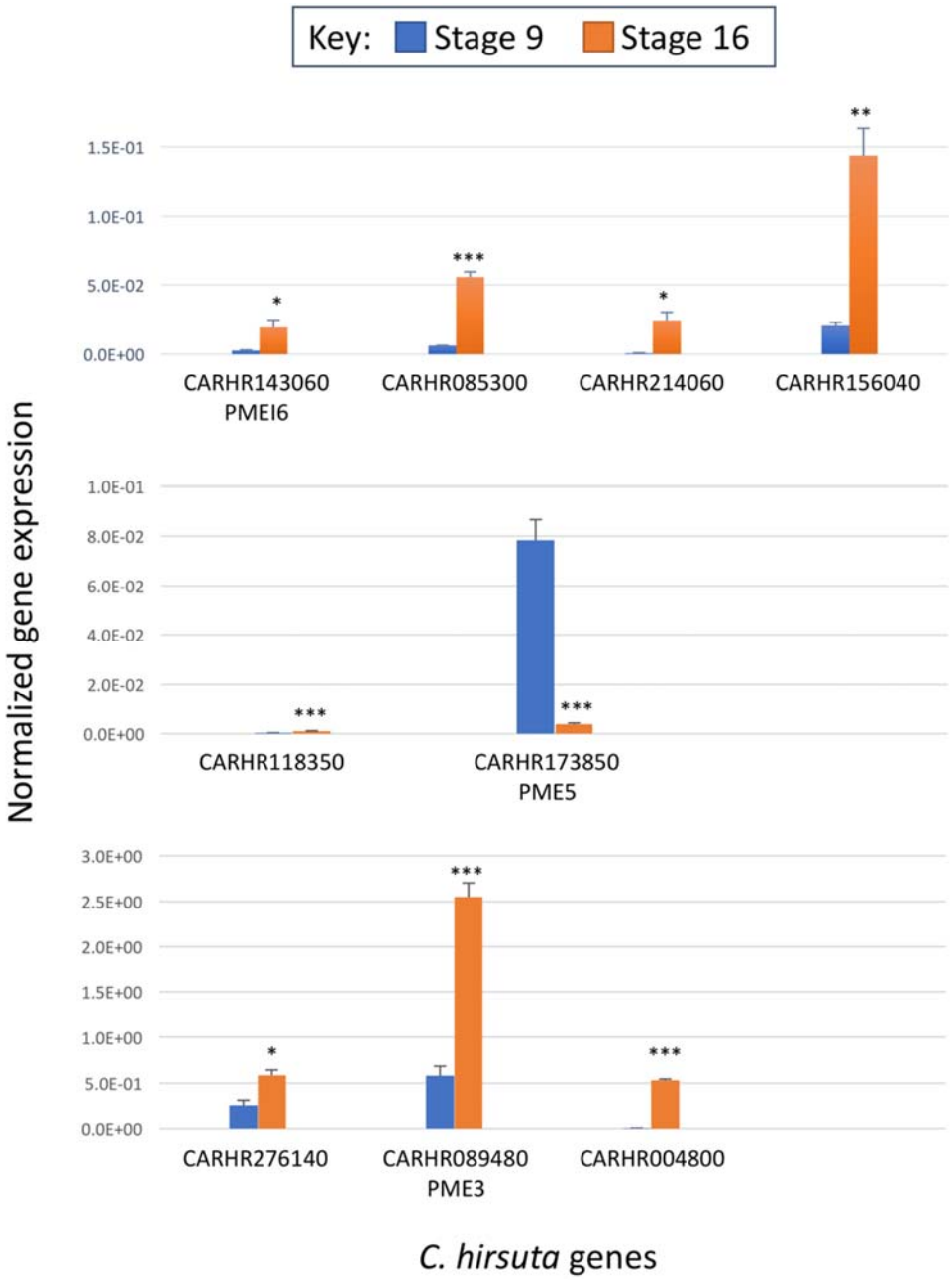

Figure 2: Validation of differential PME/I gene expression by qRT-PCR quantification in *C. hirsuta* fruit. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR143060- PMEI6, CARHR085300, CARHR214060, CARHR156040, CARHR118350, CARHR173850, CARHR276140, CARHR089480 and CARHR004800) in stage 9 and stage 16 fruit of *C. hirsuta*. Note that these *C. hirsuta* genes have orthologs in *A. thaliana.* Gene expression is compared pairwise using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

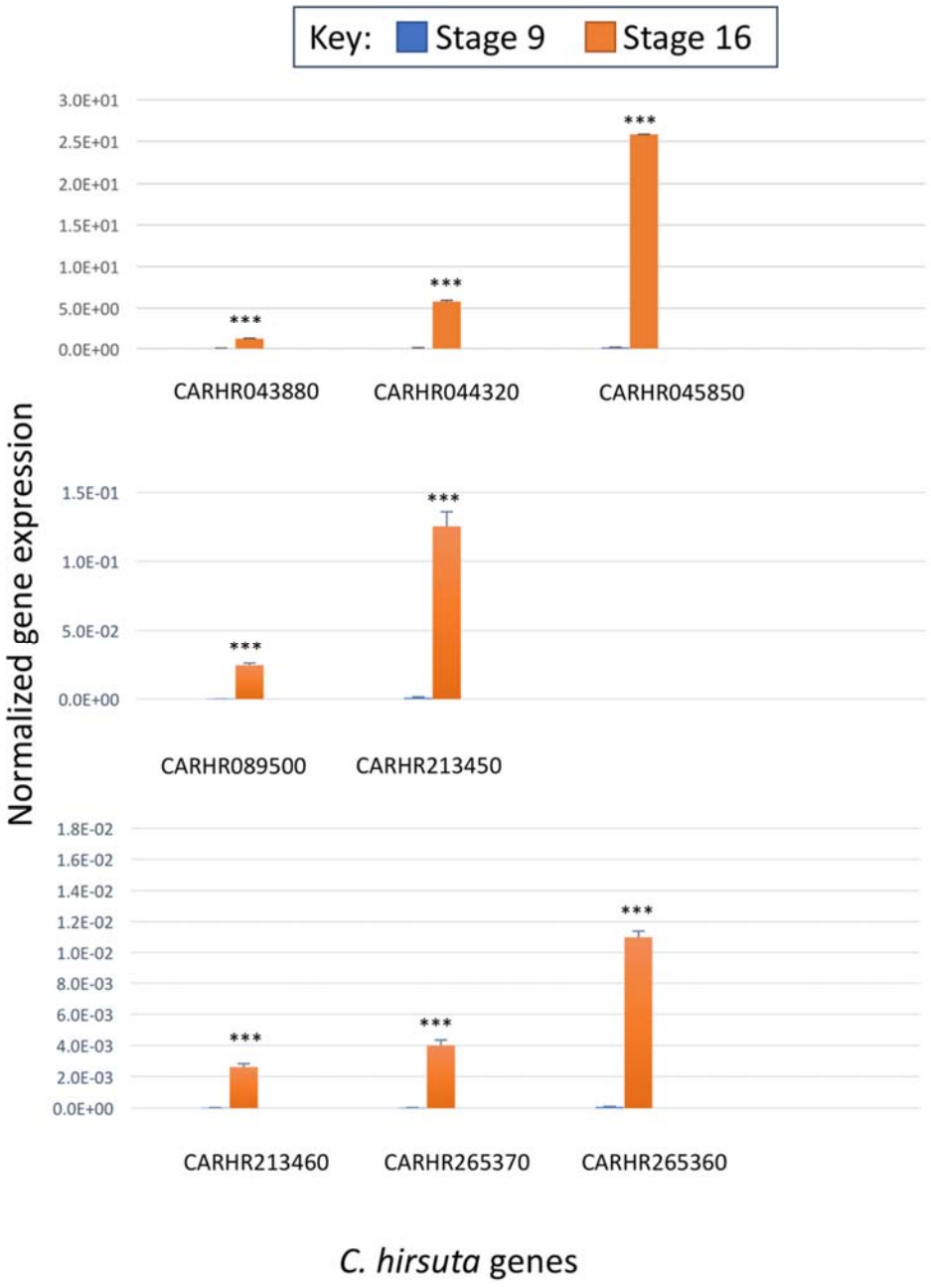

Figure 3: Validation of differential PME/I gene expression by qRT-PCR quantification in *C. hirsuta* fruit. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR043880, CARHR044320, CARHR045850, CARHR089500, CARHR213450, CARHR213460, CARHR265370 and CARHR265360) in stage 9 and stage 16 fruit of *C. hirsuta*. Note that these genes are unique to *C. hirsuta* and have no orthologs in *A. thaliana.* Gene expression is compared pairwise using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

The levels of normalized gene expression measured by qRT-PCR were on average two orders of magnitude lower than the sequence reads counted by RNAseq (Table 2). However, the relative expression levels of each gene were similar in both techniques. Therefore, the results from the two techniques agree with each other and I was able to validate a significant difference in expression for all PME/I genes identified by RNAseq.

Table 2. Comparison of qRT-PCR and RNAseq results

	qRT-PCR		RNAseq	
	stage 9	**stage 16**	**stage 9**	**stage 16**
CARHR143060 PMEI6	2,66E-03	1,94E-02	4,9	143,8
CARHR085300	6,04E-03	5,56E-02	229,6	1730,6
CARHR118350	2,47E-04	9,93E-04	13,5	266
CARHR214060	6,12E-04	2,42E-02	0,0	34,5
CARHR276140	2,61E-01	5,84E-01	115,8	806
CARHR173850 PME5	7,84E-02	3,72E-03	682,5	48,4
CARHR089480 PME3	5,84E-01	2,55E+00	700,3	6075,5
CARHR004800	2,41E-03	5,31E-01	0	36
CARHR156040	2,04E-02	1,44E-01	5,7	66,1
CARHR043880	0	1,20E+00	0	9058,8
CARHR044320	0	5,78E+00	0	22229,7
CARHR045850	1,50E-01	2,58E+01	1,3	19915,5
CARHR089500	3,30E-05	2,44E-02	0	197,3
CARHR213460	1,10E-05	2,62E-03	0	17,6
CARHR265370	2,51E-05	0,004015796	0	16,1
CARHR213450	8,65E-04	1,26E-01	0	42,7
CARHR265360	8,34E-05	1,10E-02	0	24.2

C. hirsuta fruit stages are described in Fig. 1. Normalized gene expression measured by qRT-PCR is shown as means of 3 biological replicates. Normalized gene expression measured by RNAseq is shown as read counts (see Gan *et al* 2016 for full methods).

3.2.2- Spatiotemporal dynamics of PME/I gene expression in *C. hirsuta*

In the previous section, I confirmed that 17 PME/I genes were differentially expressed between early and late stages of *C. hirsuta* fruit development. Next, I characterized the spatiotemporal expression of these 17 genes in *C. hirsuta* by qRT-PCR. To investigate more thoroughly how the expression of these genes varied throughout fruit development, I sampled fruit at stages 9, 15, 16 and 17a (Fig. 1). To investigate how the expression of these genes varied between different tissues that comprise the fruit, I dissected stage 17b fruit into seeds, valves, and remaining fruit tissues (Fig. 6). Finally, to investigate how the expression of these genes varied between different plant tissues, I sampled roots, seedlings, rosette leaves, floral shoots, and stage 16 fruit (Fig. 9).

3.2.2.1- Fruit stage-specific expression

I selected different stages of *C. hirsuta* fruit development based on the following morphological features as described in (McKim *et al.* 2017 and Roeder & Yanofsky 2006). Stage 9 flowers are unopened buds where the carpel is not yet fused (Fig. 1). In stage 15 flowers, the fruit are approximately 4 mm long, and extend beyond the other floral organs (Fig. 1). In stage 16 flowers, the fruit has elongated to reach approximately 12 mm long, and the other floral organs abscise (Fig. 1). Stage 17 is divided into stage 17a, where the fruit almost reaches its final length of approximately 20 mm, and stage 17b, where the fruit expands to reach its final length and width (Fig. 1).

The stage-specific expression of three PME/I genes (CARHR043880, CARHR044320 and CARHR045850) had been reported previously (Gan *et al*., 2016). These results showed that the expression level of each gene was highest at stage 16 (Gan *et al*., 2016). To investigate whether I could replicate these results and whether the additional 14 genes showed a similar trend in expression, I performed qRT-PCR for all 17 PME/I genes. My results confirmed the previously published results that the genes CARHR043880, CARHR044320 and CARHR045850 were most highly expressed in stage 16 fruit (Table 3, Fig. 5). In fact, the other five *C. hirsuta* genes that lack a clear ortholog in *A. thaliana* (CARHR089500, CARHR213450, CARHR213460, CARHR265370, and CARHR265360), also showed the same trend with highest expression in stage 16 fruit (Table 3, Fig. 5).

On the other hand, the nine *C. hirsuta* genes with othologous genes in *A. thaliana* (CARHR143060, CARHR085300, CARHR173850, CARHR156040, CARHR276140,

CARHR089480, CARHR004800, CARHR118350, and CARHR214060), showed more variable expression (Table 3, Fig. 4). For example, the CARHR143060 gene, which is the otholog of *PMEI6* in *A. thaliana*, was most highly expressed in stage 17 fruit (Table 1, Fig. 4). Another example is the CARHR173850 gene, which is the otholog of *PME5* in *A. thaliana*. This gene was most highly expressed in stage 9 fruit (Table 3, Fig. 4).

In summary, my results agreed with the expression data reported previously for three PME/I genes (Gan *et al*., 2016). My results showed a lower magnitude of normalized gene expression than was previously reported (Gan *et al*., 2016); however, I found a similar trend in stage-specific expression. My results extend the previously published data by finding that all eight *C. hirsuta* PME/I genes that lack a clear ortholog in *A. thaliana* showed their highest expression in stage 16 fruit (Table 3, Fig. 5). In contrast to this, the nine *C. hirsuta* PME/I genes with othologous genes in *A. thaliana*, showed more variability, although three of these genes (CARHR085300, CARHR089480 and CARHR004800) clearly had higher expression in stage 16 fruit (Table 3, Fig. 4). Taken together, the majority of PME/I genes were most highly expressed during stage 16 of fruit development, and this was a particularly obvious trend for all *C. hirsuta* PME/I genes without clear orthologues in *A. thaliana*.

Table 3. Normalized expression levels of PME/I genes quantified by qRT-PCR at different stages of *C. hirsuta* fruit development.

***C. hirsuta* fruit**

Gene Name	Stage 9	Stage 15		Stage 16		Stage 17a	
	mean	mean	P-Value	mean	P-Value	mean	P-Value
CARHR143060 PMEI6	2,66E-03 ±9,40E-05	2,78E-03 ±3,70E-04	7,79E-01	1,94E-02 ±5,30E-03	3,44E-02	2,10E-01 ±4,54E-02	1,03E-02
CARHR085300	6,04E-03 ±2,34E-04	1,42E-02 ±3,94E-03	1,09E-01	5,56E-02 ±3,90E-03	2,22E-04	2,52E-02 ±7,95E-03	7,39E-02
CARHR118350	2,47E-04 ±3,23E-05	1,56E-03 ±4,05E-04	3,15E-02	9,93E-04 ±2,59E-05	5,55E-05	1,53E-03 ±2,85E-04	1,10E-02
CARHR214060	6,12E-04 ±1,53E-04	6,42E-03 ±2,58E-03	8,78E-02	2,42E-02 ±6,03E-03	1,75E-02	2,41E-02 ±1,22E-03	4,41E-05
CARHR276140	2,61E-01 ±5,29E-02	3,21E-01 ±1,44E-01	7,14E-01	5,84E-01 ±6,13E-02	1,62E-02	5,23E-01 ±8,87E-02	6,42E-02
CARHR173850 PME5	7,84E-02 ±8,33E-03	4,87E-02 ±2,31E-03	2,64E-02	3,72E-03 ±4,89E-04	8,61E-04	5,18E-02 ±1,05E-02	1,18E-01
CARHR089480 PME3	5,84E-01 ±1,00E-01	1,41E+00 ±2,82E-01	5,15E-02	2,55E+00 ±1,54E-01	4,36E-04	3,31E-01 ±8,14E-02	1,22E-01
CARHR004800	2,41E-03 ±1,51E-03	4,81E-02 ±4,73E-02	3,89E-01	5,31E-01 ±1,43E-02	3,27E-06	6,38E-02 ±3,28E-02	1,35E-01
CARHR156040	2,04E-02 ±2,38E-03	1,12E-01 ±1,12E-02	1,33E-03	1,44E-01 ±1,98E-02	3,46E-03	7,68E-03 ±4,40E-04	6,39E-03
CARHR043880	0,00E+00	1,38E-03 ±9,97E-04	6,04E-04	1,20E+00 ±2,48E-01	6,04E-04	2,04E-01 ±9,91E-02	6,04E-04
CARHR044320	0,00E+00	7,94E-02 ±1,58E-02	6,04E-04	5,78E+00 ±2,48E-01	6,04E-04	1,08E+00 ±4,76E-01	6,04E-04
CARHR045850	1,50E-01 ±1,36E-02	2,20E+00 ±4,56E-01	1,08E-02	2,58E+01 ±1,77E+00	1,30E-04	9,72E+00 ±4,97E+00	1,26E-01
CARHR089500	3,30E-05 ±8,34E-06	4,44E-03 ±1,36E-03	3,21E-02	2,44E-02 ±1,54E-03	9,34E-05	1,10E-02 ±3,09E-03	2,39E-02
CARHR213460	1,10E-05 ±5,30E-06	3,56E-04 ±1,35E-04	6,35E-02	2,62E-03 ±2,05E-04	2,19E-04	1,28E-03 ±3,26E-04	1,78E-02
CARHR265370	2,51E-05 ±2,65E-06	1,66E-03 ±2,69E-04	3,72E-03	4,02E-03 ±3,27E-04	2,58E-04	8,02E-04 ±4,09E-04	1,30E-01
CARHR213450	8,65E-04 ±4,87E-04	2,64E-02 ±5,32E-03	8,82E-03	1,26E-01 ±1,04E-02	2,80E-04	6,50E-02 ±1,23E-02	6,39E-03
CARHR265360	8,34E-05 ±6,80E-06	6,00E-03 ±8,21E-04	1,96E-03	1,10E-02 ±3,85E-04	9,20E-06	1,64E-03 ±7,29E-04	9,93E-02

C. hirsuta fruit stages are described in Fig. 1. Gene expression is compared pairwise between stage 9 and the three later stages of fruit development using Student's t-test. Values are shown as means of 3 biological replicates ± standard error of the mean.

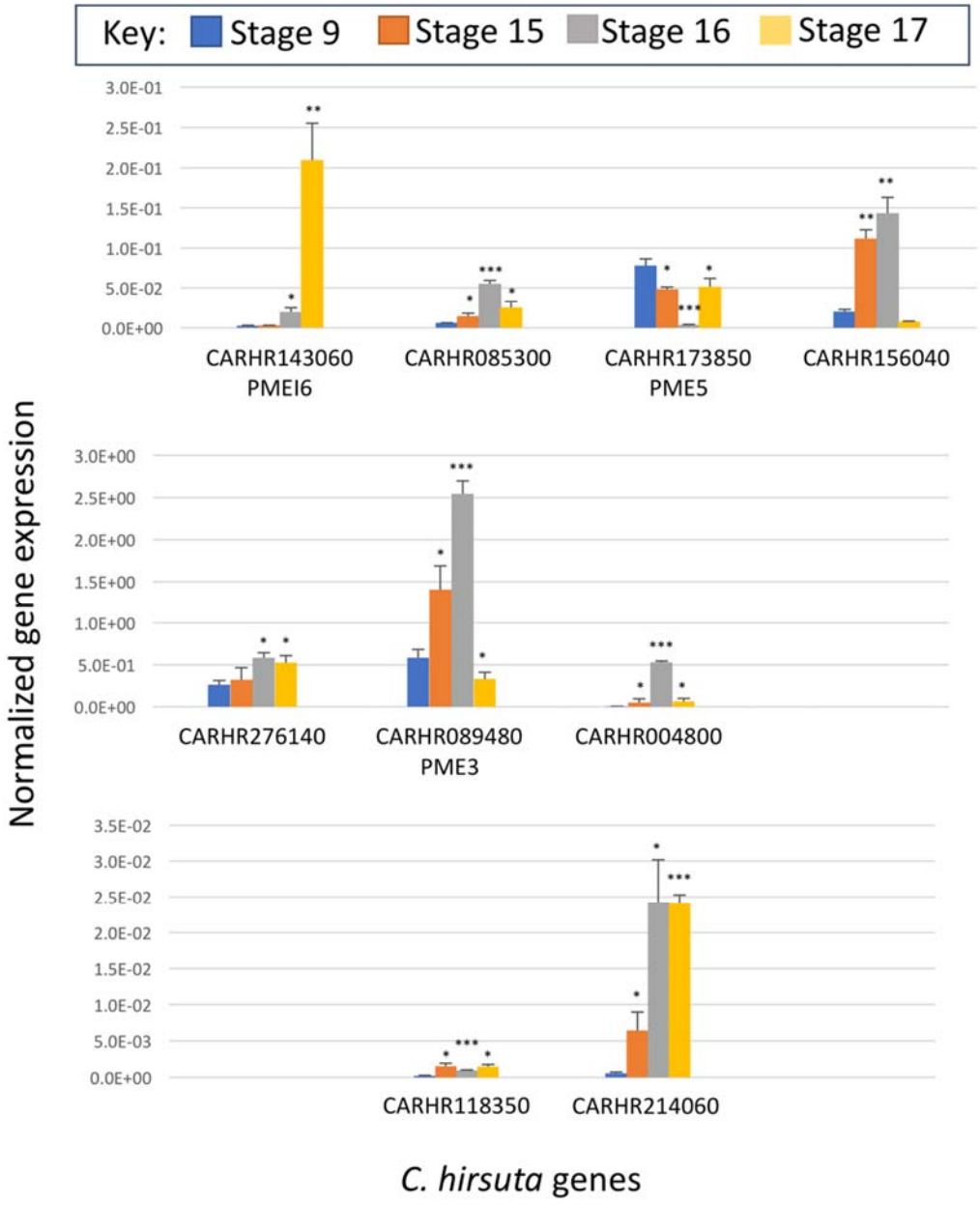

Figure 4: Normalized expression levels of PME/I genes quantified by qRT-PCR at different stages of *C. hirsuta* fruit development. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR143060- PMEI6, CARHR085300, CARHR214060, CARHR156040, CARHR118350, CARHR173850, CARHR276140, CARHR089480 and CARHR004800) in stage 9, stage 15, stage 16 and stage 17 fruit of *C. hirsuta*. Note that these *C. hirsuta* genes have orthologs in *A. thaliana.* Gene expression is compared pairwise between stage 9 and the three later stages of fruit development using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

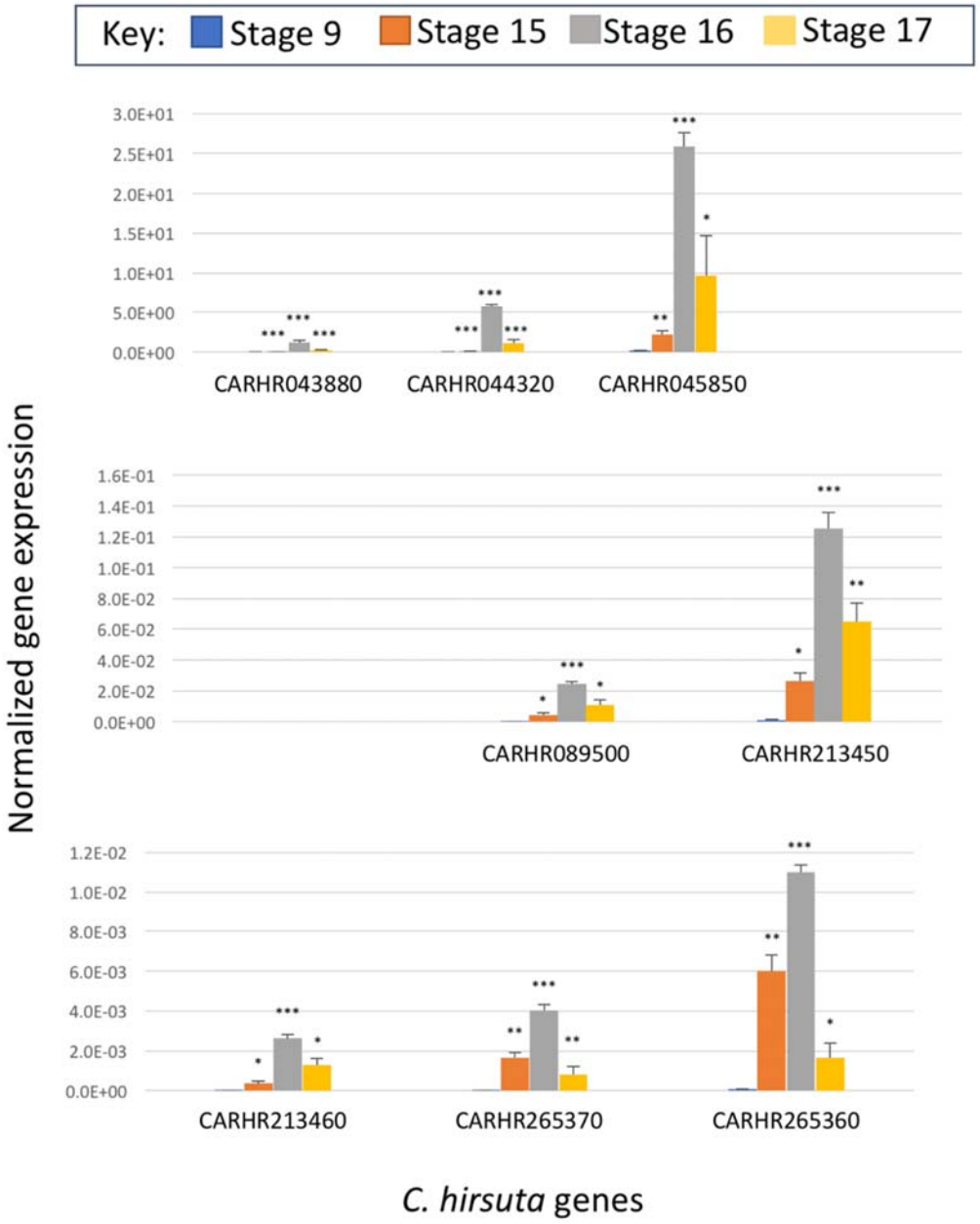

Figure 5: Normalized expression levels of PME/I genes quantified by qRT-PCR at different stages of *C. hirsuta* fruit development. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR043880, CARHR044320, CARHR045850, CARHR089500, CARHR213450, CARHR213460, CARHR265370 and CARHR265360) in stage 9, stage 15, astage 16 and stage 17 fruit of *C. hirsuta*. Note that these genes are unique for *C. hirsuta and* have no orthologs in *A. thaliana.* Gene expression is compared pairwise between stage 9 and the three later stages of fruit development using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

3.2.2.2- Fruit tissue-specific expression

Fruit comprise a mixture of fruit tissues and seeds (Esau, K. Anatomy of seed plants. John Wiley & sons). Therefore, the fruit samples that were used to quantify gene expression by RNAseq (Gan *et al*., 2016) and by qRT-PCR contained all of these tissue types. To investigate how PME/I gene expression varied between these different fruit tissues, I dissected stage 17b fruit into seeds, valves, and remaining fruit tissues (Fig. 6).

The tissue-specific expression of three PME/I genes (CARHR043880, CARHR044320 and CARHR045850) within *C. hirsuta* fruit had been reported previously (Gan *et al*., 2016). These results showed that the expression level of each gene was highest in seeds (Gan *et al*., 2016). To investigate whether I could replicate these results and whether the additional 14 genes showed a similar trend in expression, I performed qRT-PCR for all 17 PME/I genes. My results confirmed the previously published results that the genes CARHR043880, CARHR044320 and CARHR045850 were most highly expressed in seeds (Table 4, Fig. 8). In fact, another four *C. hirsuta* genes that lack a clear ortholog in *A. thaliana* (CARHR089500, CARHR213450, CARHR213460 and CARHR265370), also showed the same trend with highest expression in seeds (Table 4, Fig. 8). The CARHR265360 gene, which also lacks a clear ortholog in *A. thaliana*, showed similarly high expression in both seeds and valve tissue, with significantly lower expression in the remaining fruit tissues (Table 4, Fig. 8).

The nine *C. hirsuta* PME/I genes with othologous genes in *A. thaliana* (CARHR143060, CARHR085300, CARHR173850, CARHR156040, CARHR276140, CARHR089480, CARHR004800, CARHR118350, and CARHR214060), showed more variable expression (Table 4, Fig. 7). For example, three genes (CARHR085300, CARHR276140 and CARHR118350) were most highly expressed in fruit valve tissue (Table 4, Fig. 7). While expression of the CARHR214060 gene was highest in the rest of the fruit tissues (Table 2, Fig. 5). Another two genes (CARHR089480 and CARHR004800) were expressed significantly higher in both types of fruit tissues compared to seeds (Table 4, Fig. 7).

In summary, my results agreed with the expression data reported previously for three PME/I genes (Gan *et al*., 2016). My results showed a lower magnitude of normalized gene expression than was previously reported (Gan *et al*., 2016); however, I found a similar trend in fruit tissue-specific expression. My results extend the previously published data by finding that seven of the eight *C. hirsuta* PME/I genes that lack a clear ortholog in *A. thaliana* showed their

highest expression in seeds (Table 4, Fig. 8). In contrast to this, the nine *C. hirsuta* PME/I genes with othologous genes in *A. thaliana*, showed more variability, although three of these genes (CARHR143060, CARHR173850 and CARHR156040) had significantly higher expression in seeds (Table 4, Fig. 7). Taken together, the majority of PME/I genes were most highly expressed in seeds of mature fruit, and this was a particularly obvious trend for all *C. hirsuta* PME/I genes without clear orthologues in *A. thaliana*.

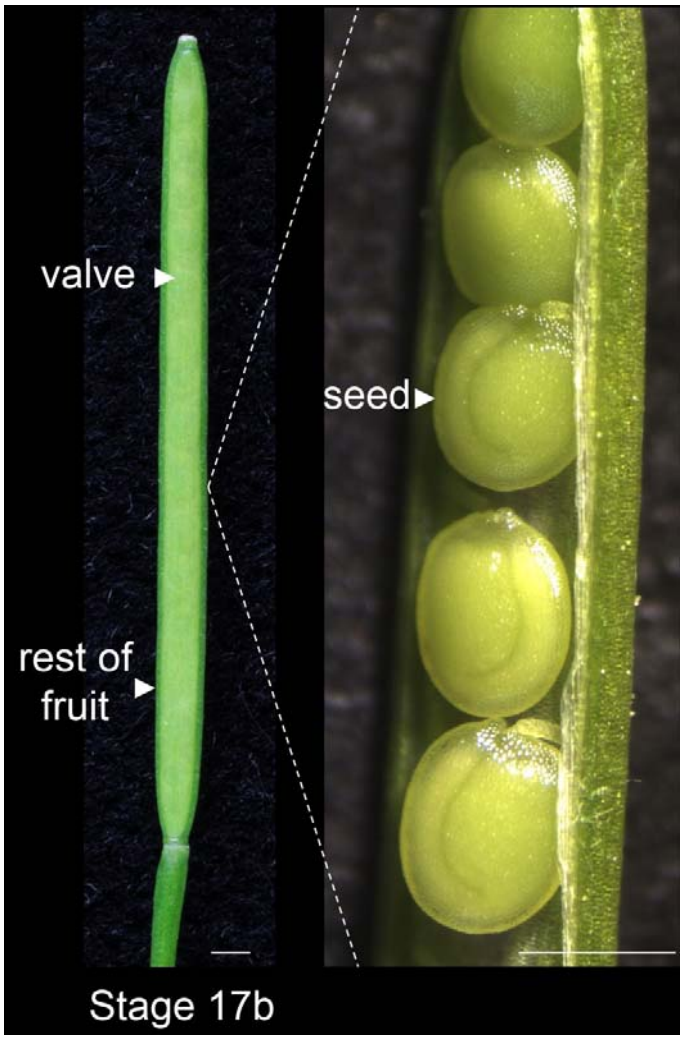

Figure 6: Different tissues of stage 17b *C. hirsuta* fruit used to localize PME/I gene expression. Note that 'rest of fruit' refers to all remaining tissues after valves and seeds have been removed. Scale bar: 1 mm.

Table 4. Normalized expression levels of PME/I genes quantified by qRT-PCR in different fruit tissues of *C. hirsuta*.

	***C. hirsuta* fruit tissues**				
Gene Name	**Seed**	**Valve**		**Rest of fruit**	
	mean	**mean**	**P-Value**	**mean**	**P-Value**
CARHR143060 PMEI6	2,65E-01 ±6,08E-02	2,20E-02 ±2,45E-03	1,63E-02	2,11E-02 ±3,16E-03	1,61E-02
CARHR085300	7,05E-05 ±1,81E-05	4,62E-02 ±1,64E-02	4,83E-02	8,43E-03 ±8,62E-04	6,34E-04
CARHR118350	1,28E-03 ±8,50E-05	2,28E-03 ±3,59E-04	5,31E-02	5,47E-04 ±1,62E-04	1,58E-02
CARHR214060	3,09E-02 ±3,80E-03	9,48E-03 ±1,33E-03	5,96E-03	4,26E-02 ±3,54E-03	8,78E-02
CARHR276140	2,67E-01 ±3,36E-02	5,77E-01 ±5,89E-02	1,03E-02	2,30E-01 ±8,63E-02	7,07E-01
CARHR173850 PME5	1,44E-01 ±2,82E-02	5,96E-04 ±3,81E-04	6,99E-03	3,22E-03 ±1,53E-03	7,49E-03
CARHR089480 PME3	2,62E-02 ±2,80E-03	4,00E-01 ± 9,12E-03	2,53E-06	3,64E-01 ±4,16E-02	1,26E-03
CARHR004800	6,47E-05 ±3,35E-05	3,60E-03 ±2,24E-03	1,90E-01	7,04E-03 ±2,26E-03	3,68E-02
CARHR156040	9,46E-03 ±2,97E-04	1,00E-03 ±6,88E-04	3,51E-04	4,09E-03 ±2,13E-03	6,73E-02
CARHR043880	4,33E-01 ±9,34E-02	7,32E-03 ±6,80E-03	1,05E-02	1,39E-02 ±5,74E-03	1,10E-02
CARHR044320	1,02E+00 ±1,74E-01	6,18E-02 ±5,75E-02	6,26E-03	4,87E-02 ±1,48E-02	5,01E-03
CARHR045850	7,86E+00 ±4,52E-01	5,52E-01 ±4,62E-01	3,49E-04	6,76E-01 ±1,42E-01	1,10E-04
CARHR089500	1,28E-02 ±3,74E-03	1,96E-04 ±1,51E-04	2,80E-02	1,45E-04 ±1,55E-05	2,76E-02
CARHR213460	3,96E-04 ±4,97E-05	1,22E-05 ±8,16E-06	3,74E-01	1,44E-05 ±4,12E-06	1,57E-03
CARHR265370	3,34E-04 ±6,47E-05	1,66E-05 ±6,29E-06	8,16E-03	7,24E-05 ±2,90E-05	2,10E-02
CARHR213450	4,12E-02 ±9,50E-03	9,54E-04 ±5,82E-04	1,34E-02	7,96E-04 ±1,61E-04	1,32E-02
CARHR265360	8,80E-04 ±4,99E-04	7,83E-04 ±6,81E-04	9,14E-01	8,81E-05 ±3,70E-05	1,89E-01

C. hirsuta fruit tissues are described in Fig. 6. Gene expression is compared pairwise between seeds and the other two fruit tissues using Student's t-test. Values shown as means of 3 biological replicates ± standard error of the mean.

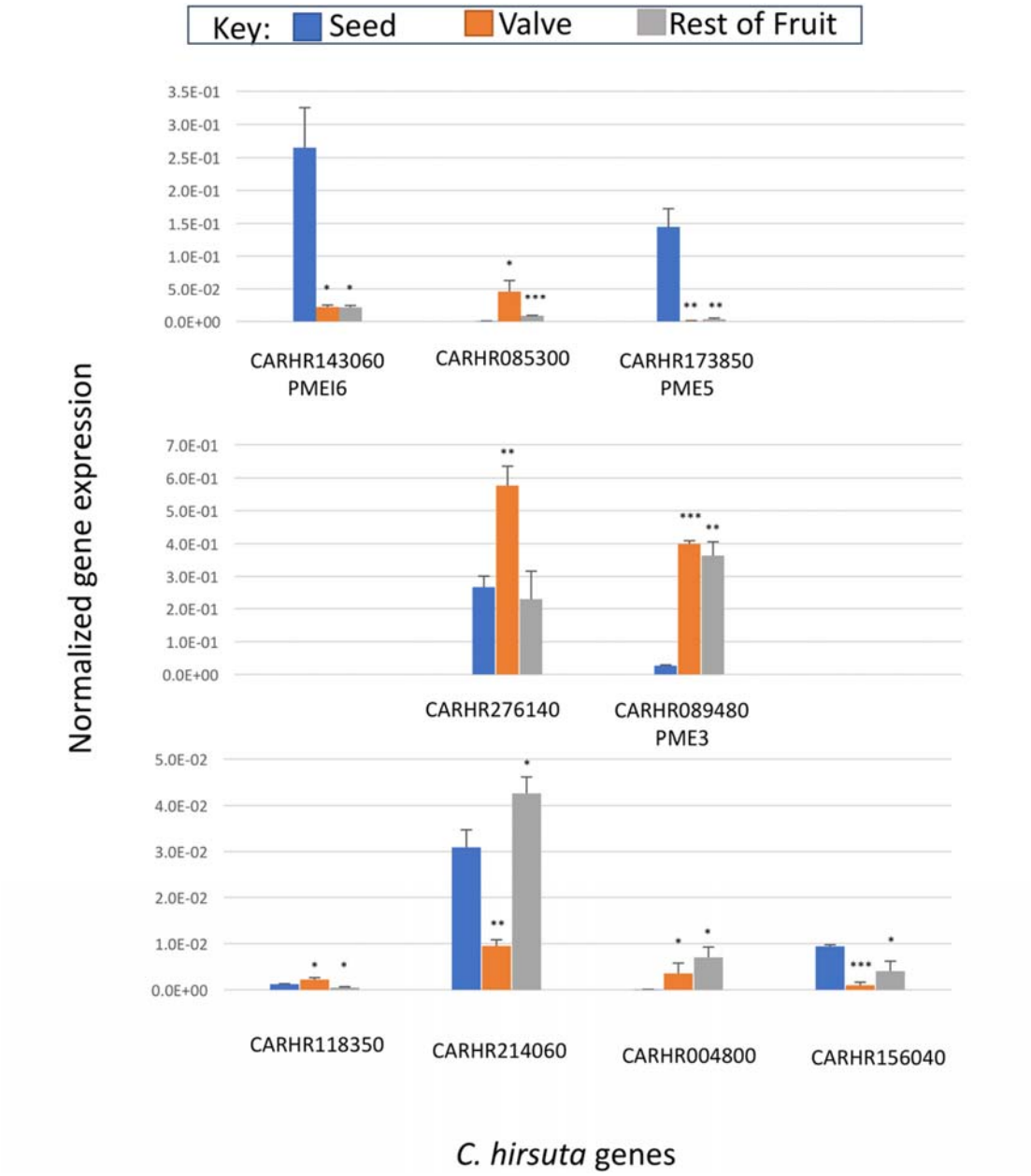

Figure 7: Normalized expression levels of PME/I genes quantified by qRT-PCR in different fruit tissues of *C. hirsuta*. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR143060- PMEI6, CARHR085300, CARHR214060, CARHR156040, CARHR118350, CARHR173850, CARHR276140, CARHR089480 and CARHR004800) in seed, valve and rest of fruit of *C. hirsuta*. Note that these *C. hirsuta* genes have orthologs in *A. thaliana.* Gene expression is compared pairwise between seeds and the other two fruit tissues using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

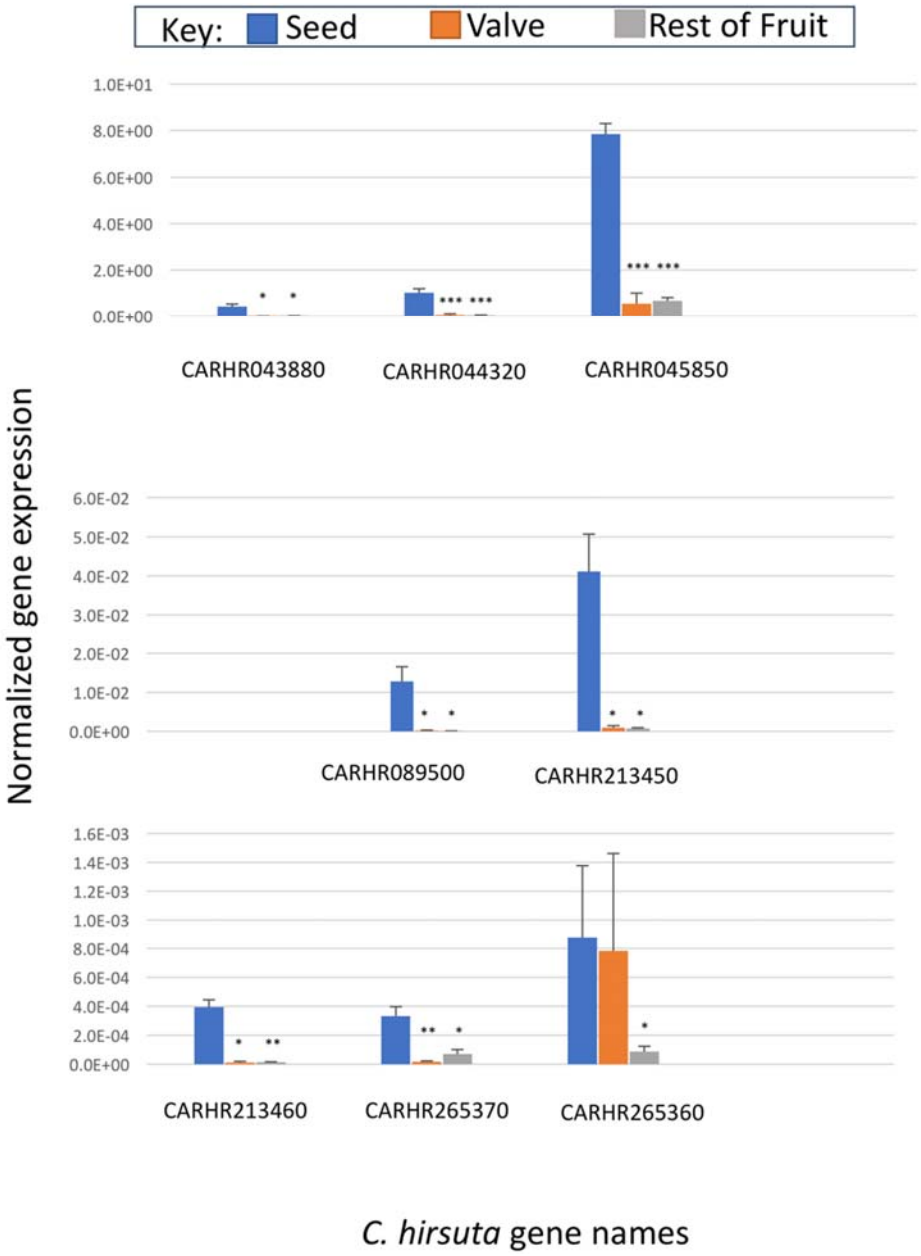

Figure 8: Normalized expression levels of PME/I genes quantified by qRT-PCR in different fruit tissues of *C. hirsuta*. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR043880, CARHR044320, CARHR045850, CARHR089500, CARHR213450, CARHR213460, CARHR265370 and CARHR265360) in seed, valve and rest of fruit of *C. hirsuta*. Note that these genes are unique for *C. hirsuta and* have no orthologs in *A. thaliana.* Gene expression is compared pairwise between seeds and the other two fruit tissues using Student's t-test. Significance levels: *** p<0.001, ** p<0.01, * p<0.05. Values shown as means of 3 biological replicates ± standard error of the mean.

3.2.2.3- Plant tissue-specific expression

To investigate how the expression of these 17 PME/I genes varied throughout plant development in *C. hirsuta*, I selected four different plant tissues to compare with fruit. For this experiment, I quantified PME/I gene expression by qPCR in seedlings, roots, rosette leaf number five, and floral shoots, in addition to stage 16 fruit (Fig. 9). Seedlings and roots were sampled from plants grown on MS media, while other tissues were sampled from plants grown on soil in long day greenhouse conditions.

I found that expression of the nine *C. hirsuta* PME/I genes with othologous genes in *A. thaliana* (CARHR143060, CARHR085300, CARHR173850, CARHR156040, CARHR276140, CARHR089480, CARHR004800, CARHR118350, and CARHR214060), varied between different tissues (Table 5, Fig. 10). For example, four genes (CARHR085300, CARHR156040, CARHR276140, and CARHR004800) showed significantly less expression in all other plant tissues compared to stage 16 fruit (Table 5, Fig. 10). While expression of three other genes (CARHR143060, CARHR118350, and CARHR173850) was significantly higher in other tissues, including leaves and floral shoots, compared to stage 16 fruit (Table 5, Fig. 10). Another two genes (CARHR214060 and CARHR089480) showed similarly high expression levels in stage 16 fruit and other tissues such as roots and leaves (Table 5, Fig. 10).

On the other hand, I found a clear trend in all eight *C. hirsuta* PME/I genes that lack a clear ortholog in *A. thaliana* (CARHR043880, CARHR044320, CARHR045850, CARHR089500, CARHR213450, CARHR213460, CARHR265370 and CARHR265360). These genes showed significantly higher expression in stage 16 fruit than any other plant tissues sampled (Table 5, Fig. 11). In fact, fruit were the only part of the plant where the expression of these genes could be detected by qRT-PCR (Table 5, Fig. 11).

In summary, my results showed a very restricted expression pattern for the eight *C. hirsuta* PME/I genes that lack a clear ortholog in *A. thaliana* (Table 5, Fig. 11). Expression of these genes was only detected in fruit. In comparison, the other nine *C. hirsuta* PME/I genes with othologous genes in *A. thaliana* were expressed in many other plant tissues, and some of these genes showed higher expression in other tissues compared to fruit.

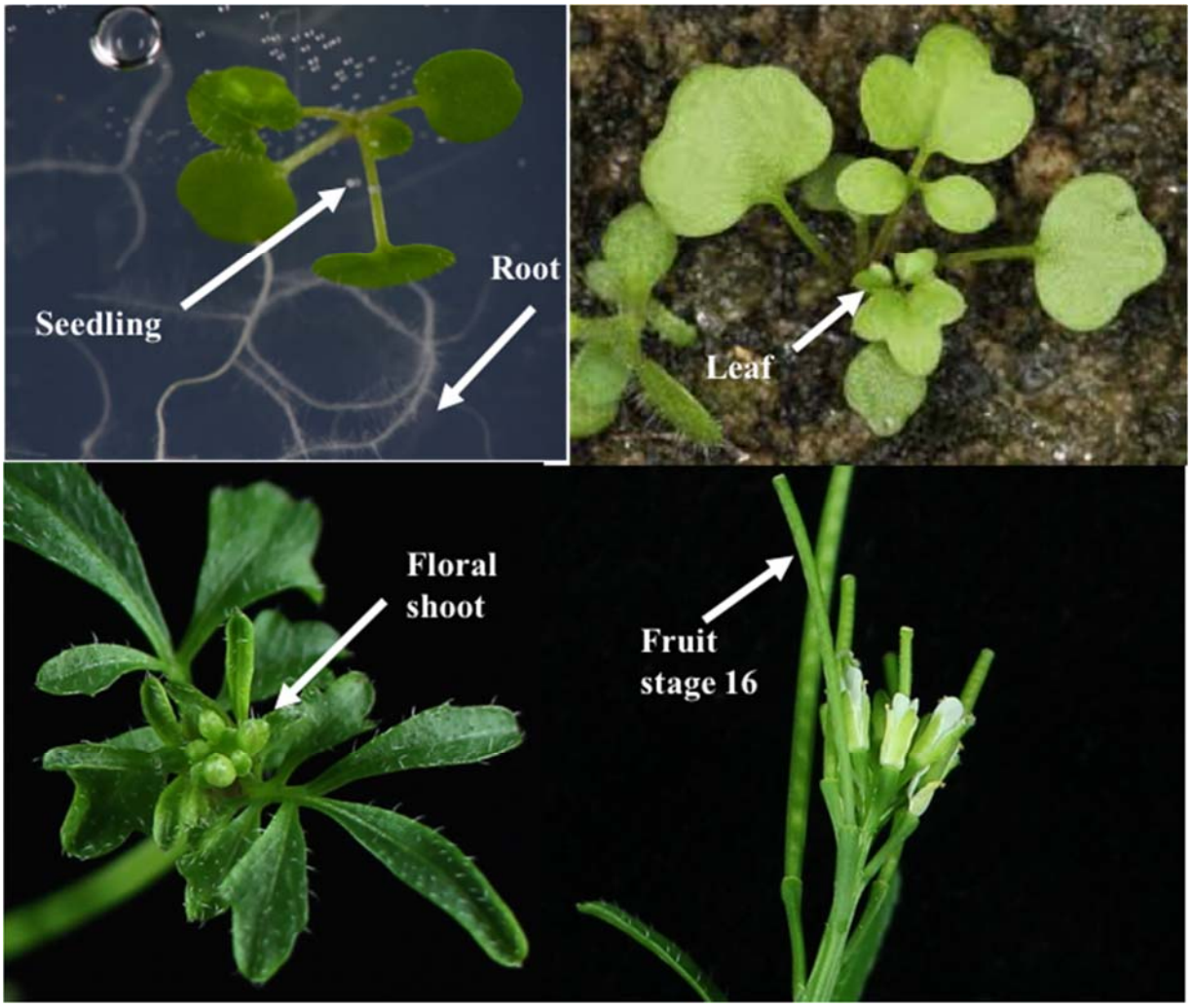

Figure 9: *C. hirsuta* plant tissues used to localize PME/I gene expression. Seeding and root samples were dissected from plants grown on MS media. Rosette leaf number five was pooled from soil-grown plants; whole floral shoots, including inflorescence meristem, cauline leaves and flowers, were dissected from soil-grown plants; and fruits at stage 16 were pooled from soil-grown plants.

Table 5. Normalized expression levels of PME/I genes quantified by qRT-PCR in different plant tissues of *C. hirsuta*.

	***C. hirsuta* Plant Tissue**								
	Fruit	**Seedling**		**Root**		**Leaf**		**Floral shoot**	
Gene Name	**mean**	**mean**	**P-Value**	**mean**	**P-Value**	**mean**	**P-Value**	**mean**	**P-Value**
CARHR143060 PME16	1,94E-02 ±5,30E-03	7,87E-03 ±5,32E-04	9,69E-02	2,76E-03 ±1,99E-04	3,51E-02	4,25E-02 ±3,67E-03	2,30E-02	3,38E-02 ±7,33E-03	1,85E-01
CARHR085300	5,56E-02 ±3,90E-03	7,77E-03 ±2,20E-03	4,35E-04	7,14E-03 ±1,98E-03	3,77E-04	4,83E-03 ±6,77E-04	2,13E-04	1,47E-02 ±3,13E-03	1,22E-03
CARHR118350	9,93E-04 ±2,59E-05	4,87E-04 ±7,91E-05	3,70E-03	2,49E-03 ±7,72E-04	1,25E-01	4,63E-03 ±4,49E-04	1,27E-03	1,98E-03 ±2,13E-04	1,02E-02
CARHR214060	2,42E-02 ±6,03E-03	2,67E-03 ±2,39E-04	2,36E-02	2,97E-02 ±8,68E-03	6,30E-01	1,85E-02 ±1,07E-03	4,07E-01	1,33E-02 ±5,31E-04	1,48E-01
CARHR276140	5,84E-01 ±6,13E-02	1,14E-01 ±1,21E-02	1,66E-03	1,91E-01 ±7,31E-02	1,46E-02	9,48E-02 ±2,68E-02	1,86E-03	4,78E-01 ±1,02E-01	4,22E-01
CARHR173850 PME5	3,72E-03 ±4,89E-04	4,80E-03 ±1,38E-03	5,03E-01	1,27E-02 ±2,08E-03	1,37E-02	6,48E-02 ±6,67E-03	7,94E-04	8,59E-02 ±6,99E-03	3,02E-04
CARHR089480 PME3	2,55E+00 ±1,54E-01	4,03E-01 ±5,22E-02	1,92E-04	4,54E-01 ±1,74E-01	8,39E-04	2,50E+00 ±1,42E-01	8,21E-01	1,57E+00 ±8,19E-02	4,93E-03
CARHR004800	5,31E-01 ±1,43E-02	9,50E-03 ±2,43E-03	3,57E-06	2,07E-02 ±1,61E-03	3,77E-06	2,16E-01 ±1,78E-02	1,60E-04	1,20E-01 ±1,50E-02	3,76E-05
CARHR156040	1,44E-01 ±1,98E-02	1,27E-02 ±2,01E-03	2,75E-03	1,16E-01 ±1,53E-02	3,25E-01	1,27E-01 ±5,87E-03	4,70E-01	1,00E-01 ±2,21E-02	2,15E-01
CARHR043880	1,20E+00 ±2,48E-01	6,04E-04	1,17E-05	1,58E-04 ±1,27E-04	1,17E-05	3,48E-05 ±2,28E-05	1,17E-05	4,71E-04 ±3,77E-04	1,17E-05
CARHR044320	5,78E+00 ±2,48E-01	6,04E-04	5,48E-03	1,69E-04 ±9,00E-05	5,48E-03	8,79E-04 ±2,49E-04	5,48E-03	2,55E-05 ±1,42E-05	5,48E-03
CARHR045850	2,58E+01 ±1,77E+00	1,30E-04	2,12E-03	7,55E-03 ±2,90E-03	2,11E-03	8,64E-03 ±3,64E-03	2,11E-03	3,67E-01 ±5,29E-02	2,18E-03
CARHR089500	2,44E-02 ±1,54E-03	1,08E-05 ±1,64E-06	9,30E-05	9,31E-06 ±2,74E-06	9,30E-05	4,56E-05 ±9,85E-06	9,36E-05	4,57E-05 ±2,86E-05	9,36E-05
CARHR213460	2,62E-03 ±2,05E-04	1,56E-06 ±1,55E-06	2,16E-04	8,65E-11 ±8,65E-11	2,15E-04	6,05E-08 ±1,20E-08	2,19E-03	2,00E-06 ±1,19E-06	2,16E-04
CARHR265370	4,02E-03 ±3,27E-04	5,91E-05 ±1,39E-05	2,68E-04	1,07E-05 ±1,46E-06	2,54E-04	1,83E-05 ±3,56E-06	2,56E-04	2,05E-05 ±8,40E-06	2,57E-04
CARHR213450	1,26E-01 ±1,04E-02	8,44E-05 ±3,12E-06	2,72E-04	1,15E-04 ±3,95E-05	2,72E-04	1,03E-04 ±3,51E-05	2,72E-04	4,97E-04 ±3,87E-04	2,76E-04
CARHR265360	1,31E-02 ±6,89E-04	2,73E-06 ±2,31E-06	4,57E-05	1,30E-05 ±9,43E-06	4,58E-05	1,03E-06 ±3,79E-07	4,57E-05	1,05E-04 ±4,93E-05	4,76E-05

C. hirsuta plant tissues are described in Fig. 9. Gene expression is compared pairwise between fruits and the other four plant tissues using Student's t-test. Values shown as means of 3 biological replicates ± standard error of the mean.

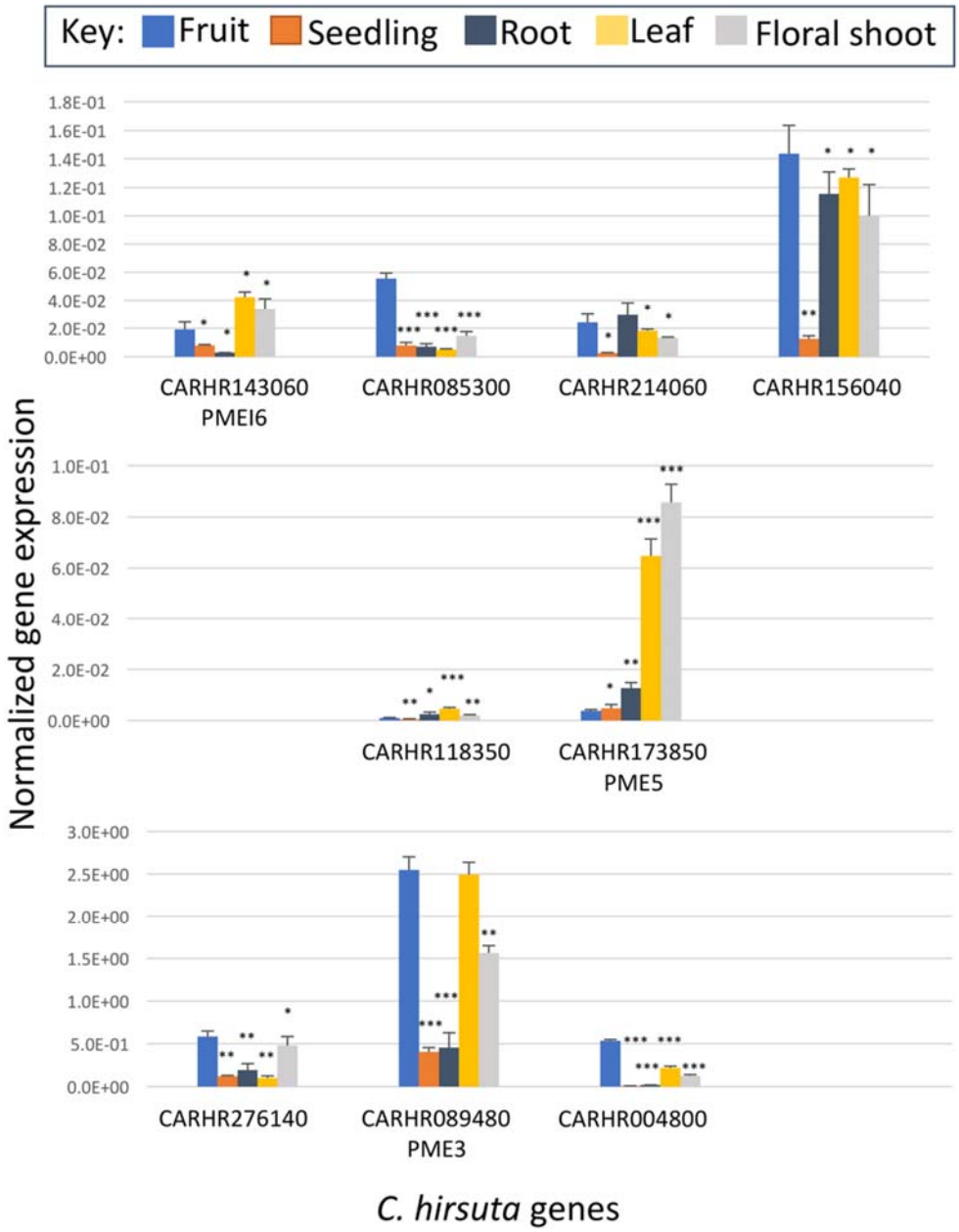

Figure 10: Normalized expression levels of PME/I genes quantified by qRT-PCR in different plant tissues of *C. hirsuta*. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR143060- PMEI6, CARHR085300, CARHR214060, CARHR156040, CARHR118350, CARHR173850, CARHR276140, CARHR089480 and CARHR004800) in fruit, seedling, root, leaf and floral shoot of *C. hirsuta*. Note that these *C. hirsuta* genes have orthologs in *A. thaliana.* Gene expression is compared pairwise between fruits and the other four plant tissues using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

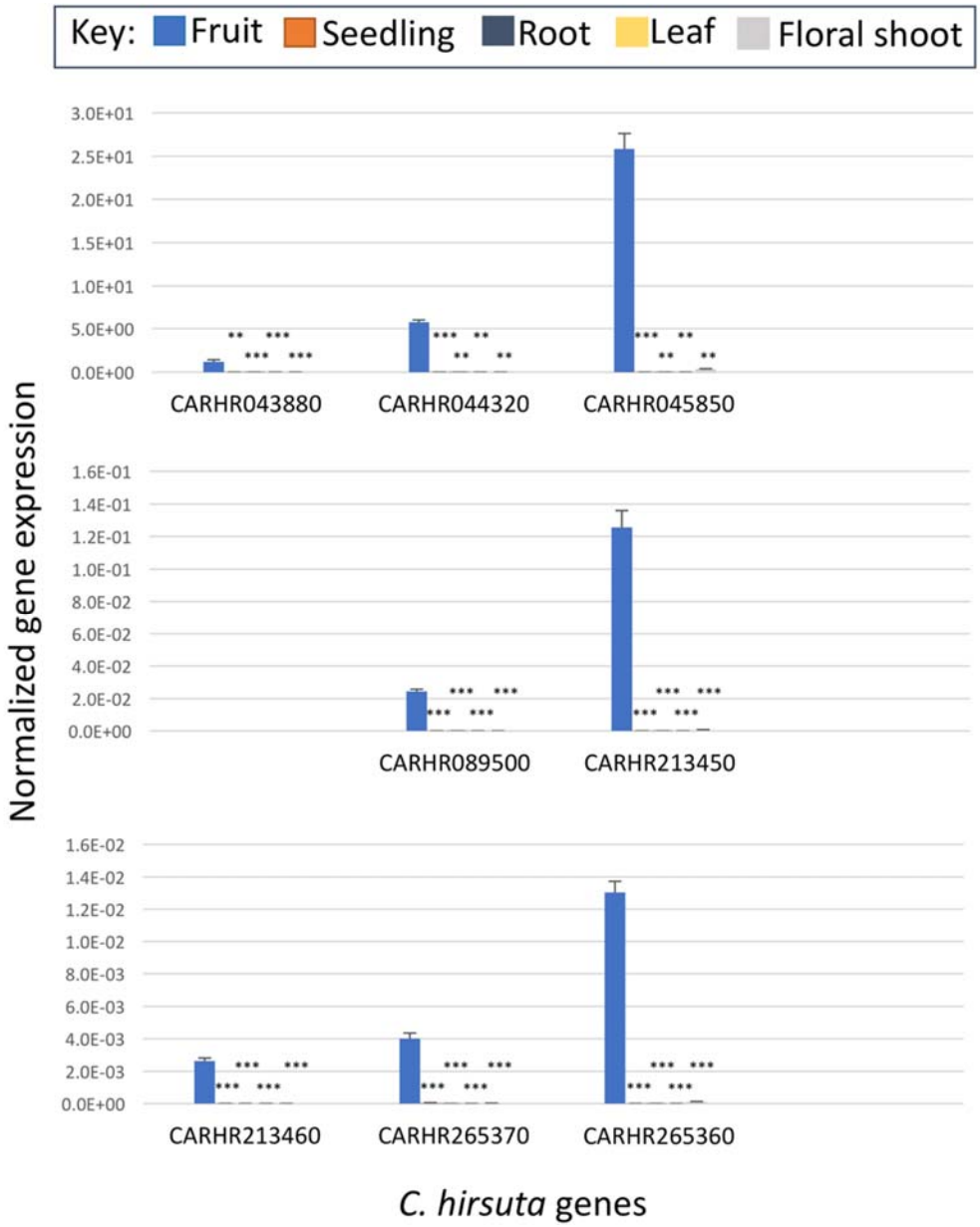

Figure 11: Normalized expression levels of PME/I genes quantified by qRT-PCR in different plant tissues of *C. hirsuta*. Normalized expression levels of *C. hirsuta* PME/I genes (CARHR043880, CARHR044320, CARHR045850, CARHR089500, CARHR213450, CARHR213460, CARHR265370 and CARHR265360) in fruit, seedling, Root, leaf and floral shoot meristem of *C. hirsuta*. Note that these genes are unique for *C. hirsuta and* have no orthologs in *A. thaliana.* Gene expression is compared pairwise between fruits and the other four plant tissues using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

3.3- Summary of PME/I gene expression

In summary, I validated the differential expression of all 17 PME/I genes that were previously identified by RNAseq as being differentially expressed during *C. hirsuta* fruit development (Gan *et al*.,. 2016) (Table 6). I also confirmed the previously reported qRT-PCR data for three of these genes (CARHR043880, CARHR044320 and CARHR045850), which found the highest levels of expression during stage 16 of fruit development and in the seeds of stage 17b fruit. I extended these findings by showing that only the eight *C. hirsuta* PME/I genes that lack a clear ortholog in *A. thaliana* have such a restricted expression pattern. Expression of these genes is restricted to fruit tissues and highest during stage 16 of fruit development (Table 6). Moreover, expression of these genes is higher in the seeds than other fruit tissues (Table 6). In comparison, the nine *C. hirsuta* PME/I genes with othologous genes in *A. thaliana* showed more variable expression between different fruit tissues, between different stages of fruit development, and between different plant tissues (Table 6). Therefore, my results indicate a striking association between genes that are unique to *C. hirsuta*, without clear orthologues in *A. thaliana*, having a very restricted pattern of gene expression. Based on these results, it will be interesting to investigate whether these genes have a function in *C. hirsuta* seed development.

Table 6. Summary of validation of RNAseq results and localization of PME/I gene expression in *C. hirsuta* fruit.

	Validation	Localization		
chi_gene	**RNA seq data**	**Fruit Stages**	**Fruit Tissues**	**Plant tissues**
CARHR143060	√	stage 17	seed	Leaf
CARHR085300	√	stage 16	valve	fruit
CARHR118350	√	stage 15	valve	leaf
CARHR214060	√	stage 16	rest of fruit	root
CARHR276140	√	stage 16	valve	fruit
CARHR173850	√	stage 9	seed	floral shoot
CARHR089480	√	stage 16	valve	leaf
CARHR004800	√	stage 16	rest of fruit	leaf
CARHR156040	√	stage 16	seed	Fruit
CARHR043880	√	stage 16	seed	Fruit
CARHR044320	√	stage 16	seed	Fruit
CARHR045850	√	stage 16	seed	Fruit
CARHR089500	√	stage 16	seed	Fruit
CARHR213460	√	stage 16	seed	Fruit
CARHR265370	√	stage 16	seed	Fruit
CARHR213450	√	stage 16	seed	Fruit
CARHR265360	√	stage 16	seed	Fruit

Chapter 4

Results

Analysis of PME/I gene function in *C. hirsuta*

4. Analysis of PME/I gene function in *C. hirsuta*

4.1- Introduction

A set of 17 PME/I genes were previously identified as differentially expressed during explosive seed dispersal in *C. hirsuta* (Gan *et al*. 2016). Of these, 7-8 *C. hirsuta* genes do not have clear orthologues in *A. thaliana* based on reciprocal best BLAST, analysis of expanded gene families, and phylogenetic analysis (Gan *et al*. 2016). These genes are annotated in the *C. hirsuta* genome based on conserved protein domains including pectin methylesterase inhibitor domain (IPR006501), pectin methylesterase catalytic (IPR000070) and pectin lyase domains (IPR011050, IPR012334), and pectin methylesterase active site domain (IPR018040). Based on these annotations, the products encoded by these 7-8 genes are likely to modify pectin methylesterase activity in the cell wall. However, the precise function of these genes is unknown. In the previous chapter, I found that the expression of these 8 PME/I genes is specifically localized to seeds during stage 16 of fruit development. The specificity of this expression suggests that these genes may function in seed development or dispersal in *C. hirsuta.* Therefore, the goal of this chapter is to analyze the function of these novel PME/I genes in *C. hirsuta*.

In this chapter, I describe the approach I took to knock down the function of 7 PME/I genes in *C. hirsuta* using artificial microRNAs (amir). I describe the generation and characterization of transgenic lines, which provide the necessary tools to determine the function of six of these novel PME/I genes in *C. hirsuta*. Moreover, I describe amir-PMEI6 transgenic lines that knock down the expression of the *C. hirsuta* ortholog of *PMEI6*. *PMEI6* is required for mucilage release during seed germination, and is one of the few *PMEI* genes with a well-described developmental function in *A. thaliana* (Saez-Aguayo *et al*., 2013). By developing these genetic tools to study PME/I gene function in *C. hirsuta*, my work provides a way forward to understand what role, if any, PME/I genes play in explosive seed dispersal.

4.2- Results

4.2.1- Construction of amir-PME/I transgenic lines

I chose to analyze the function of six PME/I genes that are differentially expressed during *C. hirsuta* fruit development and are present in the genome of *C. hirsuta* but not *A. thaliana*. To this

end, I designed amirs to silence multiple genes, where genes had sufficiently high sequence similarity, using the Web MicroRNA Designer tool (available at weigelworld.org). I designed two amirs (amir-4a and amir-3a) that target the same three genes (CARHR043880, CARHR044320, CARHR045850) (Table 1). Amir-4a additionally targets the duplicate gene CARHR045840, which is not expressed at detectable levels in *C. hirsuta* fruit (Gan *et al.* 2016). I designed two amirs (amir-3c and amir-2a) that target the same two genes (CARHR265370 and CARHR213460). Amir-3c also targets a third gene CARHR089500 (Table 1). Additionally, I chose to analyze the function of the *C. hirsuta PMEI6* gene (CARHR143060) because the orthologous gene in *A. thaliana* has a clear mutant phenotype (Saez-Aguayo *et al.* 2013). I designed amir-PMEI6 to target this single gene (Table 1).

I constructed vectors that constitutively or inducibly express these amiRNAs. I used the CaMV *35S* promoter for constitutive expression, and a two-component system for dexamethasone-inducible expression (Table 1). For inducible amiRNA expression, I used the vector *pOPIn2-AtRPS5a::LhGR2* to constitutively express the LhGR2 fusion protein, which trans-activates amiRNA expression upon dexamethasone induction (Moore *et al*., 2002). I used *Agrobacterium* floral dip to transform these amiRNA constructs into *C. hirsuta* wild type plants and generated multiple independent transgenic lines for each construct (Table 1).

Table 1. **Artificial miRNA constructs and their target gene(s). The number of independent transgenic lines generated for each construct is indicated.**

	Target genes	**amir construct**	**# independent transgenic lines**
amir-PMEI6	CARHR143060	AtPRS5a>GR>amir-PMEI6	17
		35S::amirPMEI6	18
amir-4a /3a	CARHR043880 CARHR044320 CARHR045850	AtPRS5a>GR>amir-4a/3a	12
		35S::amir-4a/3a	18
amir-3c /2a	CARHR089500 CARHR213460 CARHR265370	AtPRS5a>GR>amir-3c /2a	9
		35S::amir-3c/2a	31

4.2.2- Analysis of first transgenic generation (T1)

I will consider amir-4a and amir-3a transgenic lines together as both amiRNAs target the same three PME/I genes (CARH043880, CARHR044320 and CARHR045850). A total of 12 independent inducible amir transgenic lines were resistant to Basta (Glufosinate) selection, and 18 constitutive amir transgenic lines were resistant to hygromycin selection (Table 1).

I will consider amir-3c and amir-2a transgenic lines together as both amiRNAs target the same two PME/I genes (CARHR265370, CARHR213460), and amir-3c targets one additional PME/I gene (CARHR089500). A total of 9 independent inducible amir transgenic lines were resistant to Basta (Glufosinate) selection, and 31 independent constitutive amir transgenic lines were resistant to hygromycin selection (Table 1).

Amir-PMEI6 targets the *PMEI6* gene (CARHR143060). A total of 17 independent inducible amir transgenic lines were resistant to Basta (Glufosinate) selection, and 19 independent constitutive amir transgenic lines were resistant to hygromycin selection (Table 1).

In summary, I generated multiple independent transgenic lines for each construct. All plants that expressed the amir-PME/I transgenes constitutively were viable and fertile. Therefore, I chose to continue characterizing the constitutive amir lines and harvest T2 seed of the inducible amir lines to archive for future research.

4.2.3- Analysis of second transgenic generation (T2)

To start characterizing the constitutive amir transgenic lines, I analysed segregation of the hygromycin selectable marker in the T2 generation. I plated approximately 60 to 100 seeds of each transgenic line on MS media supplemented with hygromycin in order to identify lines that inherited and expressed the transgene, and contained a single transgene locus. I identified 27 lines that were not hygromycin resistant in the T2 generation. These included 26 lines of amir-3c and amir-2a, and one line of amir-4a. To identify lines that contain a single transgene locus, I used a Chi-squared test to test the goodness of fit between the observed segregation ratio of resistant to sensitive plants to an expected 3:1 ratio for a single dominant locus. My results showed that segregation of the hygromycin marker fit a 3:1 ratio for the majority of transgenic lines (Table 2). I did not quantify segregation of the hygromycin marker in 17 PMEI6-amir lines that I generated later in my project (included in Table 1), but I estimated that the segregation ratio of resistant to

sensitive plants in these lines was 3:1. Therefore, I continued to characterize all constitutive amir lines that were likely to contain a single transgene locus.

Table 2. Segregation ratio of hygromycin resistant to sensitive plants in T2 progeny of *35S::amir-PME/I* lines, tested for goodness of fit to a 3:1 ratio by Chi-squared test.

Transgenic lines	Observed	x2 statistic	Accept null
35s::PMEI6-amir-T2_1	55 (64)	4,08	No
35s::4a-amir-T2-1	48 (56)	3,43	Yes
35s::4a-amir-T2-2	70 (70)	23,33	No
35s::4a-amir-T2-3	39 (40)	10,80	No
35s::4a-amir-T2-4	20 (58)	50,78	No
35s::4a-amir-T2-5	62 (73)	3,84	Yes
35s::4a-amir-T2-6	70 (91)	0,18	Yes
35s::4a-amir-T2-7	85 (102)	3,78	Yes
35s::4a-amir-T2-8	96 (120)	1,60	Yes
35s::4a-amir-T2-9	68 (88)	0,24	Yes
35s::4a-amir-T2-10	90 (100)	12,00	No
35s::4a-amir-T2-11	68 (89)	0,09	Yes
35s::4a-amir-T2-12	70 (86)	1,88	Yes
35s::4a-amir-T2-13	52 (76)	1,75	Yes
35s::4a-amir-T2-14	71 (95)	0,00	Yes
35s::4a-amir-T2-15	51 (82)	7,17	No
35s::4a-amir-T2-16	76 (96)	0,89	Yes
35s::-3a-amir-T2-1	55 (66)	2,44	Yes
35s::2a-amir-T2-1	56 (76)	0,07	Yes
35s::2a-amir-T2-3	33 (42)	0,29	Yes
35s::2a-amir-T2-4	51 (60)	3,20	Yes
35s::3c-amir-T2-1	40 (52)	0,10	Yes
35s::3c-amir-T2-4	50 (62)	1,05	Yes

Observed resistant plants with total plants shown in brackets, expected ratio of resistant plants = 0.25, alpha level of significance = 0.05, degrees of freedom = 1, critical value in x2 distribution table = 3.841.

4.2.4- Determining transgene copy number and zygosity (T3)

I determined the number of transgene copies and zygosity in a subset of *35S::amir-PME/I* lines in the T3 generation. I outsourced this analysis to iDNA Genetics, Norwich, UK, where they use a multiplex PCR method called *g-Count*. My results showed that two out of five *35S::4a-amir* lines that were analysed had a single T-DNA insertion (Table 3). All other *35S::4a-amir*, *35S::2a-amir* and *35S::3c-amir* lines had multiple T-DNA insertions (Table 3). I could identify homozygous individuals in almost every independent line.

In summary, I determined the heritable expression of amir-PME/I transgenes, and the segregation of the transgene locus for each transgenic line. I also determined the number of transgene copies and zygosity of the transgene locus for a subset of lines. I identified *35S::4a-amir-4* individuals that were potentially homozygous for a single copy of the transgene and used seeds of this individual for subsequent analysis of pectin methylesterase activity in section 4.4.

Table 3. Transgene copy number determined in a subset of *35S::amir-PME/I* lines.

Transgenic line	Transgene copy number
35S::4a-amir-4	1 copy
35S::4a-amir-6	3-4 copies
35S::4a-amir-8	1 copy
35S::4a-amir-10	inconclusive[1]
35S::4a-amir-13	3-4 copies
35S::2a-amir-1	3-4 copies[2]
35S::2a-amir-4	8 copies
35S::-3c-amir-1	3-4 copies
35S::3c-amir-4	4 or 8 copies

Transgene copy number was determined in T3 plants by iDNA Genetics, Norwich, UK.

[1] Results for this line showed that two segregating families had different copy numbers, family 10 had 1 copy and family 14 had 5 copies. Therefore, it is not clear what the correct copy number for line *35S::amir-4a-10* is.

[2] Results for this line showed that two segregating families had different copy numbers, family 2 had 3 copies and family 9 had 4 copies. Therefore, the correct copy number for line *35S::amir-2a-1* is either 3 or 4 copies.

4.2.5- PME/I gene expression analysis in transgenic plants

To assess whether the amir-PME/I transgenes were efficiently silencing target gene expression, I used qRT-PCR to quantify gene expression levels in transgenic lines compared to wild type. I outline the methodology that I followed to prioritise which transgenic lines to evaluate by qRT-PCR in Figure 1.

Final qRT-PCR analyses were performed using three biological replicates of stage 16 fruit pooled from 8-10 individual T2 plants per replicate per transgenic line. Results are presented as the mean expression value of these three biological replicates with the standard error associated with this mean. Therefore, the error incorporates technical error, which was minimized by using the mean value of three technical replicates for each sample, and biological error, which includes the variability in expression between heterozygous and homozygous genotypes in pooled samples, and differences in growth and development between individual fruits and individual plants. qRT-PCR results were analysed as in the previous chapter using the $2^{-\Delta\Delta CT}$ method (Pfaffl et al 2001) and normalizing PME/I gene expression to the housekeeping gene Clatherin.

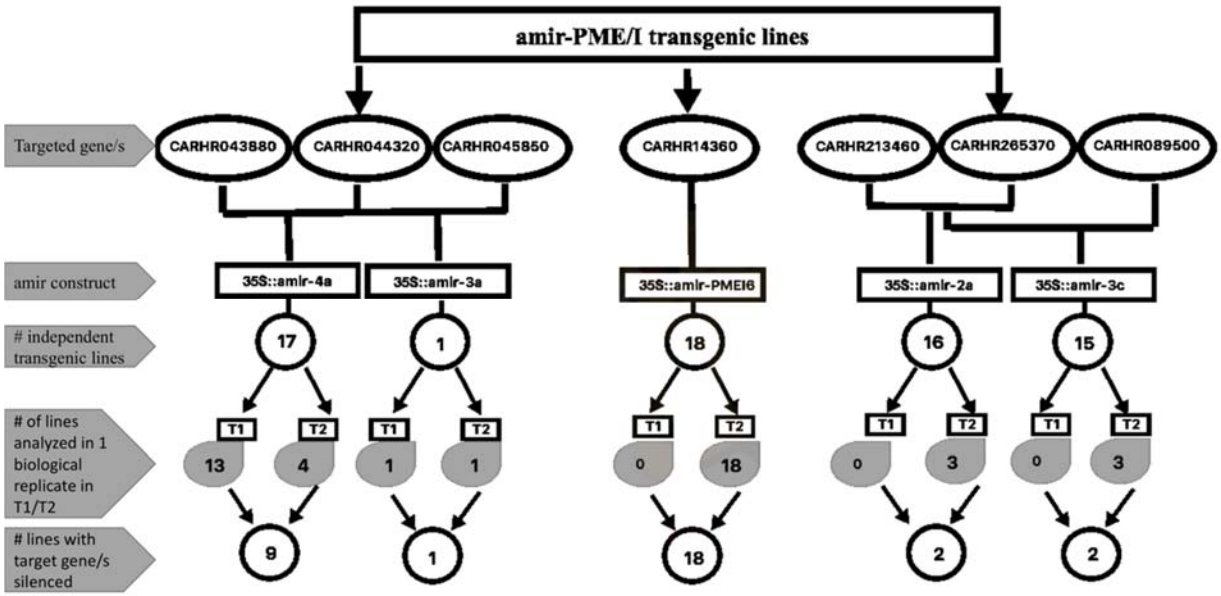

Figure 1: Outline of methodology to prioritize *35S::amir-PME/I* lines for gene expression analysis by qRT-PCR.

4.2.5.1- *35S::amir-4a* transgenic lines

The 35S::amir-4a construct targets three PME/I genes (CARH043880, CARHR044320 and CARHR045850). Therefore, I first measured expression of these three genes by qRT-PCR using a single biological replicate in *35S::amir-4a* lines. I analyzed 13 lines in the T1 generation and 4 lines in the T2 generation (Fig. 1). My results showed that 10 lines had reduced expression of gene CARH043880, 13 lines had reduced expression of CARHR045850, and 13 lines had reduced expression of CARHR044320. Therefore, from this preliminary analysis I identified nine *35S::amir-4a* lines where the expression of all three PME/I genes was reduced compared to wild type.

Next, I determined the expression of all three PME/I genes (CARH043880, CARHR044320 and CARHR045850) in three biological replicates of these nine *35S::amir-4a* lines in the T2 generation. In this qRT-PCR experiment, I confirmed that all nine *35S::amir-4a* lines had significantly reduced expression of all three PME/I genes compared to wild type (Table 4, Fig. 2, Fig. 3). One exception was line *35S::amir-4a-16* which did not have significantly reduced expression of CARHR045850 (Table 4, Fig. 2, Fig. 3). Lines *35S::amir-4a-1*, *35S::amir-4a-8* and *35S::amir-4a-10* showed the most significant reduction in expression of all three PME/I genes (Table 4, Fig. 2, Fig. 3). Therefore, these three lines represent the most promising transgenic lines to use for phenotypic analyses.

Table 4. Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-4a* transgenic lines (T2 generation) in *C. hirsuta*.

	***C. hirsuta* gene name**					
T2 line	**CARHR043880**		**CARHR044320**		**CARHR045850**	
	Normalized gene expression	**P-Value**	**Normalized gene expression**	**P-Value**	**Normalized gene expression**	**P-Value**
Wild Type	26,96 ± 1,01		19,65 ± 3,60		43,95 ± 6,22	
T2-35s::4a-1	0,48 ± 0,08	1,27E-05	0,75 ± 0,15	6,32E-03	11,72 ± 2,35	8,35E-03
T2-35s::4a-2	1,02 ± 0,24	1,52E-05	5,94 ± 0,30	1,92E-02	28,24 ± 4,53	1,11E-01
T2-35s::4a-4	0,72 ± 0,34	1,62E-05	2,21 ± 1,86	1,26E-02	16,14 ± 10,08	7,86E-02
T2-35s::4a-6	2,02 ± 0,51	2,49E-05	2,66 ± 0,19	9,22E-03	25,14 ± 0,64	3,96E-02
T2-35s::4a-8	0,26 ± 0,06	1,22E-05	0,16 ± 0,02	5,65E-03	3,41 ± 0,39	2,88E-03
T2-35s::4a-9	4,41 ± 2,53	1,16E-03	5,88 ± 0,86	2,05E-02	32,58 ± 6,44	2,73E-01
T2-35s::4a-10	0,68 ± 0,22	1,42E-05	0,34 ± 0,05	5,84E-03	6,24 ± 0,79	3,84E-03
T2-35s::4a-13	5,12 ± 0,93	9,12E-05	3,17 ± 0,14	1,02E-02	26,22 ± 3,39	6,65E-02
T2-35s::4a-16	6,95 ± 1,99	8,55E-04	6,82 ± 2,24	3,89E-02	40,13 ± 11,65	7,87E-01

Gene expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Values shown as means of 3 biological replicates ± standard error of the mean.

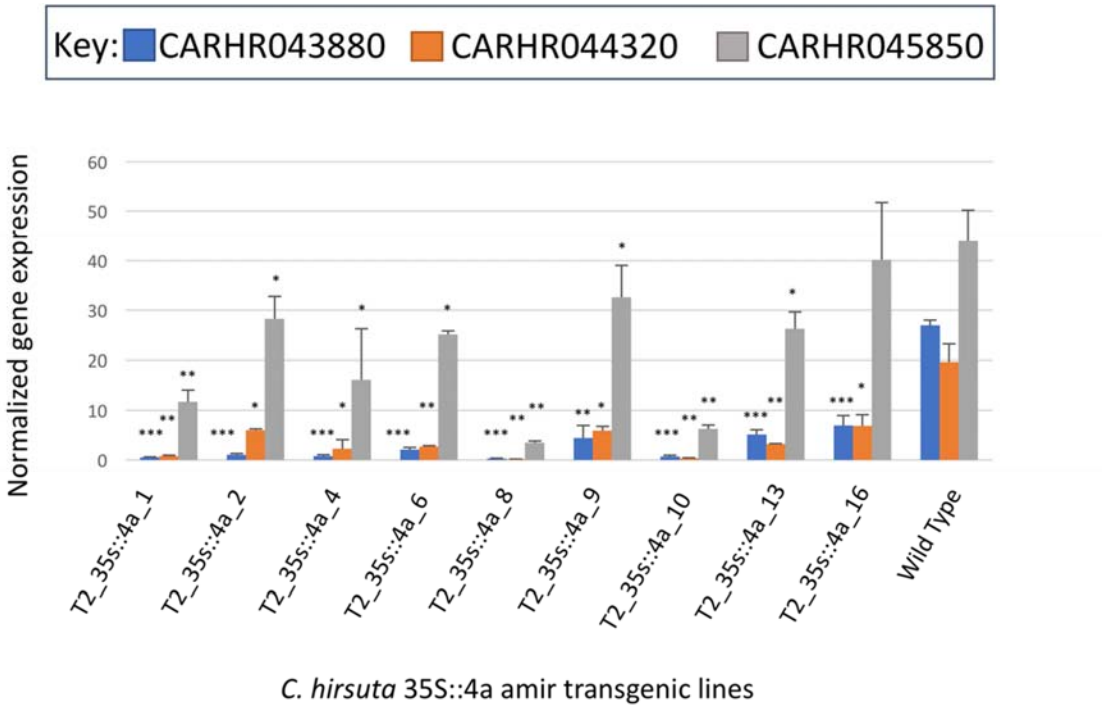

Figure 2: Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-4a* transgenic lines (T2 generation) in *C. hirsuta*. Nine independent transgenic lines of 35S::amir-4a where three *C. hirsuta* genes (CARHR043880, CARHR045850 and CARHR044320) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and have no orthologs in *A. thaliana.* Gene expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

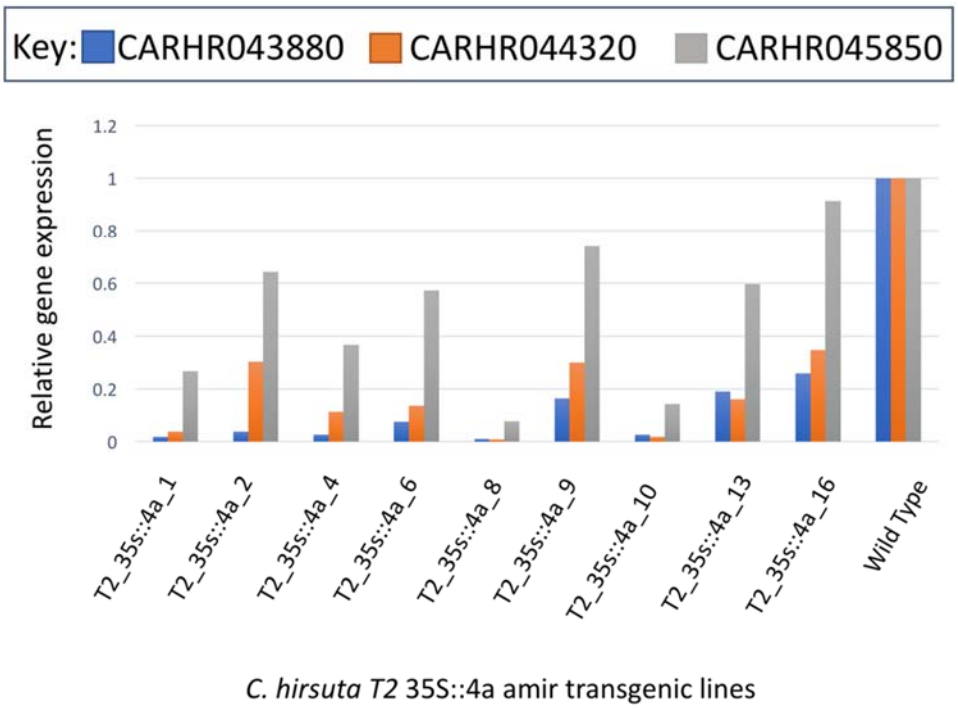

Figure 3: Expression of 3 PME/I genes quantified by qRT-PCR in *35S::amir-4a* transgenic lines (T2 generation) in *C. hirsuta* relative to wild type. Nine independent transgenic lines of 35S::amir-4a where three *C. hirsuta* genes (CARHR043880, CARHR045850 and CARHR044320) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and have no orthologs in *A. thaliana.* Expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Values shown as means of 3 biological replicates.

4.2.5.2- *35S::amir-3a* transgenic lines

The 35S::amir-3a construct targets the same three PME/I genes as amir-4a (CARH043880, CARHR044320 and CARHR045850). I only recovered one *35S::amir-3a* line. I first measured expression of the three PME/I genes in this line by qRT-PCR using a single biological replicate in the T1 generation (Fig. 1). My results showed that expression of all three genes: CARH043880, CARHR045850, and CARHR044320, was reduced in this *35S::amir-3a* line. I confirmed this qRT-PCR result in *35S::amir-3a* by using a single biological replicate in the T2 generation (Fig. 1).

Next, I determined the expression of all three PME/I genes (CARH043880, CARHR044320 and CARHR045850) in three biological replicates of *35S::amir-3a* in the T2 generation. In this qRT-PCR experiment, I showed that all three PME/I genes had significantly reduced expression compared to wild type (Table 5, Fig. 4, Fig. 5). Therefore, this *35S::amir-3a* line is promising to use for phenotypic analyses.

Table 5. Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-3a-1* transgenic line (T2 generation) in *C. hirsuta.*

	***C. hirsuta* gene name**					
T2 line	**CARHR043880**		**CARHR044320**		**CARHR045850**	
	Normalized gene expression	**P-Value**	**Normalized gene expression**	**P-Value**	**Normalized gene expression**	**P-Value**
Wild Type	26,96 ± 1,01		19,65 ± 3,60		43,95 ± 6,22	
T2-35s::3a-1	0,12 ± 0,03	1,19E-05	0,12 ± 0,03	5,61E-03	0,82 ± 0,06	2,27E-03

Gene expression is compared pairwise using Student's t-test. Values shown as means of 3 biological replicates ± standard error of the mean.

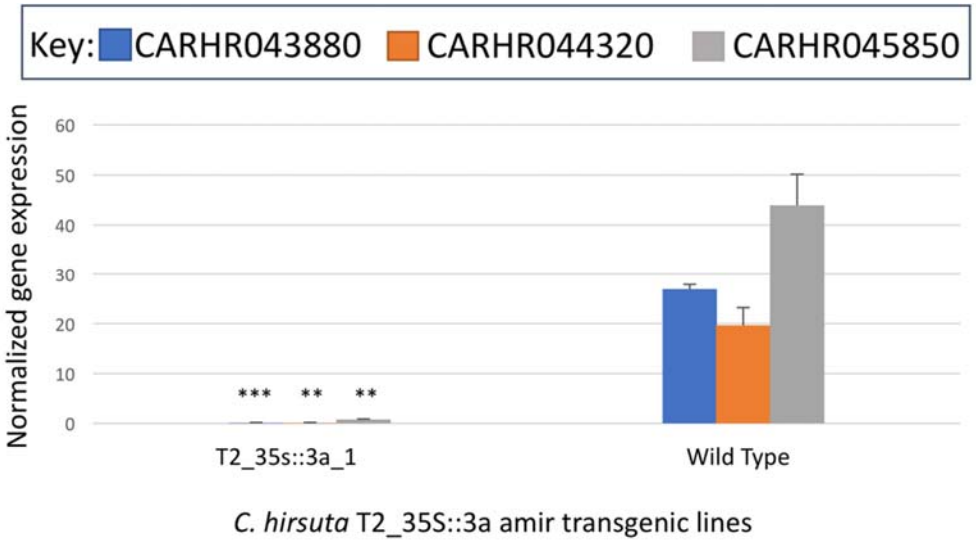

Figure 4: Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-3a* transgenic line (T2 generation) in *C. hirsuta*. One transgenic line (T2_1) of 35S::amir-3a, where three *C. hirsuta* genes (CARHR043880, CARHR045850 and CARHR044320) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and have no orthologs in *A. thaliana.* Gene expression is compared pairwise using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

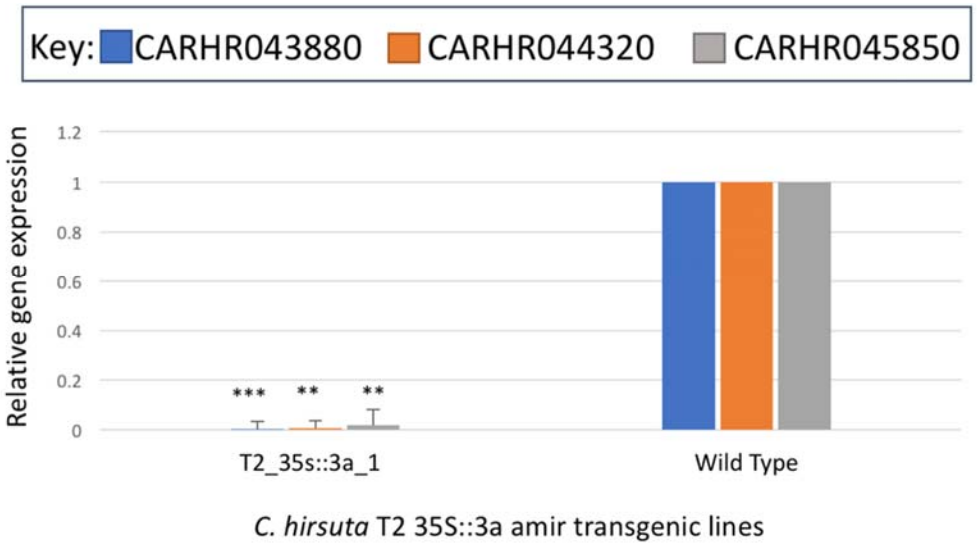

Figure 5: Expression of 3 PME/I genes quantified by qRT-PCR in *35S::amir-3a-1* transgenic line (T2 generation) in *C. hirsuta* relative to wild type. One transgenic line (T2_1) of *35S::amir-3a*, where three *C. hirsuta* genes (CARHR043880, CARHR045850 and CARHR044320) are targeted for silencing. Expression is analyzed in stage 16 fruits. Expression is compared pairwise using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

In summary, I identified ten independent transgenic lines that show significantly reduced expression of three PME/I genes: CARH043880, CARHR044320 and CARHR045850. These genes are present in *C. hirsuta* but do not have clear orthologues in *A. thaliana* (Fig. 6). Therefore, the *35S::amir-4a* and *35S::amir-3a* transgenic lines that I generated here, provide a first means to evaluate the function of these novel genes in *C. hirsuta.* A particularly promising line for further analysis is *35S::amir-3a-1*, which has less than 2% of wild-type expression levels of all three genes.

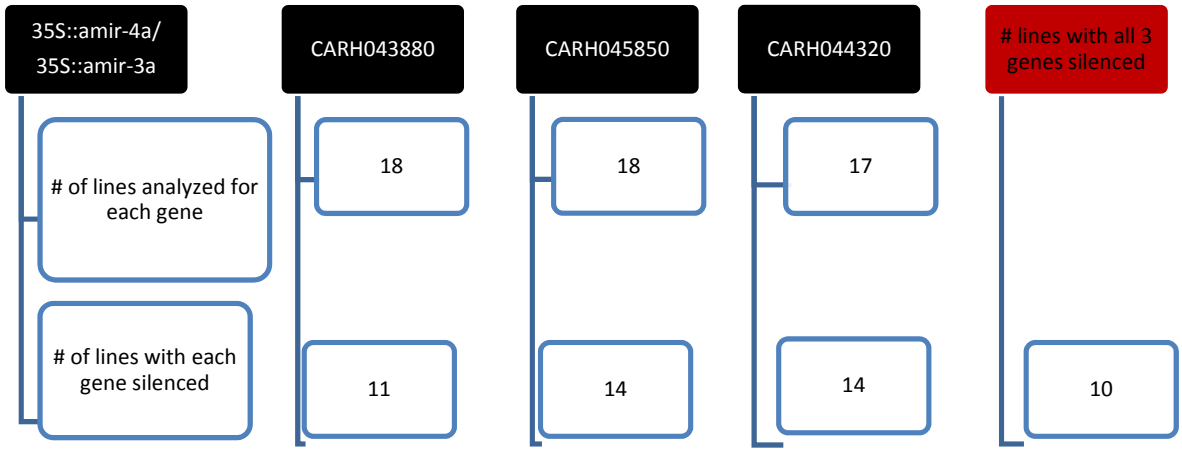

Figure 6: Summary of *35S::amir-4a* and *35S::amir-3a* transgenic lines, which target the same three PME/I genes in *C. hirsuta*.

4.2.5.3- *35S::amir-3c* transgenic lines

The 35S::amir-3c construct targets a different set of three PME/I genes (CARHR089500, CARHR213460 and CARHR265370). I only recovered 3 of the original 15 T1 lines isolated for *35S::amir-3c*. I first measured expression of the three PME/I genes targeted by amir-3c by qRT-PCR using a single biological replicate. I analyzed all three *35S::amir-3c* lines in the T2 generation (Fig. 1). My results showed that two lines had reduced expression of all three PME/I genes CARHR089500, CARHR213460 and CARHR265370. Genes CARHR213460 and CARHR265370 were expressed at very low levels (also see RNAseq results in Fig. 6 introduction). Therefore, from this preliminary analysis I identified two *35S::amir-3c* lines where the expression of three PME/I genes was reduced compared to wild type.

Next, I determined the expression of these three targeted PME/I genes (CARHR089500, CARHR213460 and CARHR265370) in three biological replicates of these two *35S::amir-3c* lines in the T2 generation. In this qRT-PCR experiment, I confirmed that both *35S::amir-3c* lines had significantly reduced expression of both CARHR213460 and CARHR265370 compared to wild type (Table 6, Fig. 7, Fig. 8). Expression of the third gene, CARHR089500, was significantly reduced in *35S::amir-3c-1*, but not in *35S::amir-3c-4* (Table 6, Fig. 7, Fig. 8). Therefore, line *35S::amir-3c-1* is the most promising transgenic line to use for phenotypic analyses. For line *35S::amir-3c-1,* expression of CARHR265370 was reduced to 30% and CARHR213460 expression was reduced to 18% and the expression of CARHR089500 was reduced to 70% of wild-type levels.

Table 6. Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-3c* transgenic lines (T2 generation) in *C. hirsuta*

	***C. hirsuta* gene name**					
T2 line						
	Normalized gene expression	**P-Value**	**Normalized gene expression**	**P-Value**	**Normalized gene expression**	**P-Value**
	CARHR089500		**CARHR265370**		**CARHR213460**	
Wild Type	1,82E-02 ± 7,68E-03		2,89E-03 ±2,21E-04		1,99E-03 ±9,06E-05	
T2-35s::2a-1	8,98E-03 ± 5,29E-04	2,98E-01	9,08E-04 ±5,80E-05	3,14E-01	3,50E-04 ±3,46E-05	7,19E-05
T2-35s::2a-4	1,17E-02 ± 1,98E-03	4,57E-01	1,42E-03 ±9,08E-05	4,91E-01	4,26E-04 ±3,95E-05	9,38E-05

Gene expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Values shown as means of 3 biological replicates ± standard error of the mean.

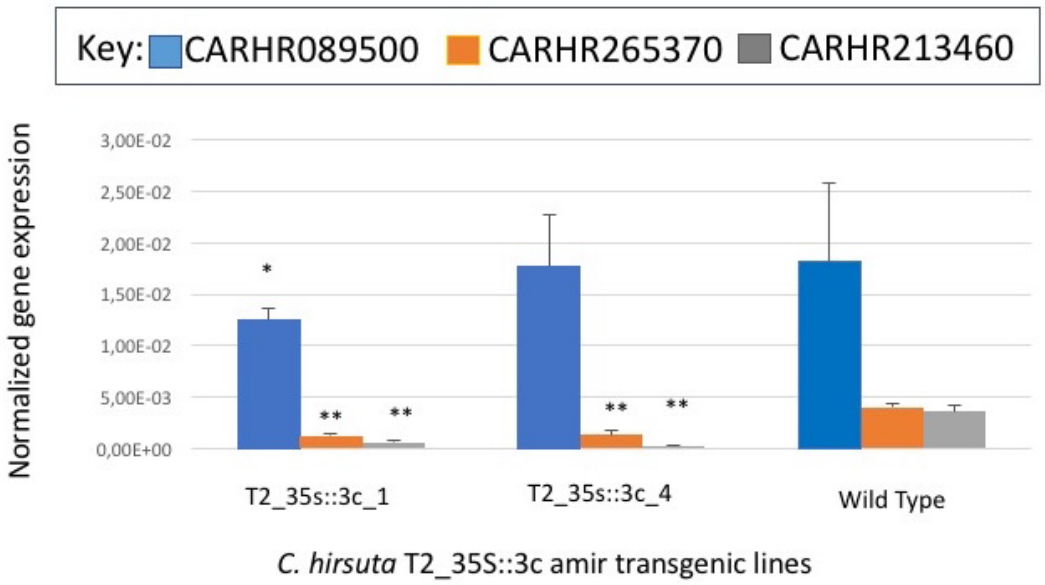

Figure 7: Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-3c* transgenic lines (T2 generation) in *C. hirsuta*. Two independent transgenic lines (T2-1, T2-4) of 35S::amir-3c where three *C. hirsuta* genes (CARHR089500, CARHR213460 and CARHR265370) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and have no orthologs in *A. thaliana*. Gene expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

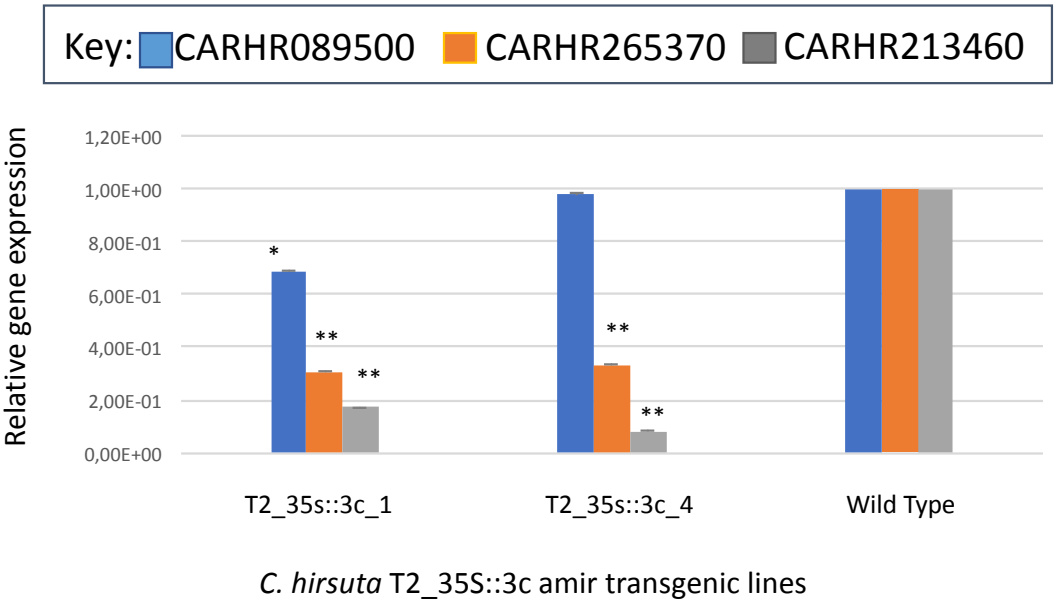

Figure 8: Expression of 3 PME/I genes quantified by qRT-PCR in *35S::amir-3c* transgenic lines (T2 generation) in *C. hirsuta* relative to wild type. Two independent transgenic lines (T2-1, T2-4) of *35S::amir-3c* where three *C. hirsuta* genes (CARHR089500, CARHR213460 and CARHR265370) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and have no orthologs in *A. thaliana*. Expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

4.2.5.4- *35S::amir-2a* transgenic lines

The 35S::amir-2a construct targets two of the same PME/I genes as amir-3c (CARHR265370 and CARHR213460). I only recovered three of the 16 *35S::amir-2a* T1 lines that I generated. I first measured expression of the two PME/I genes targeted by amir-2a in these three lines by qRT-PCR using a single biological replicate in the T2 generation (Fig. 1). My results showed that two lines had reduced expression of both PME/I genes (CARHR265370 and CARHR213460) (Table 7, Fig. 9). As mentioned above, both genes were expressed at very low levels. I also decided to measure expression of the more highly expressed gene CARHR089500 in these lines, reasoning that this gene shows sequence similarity to CARHR265370 and CARHR213460 and may be unintentionally targeted by amir-2a. I found that CARHR089500 levels were reduced in two of the three *35S::amir-2a* lines. Therefore, from this preliminary analysis I identified two *35S::amir-2a* lines where the expression of CARHR265370, CARHR213460 and CARHR089500 was reduced compared to wild type.

I went on to determine the expression of CARHR265370, CARHR213460 and CARHR089500 in three biological replicates of two *35S::amir-2a* lines in the T2 generation. In this qRT-PCR experiment, I showed that CARHR265370, CARHR213460 and CARHR089500 expression was significantly reduced in the fruit of *35S::amir-2a-1* and *35S::amir-2a-4* compared to wild type (Table 7, Fig. 9, Fig. 10). Although the reduction in CARHR089500 expression was significant, it was only reduced to 50% of wild-type levels, CARHR265370 reduced to 30% of wild-type levels and CARHR213460 reduced to 18% of wild-type levels (Table 9). Therefore, amir-2a was not very effective at silencing expression of the target genes CARHR265370 and CARHR213460 or the unintentional target gene CARHR089500. However, *35S::amir-2a-1* line is the most promising line to use for phenotypic analyses.

Table 7. Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-2a* transgenic lines (T2 generation) in *C. hirsuta.*

	***C. hirsuta* gene name**					
T2 line						
	Normalized gene expression	**P-Value**	**Normalized gene expression**	**P-Value**	**Normalized gene expression**	**P-Value**
	CARHR089500		**CARHR265370**		**CARHR213460**	
Wild Type	1,82E-02 ± 7,68E-03		2,89E-03 ±2,21E-04		1,99E-03 ±9,06E-05	
T2-35s::2a-1	8,98E-03 ± 5,29E-04	2,98E-01	9,08E-04 ±5,80E-05	3,14E-01	3,50E-04 ±3,46E-05	7,19E-05
T2-35s::2a-4	1,17E-02 ± 1,98E-03	4,57E-01	1,42E-03 ±9,08E-05	4,91E-01	4,26E-04 ±3,95E-05	9,38E-05

Expression of CARHR265370 is determined from 1 biological replicate. Expression of CARHR089500 is shown as means of 3 biological replicates ± standard error of the mean, and compared pairwise between wild type and each independent transgenic line using Student's t-test.

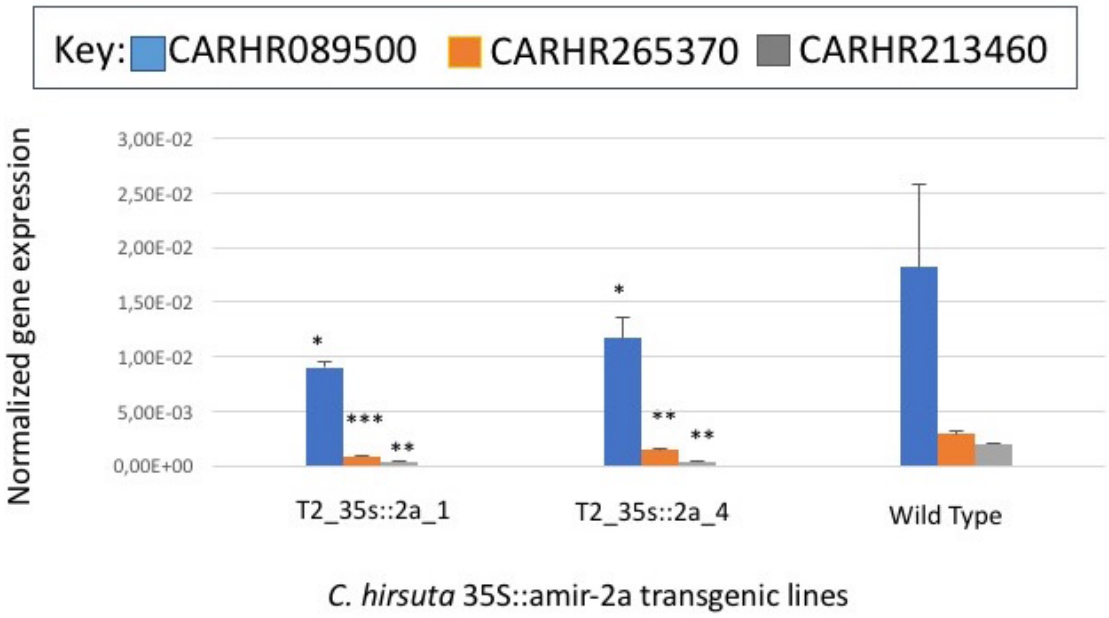

Figure 9: Normalized expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-2a* transgenic lines (T2 generation) in *C. hirsuta* relative to wild type. Two independent transgenic lines (T2-1, T2-4) of *35S::amir-2a* where the three *C. hirsuta* genes (CARHR265370, CARHR213460 and CARHR089500) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and has no ortholog in *A. thaliana*. Gene expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

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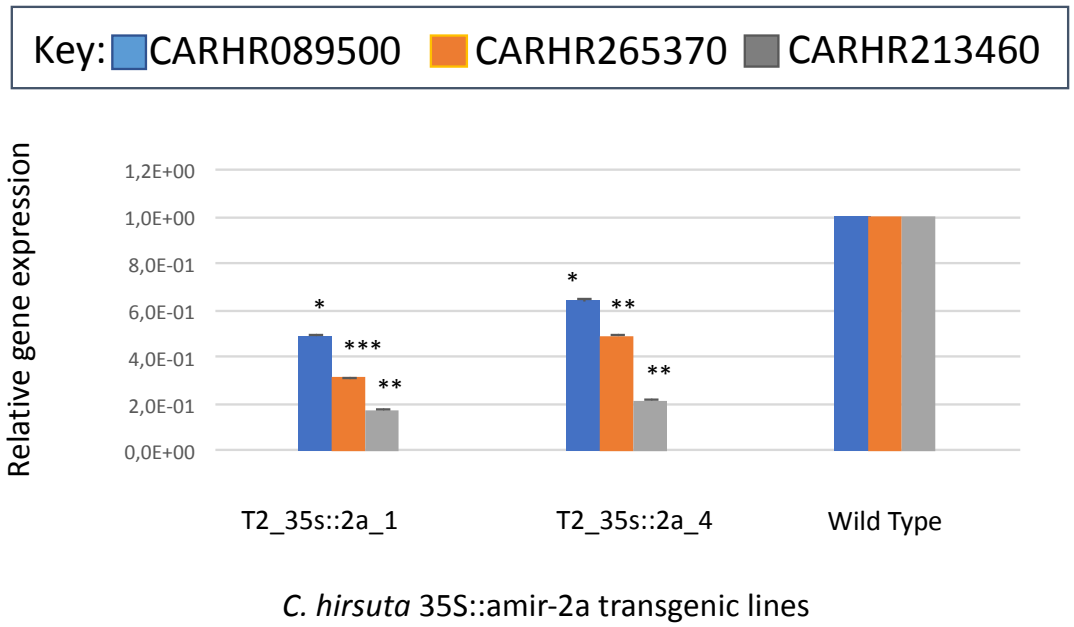

Figure 10: Expression levels of 3 PME/I genes quantified by qRT-PCR in *35S::amir-2a* transgenic lines (T2 generation) in *C. hirsuta* relative to wild type. Two independent transgenic lines (T2-1, T2-4) of *35S::amir-2a* where the three *C. hirsuta* genes (CARHR265370, CARHR213460 and CARHR089500) are targeted for silencing. Expression is analyzed in stage 16 fruits. Note that these genes are unique to *C. hirsuta* and has no ortholog in *A. thaliana.* Expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

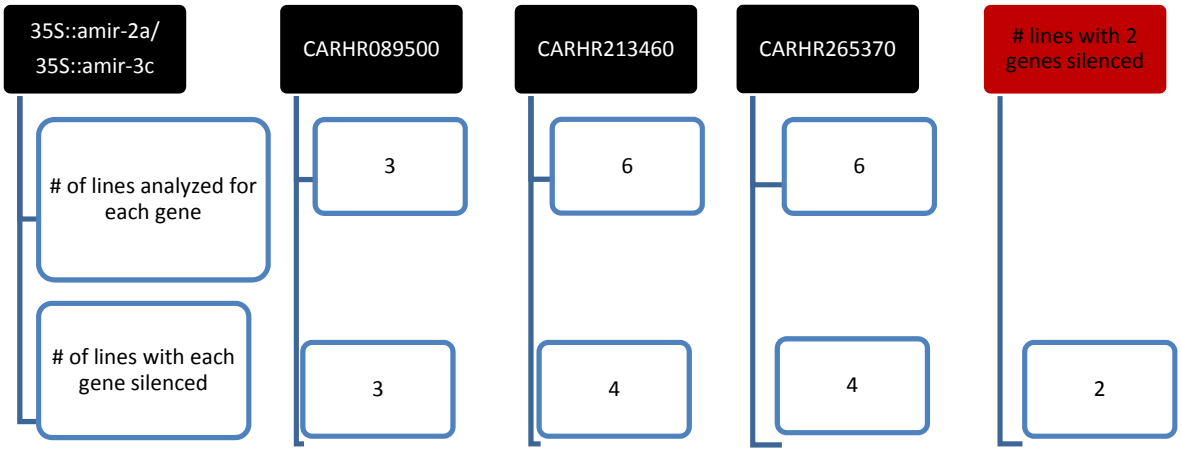

Figure 11. Summary of *35S::amir-2a* and *35S::amir-3c* transgenic lines, which target the same two genes in *C. hirsuta*.

4.2.5.5- 35S::amir-PMEI6 transgenic lines

The 35S::amir-PMEI6 construct targets the *C. hirsuta* ortholog of *PMEI6* (CARHR143060). Therefore, I measured CARHR143060 expression by qRT-PCR using a single biological replicate in *35S::amir-PMEI6* lines. I analyzed 18 lines in the T2 generation (Fig. 1). My results showed that all 18 lines had reduced expression of CARHR143060 compared to wild type. Therefore, from this preliminary analysis I selected 11 *35S::amir-PMEI6* lines with the lowest levels of CARHR143060 expression.

Next, I determined the expression of CARHR143060 in three biological replicates of these eleven *35S::amir-PMEI6* lines in the T2 generation. In this qRT-PCR experiment, I confirmed that all eleven *35S::amir-PMEI6* lines had significantly reduced expression of CARHR143060 compared to wild type (Table 8, Fig. 12, Fig. 13). Lines *35S::amir-PMEI6-1, 35S::amir-PMEI6-4, 35S::amir-PMEI6-5, 35S::amir-PMEI6-12, 35S::amir-PMEI6-16* showed the most significant reduction in CARHR143060 expression (Table 8, Fig. 12, Fig. 13). Therefore, these lines represent the most promising transgenic lines to use for phenotypic analyses.

Table 8. Normalized expression levels of PMEI6 quantified by qRT-PCR in 35S::amir-PMEI6 transgenic lines (T2 generation) in *C. hirsuta.*

***C. hirsuta* gene name**

T2 line	CARHR143060- PMEI6	
	Normalized gene expression	**P-Value**
Wild Type	2,86E-02 ± 4,94E-03	
T2-35s::PMEI6-3	1,28E-02 ± 4,09E-03	6,97E-02
T2-35s::PMEI6-4	1,28E-03 ± 6,59E-05	5,23E-03
T2-35s::PMEI6-5	1,75E-03 ± 9,73E-05	5,56E-03
T2-35s::PMEI6-7	1,18E-02 ± 2,61E-03	4,00E-02
T2-35s::PMEI6-12	1,31E-03 ± 3,78E-04	5,30E-03
T2-35s::PMEI6-13	1,15E-02 ± 4,33E-03	5,97E-02
T2-35s::PMEI6-16	1,23E-03 ± 2,99E-04	5,22E-03
T2-35s::PMEI6-17	1,47E-02 ± 4,56E-03	1,07E-01
T2-35s::PMEI6-18	6,67E-03 ± 1,57E-03	1,34E-02
T2-35s::PMEI6-20	2,81E-03 ± 4,88E-04	6,54E-03
T2-35s::PMEI6-1	1,15E-03 ± 1,17E-04	5,14E-03

Gene expression is compared pairwise using Student's t-test. Values shown as means of 3 biological replicates ± standard error of the mean.

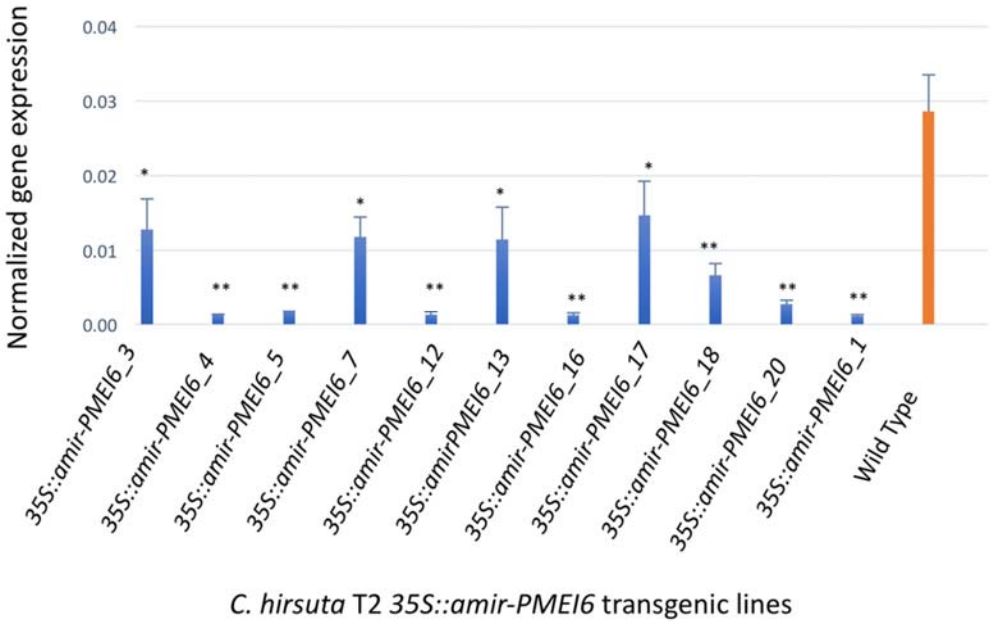

Figure 12: Normalized expression levels of CARHR143060 quantified by qRT-PCR in *35S::amir-PMEI6* transgenic lines (T2 generation) in *C. hirsuta*. Eleven independent transgenic lines of *35S::amir-PMEI6* where a single *C. hirsuta* gene (CARHR143060) is targeted for silencing. Expression is analyzed in stage 16 fruits. Note that this gene has orthologs in *A. thaliana.* Gene expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

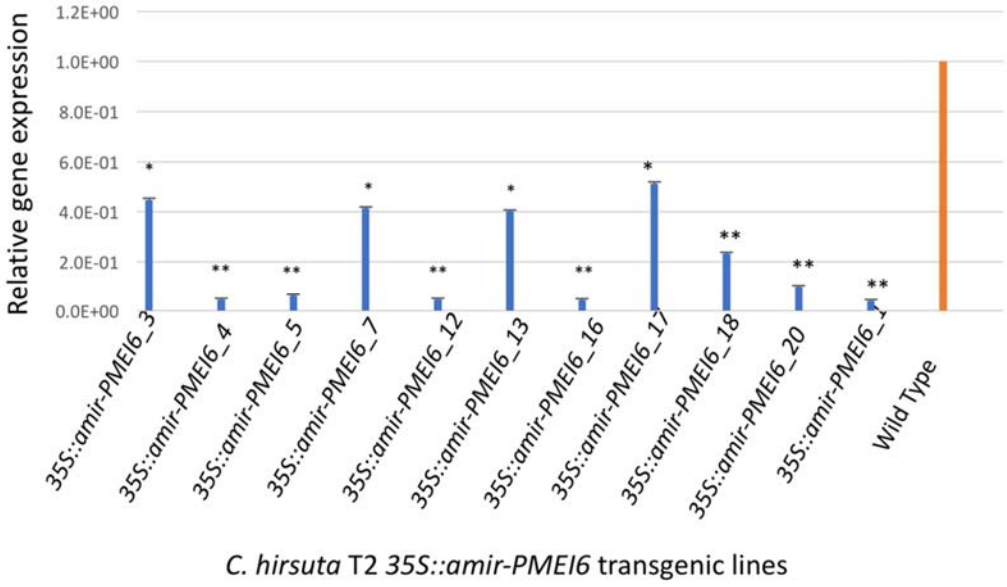

Figure 13: Expression of CARHR143060 quantified by qRT-PCR in *35S::amir-PMEI6* transgenic lines (T2 generation) in *C. hirsuta* relative to wild type. Eleven independent transgenic lines of *35S::amir-PMEI6* where a single *C. hirsuta* gene (CARHR143060) is targeted for silencing. Expression is analyzed in stage 16 fruits. Note that this gene has orthologs in *A. thaliana.* Expression is compared pairwise between wild type and each independent transgenic line using Student's t-test. Significance levels: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Values shown as means of 3 biological replicates ± standard error of the mean.

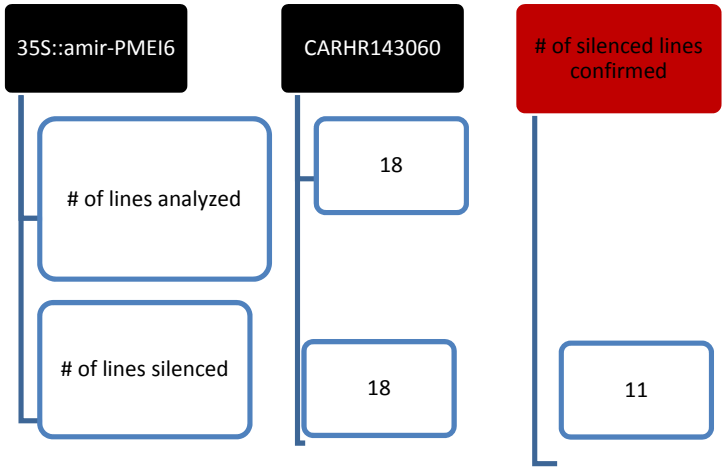

Figure 14: Summary of *35S::amir-PMEI6* transgenic lines in *C. hirsuta*.

4.3-Summary of artificial miRNA approach

In summary, I designed four artificial miRNAs to target two sets of three *C. hirsuta* PME/I genes that do not have clear orthologues in *A. thaliana*. I generated transgenic lines that express these constructs and identified lines with reduced expression of these PME/I genes. I achieved very different results for the two sets of genes. I achieved very efficient silencing of CARH043880, CARHR044320 and CARHR045850 genes in the *35S::amir-3a-1* transgenic line. Gene expression was reduced to less than 2% of wild-type levels for all three genes in this line (Table 9). Additionally, the *35S::amir-4a-8* line showed similarly low expression of CARH043880 and CARHR044320, but the expression level of CARHR045850 was almost 10% of wild-type levels. The reduction of gene expression achieved in these lines is very suitable to assess gene function using phenotypic analyses.

On the other hand, I did not achieve very efficient silencing of CARHR089500, CARHR213460 and CARHR265370 genes in the *35S::amir-3c* and *35S::amir-2a* transgenic lines. In the most promising line that I characterized, *35S::amir-2a-1*, CARHR265370 expression was reduced to 30% and CARHR213460 expression was reduced to 18% and CARHR089500 expression was reduced to 50% of wild-type levels (Table 9). This reduction in gene expression is unlikely to be sufficient to allow gene function to be assessed by phenotypic analyses. In the following discussion section, I will discuss possible reasons why these two sets of genes showed a difference in silencing efficiency by artificial miRNAs.

In addition, I generated *35S::amir-PMEI6* transgenic lines that efficiently silenced expression of the *PMEI6* ortholog in *C. hirsuta* (CARHR143060). In the *35S::amir-PMEI6-1* transgenic line, expression of CARHR143060 was reduced to 2 % of wild-type levels (Table 9). This reduction of gene expression is very suitable to assess gene function using phenotypic analyses.

Table 9. Summary of amir-PME/I lines with the most efficient gene silencing.

***C. hirsuta* gene**	**Best amir line**	**% of wild-type expression levels**
CARHR143060	*35S::amirPMEI6-1*	2%
CARHR043880	*35S::amir-3a-1*	1%
CARHR044320		2%
CARHR045850		1%
CARHR089500	*35S::amir-2a-1*	50%
CARHR213460		18%
CARHR265370		30%

4.4- Determining PME activity in *35S::amir-4a* seeds

An important prediction about modifying PME/I gene expression is that this should affect PME enzymatic activity in the plant cell wall. Moreover, I can make the following specific predictions based on my results so far: (1) Reduced PMEI gene expression in amir-PMEI transgenic plants is predicted to cause an increase in PME activity. (2) The PMEI genes that are targeted by these amir transgenes show seed-specific expression, therefore, I predict that this is an appropriate tissue in which to assay PME activity.

To determine PME activity in protein extracts of T3 *35S::amir-4a-1* seeds, I used ruthenium red-stained gel assays as previously described (Gan *et al*., 2016). I used several control samples in this experiment to allow me to interpret the relative PME activity in *35S::amir-4a-1* seeds compared to wild-type seeds. I observed PME activity in all seed samples relative to a no protein control that showed no ruthenium red staining (Fig. 15). Moreover, I replicated previously published results showing reduced PME activity in *35S::PMEI6* seeds compared to Col-0 seeds in *A. thaliana* (Saez-Aguayo *et al*., 2013), and reduced PME activity in *C. hirsuta* seeds compared to *A. thaliana* seeds (Gan *et al*., 2016) (Fig. 15).

In my samples of interest, I observed increased PME activity in *35S::amir-4a-1* seeds compared to wild-type *C. hirsuta* Ox seeds (Fig. 15). I observed a similar increase in PME activity in *pmei6-1* mutant seeds compared to wild-type Col-0 seeds in *A. thaliana* (Fig. 15), as previously reported (Saez-Aguayo *et al*., 2013). Furthermore, I observed the same relative PME activity between samples using both 20 µg and 80 µg protein, which gives me confidence that this result is reproducible. Therefore, I conclude that the reduced expression of CARH043880, CARHR044320 and CARHR045850 genes in *35S::amir-4a-1* seeds is associated with increased PME activity. This result also confirms that these three *C. hirsuta* genes, which do not have clear orthologues in *A. thaliana*, act as inhibitors of PME activity (PMEIs). This result is presented in a qualitative format but can the intensity of staining can also be quantified as reported previously (Gan *et al*., 2016; Saez-Aguayo *et al*., 2013).

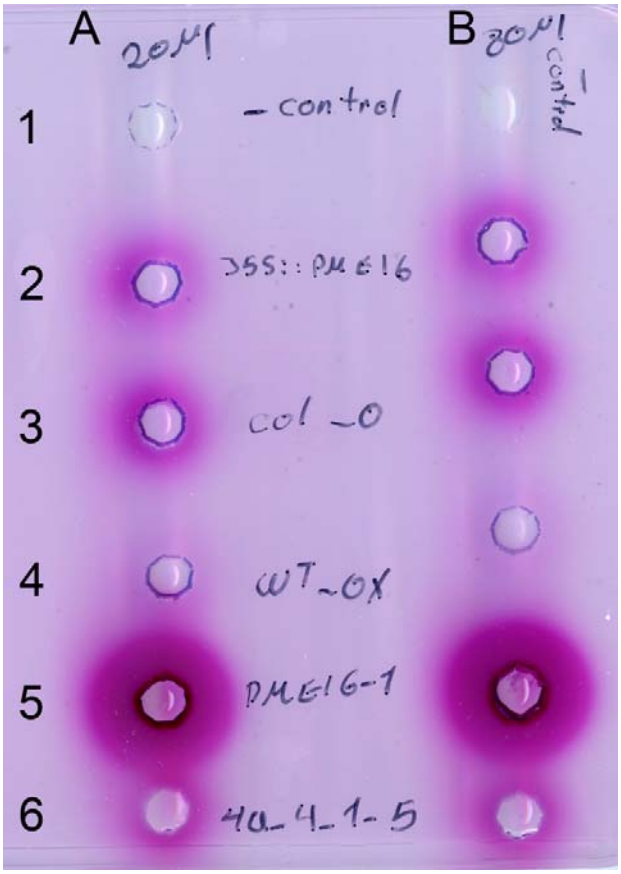

Figure 15: Pectin methylesterase (PME) activity assay. Relative PME activity determined by ruthenium red-stained gel assays in seed protein extracts of the following genotypes: (1) Control contains no protein. (2) *A. thaliana 35S::PMEI6*. (3) *A. thaliana* Col-0. (4) *C. hirsuta* Ox. (5) *A. thaliana pmei6-1*. (6) *C. hirsuta 35S::amir-4a-4*. (A) 20 µg seed protein extracts. (B) 80 µg seed protein extracts.

Chapter 5

General discussion

5.1- Summary

In this project, I characterized the expression dynamics of a group of 17 genes that are predicted to control pectin methylesterase activity in the cell wall. These genes were previously identified as differentially expressed during fruit development specifically in *C. hirsuta*, but not in *A. thaliana* (Gan *et al.* 2016). This was an interesting result as it suggested that the species-specific expression of cell wall-remodeling genes may be associated with the dramatically different seed dispersal strategies of *C. hirsuta* and *A. thaliana*. Here, I found that eight genes unique to *C. hirsuta*, with no clear orthologues in *A. thaliana*, were spatially and temporally restricted in their expression. All eight of these genes were expressed only in seeds during stage 16 of fruit development. I designed artificial microRNAs to target individual or groups of these pectin methylesterase inhibitor genes in *C. hirsuta*. I showed that I could very efficiently silence three of these genes in a single plant using just one artificial microRNA. Expression of all three genes was reduced to only 1-2% of wild-type levels in plants that expressed either the *35S::amir-3a* or *35S::amir-4a* transgenes. Expression of the *C. hirsuta PMEI6* gene was similarly reduced in plants expressing *35S::amir-PMEI6*. Moreover, I determined that pectin methylesterase enzymatic activity was reduced in *35S::amir-4a* seeds. This result confirms that the reduced expression of CARH043880, CARHR044320 and CARHR045850 genes in *35S::amir-4a* seeds is associated with increased PME activity. This result also confirms that these three *C. hirsuta* genes, which do not have clear orthologues in *A. thaliana*, act as inhibitors of pectin methylesterase activity (PMEIs). Therefore, the transgenic plants that I generated in this project can be used to characterize the function of cell wall-remodeling genes in explosive seed dispersal.

5.2- Seed-specific expression of *C. hirsuta*-specifc PMEI genes

All plant cells have a cell wall, and pectin is a major component of all cell walls. Furthermore, the composition of pectin domains and the crosslinking reactions that occur between them, influence cell wall biomechanics. For example, highly methylesterified pectins do not form stiff gels, and may make the wall more pliant. Whereas demethylesterified pectins can from stiff gels through Ca^{2+}-mediated crosslinking. These different properties of pectin are determined in every cell wall by the action of pectin methylesterases (PME). These PMEs are secreted by plant cells into the wall space where they hydrolyse pectin methylesters to unmask carboxyl-based crosslinking sites. This PME activity is inhibited by another class of proteins that are secreted into the wall space

called PME inhibitors (PMEI). Therefore, PME activity is regulated in the wall of every cell and influences biomechanical properties of the cell wall that are potentially important for growth and differentiation.

However, cell differentiation and growth patterns are highly specific to different tissues and different organs of the plant. Therefore, it is interesting to understand whether PME activity is regulated generally or specifically in different tissues. A recent study analyzed PME/I transcripts and pectin epitopes in garden cress seeds and found that PME activity is spatially and temporally regulated during seed germination (Scheler *et al.,* Plant Physiology 2015). However, another study found no obvious differences in the degree of pectin methylation related to organ formation at the shoot apical meristem in *A. thaliana* (Yang *et al.,* Current Biology 2016). Therefore, there is currently no consensus about whether PME activity is regulated to ensure consistency across tissues or to achieve specificity in different tissues.

My results showed a clear difference in expression between *C. hirsuta* PME/I genes that do have orthologues in *A. thaliana* and those that don't. The expression of all eight genes that are unique to *C. hirsuta*, without clear orthologues in *A. thaliana*, was limited to seeds during stage 16 of fruit development. Moreover, I could demonstrate that the reduced expression of three of these genes (CARH043880, CARHR044320 and CARHR045850) in *35S::amir-4a* seeds, was associated with increased pectin methylesterase enzymatic activity This suggests that that PME activity is spatially and temporally regulated during seed development in *C. hirsuta*.

5.3- Other functions for PME/I genes in *C. hirsuta* fruit development

Based on differential gene expression, it is possible that the PME/I genes studied here may have a function in *C. hirsuta* fruit development that they do not have in *A. thaliana* fruit development (Gan et al 2016). The eight *C. hirsuta*-specific PMEI genes, discussed above, are likely to function in seeds because their expression is mostly restricted to seeds. But the nine PME/I genes with *A. thaliana* orthologues do not show such a restricted pattern of gene expression. For example, the CARHR143060 gene, which is the otholog of *PMEI6* in *A. thaliana*, was expressed at high levels in leaf tissue. But during fruit development, it was most highly expressed at stage 17 and in seeds of stage 17b fruit, which fits with the function of *PMEI6* in seed mucilage release in *A. thaliana* (Saez-Aguayo *et al.,* 2013). I identified several lines of *35S::amir-PMEI6* in *C. hirsuta* where expression of the CARHR143060 gene was significantly reduced. Future analysis of these lines

can be used to assess whether or not the CARHR143060 gene is required for seed mucilage release in *C. hirsuta*. If so, then it is possible that the up-regulation of CARHR143060 expression occurs earlier in *C. hirsuta* fruit development than that of *PMEI6* in *A. thaliana,* thereby resulting in differential gene expression between stage 9 and 16 fruit, specifically in *C. hirsuta* but not *A. thaliana* (Gan *et al.,* 2016). This would suggest that seed maturation is precocious in *C. hirsuta* compared to *A. thaliana*, as seeds are dispersed at an earlier stage of fruit development in *C. hirsuta* than in *A. thaliana*.

The CARHR173850 gene was the only differentially expressed gene identified in the previous study that was significantly down-regulated during *C. hirsuta* fruit development (Gan et al 2016). This gene is the othologue of *PME5* in *A. thaliana*, which has a distinctive, spotted pattern of expression in the shoot meristem of *A. thaliana*, reminiscent of cell cycle-related genes (Peaucelle *et al.,* Development 2011). CARHR173850 was most highly expressed in stage 9 carpels, which fits with a function in cell division as cells are actively dividing in these carpels, whereas subsequent stages of fruit development involve cell expansion. CARHR173850 had higher expression in leaves and floral shoots compared to stage 16 fruit, which also suggests that its expression is higher in tissues with more active cell division.

5.4- Efficient gene silencing by artificial miRNAs

My results show that it is possible to achieve very efficient gene silencing in *C. hirsuta* using amiRNAs, as was previously shown (Gan *et al.,* 2016). I achieved similar efficiency with amir-PMEI6, which targets a single gene, and amir-4a/amir-3a, which target multiple genes. Therefore, it is possible to design amiRNAs that silence multiple gene targets as efficiently as a single gene. In comparison to this, amir-2a/amir-3c produced very inefficient gene silencing. It is not straight-forward to predict this difference in efficiency by scrutinizing the amiRNA design. As mentioned in chapter 2, the WMD quality ranking of amiRNA sequences is based on many different criteria. One criterion is the hybridization energy of pairing between the amiRNA to its intended target(s). For amir-PMEI6, which gave efficient silencing, this hybridization energy was almost as high as for a perfect complement (Table 1, chapter 2). For amir-2a and amir-3c by contrast, this hybridization energy was lower than for a perfect complement (Table 1, chapter 2). However, for amir-4a, which gave efficient silencing, this hybridization energy was also low (Table 1, chapter 2). In fact, the WMD ranking for amir-4a was lower than for any of the other amiRNA sequences

used in this project (Table 1, chapter 2), yet it elicited efficient gene silencing *in planta*. In summary, it is difficult to identify the precise reasons why amir-2a and amir-3c produced inefficient gene silencing.

A common feature of the three PME/I genes targeted by amir-2a/amir-3c (CARHR089500, CARHR213460 and CARHR265370) is their low level of expression in wild-type fruit. RNAseq is a very sensitive technique to accurately measure gene expression and showed that while these genes were differentially expressed, their read counts were low (Fig. 6, chapter 1, Gan *et al*., 2016). This low level of gene expression may confound a technique such amiRNA that is based on the degradation of endogenous mRNA transcripts. It certainly confounds the use of qRT-PCR to measure expression differences of these genes in transgenic versus wild-type plants. Therefore, the low level of expression of CARHR089500, CARHR213460 and CARHR265370 genes may have hampered my ability to generate amiRNAs that could efficiently silence these genes.

Another feature of two of the three PME/I genes targeted by amir-2a/amir-3c, is that they are tandemly duplicated genes in *C. hirsuta*. CARHR265370 and CARHR265360 are adjacent genes that evolved by tandem duplication, as are CARHR213460 and CARHR213450 (Fig. 5, chapter 1, Gan *et al.,* 2016). This feature restricted my choice of available sequences for qRT-PCR primer design as I tried to ensure that primer pairs were specific for a single gene. Coupled with the low expression of these genes, sub-optimal primers may have further hampered my ability to analyze gene silencing in *35S::amir-2a* and *35S::amir-3c* lines.

A distinguishing feature of the *35S::amir-2a* and *35S::amir-3c* lines that I generated, was that the majority of T1 lines could not be recovered in the T2 generation. This was not the case for *35S::amir-4a*, *35S::amir-3a* or *35S::amir-PMEI6* lines. All of these transgenes were constructed in the same vector backbone with a hygromycin selectable marker gene so it is unlikely that I had technical difficulties with plant selection. However, it is formally possible that I mis-scored the hygromycin resistance in many *35S::amir-2a* and *35S::amir-3c* T1 lines. However, it is also possible that these lines showed higher rates of transgene silencing than the other more efficient amiRNA constructs.

5.5- Future perspectives

The role of cell wall-remodelling genes in plant development and differentiation is currently a topic of active research and international interest. In this project, I followed up a recent finding that the species-specific expression of cell wall-remodeling genes may be associated with explosive seed dispersal in *C. hirsuta* (Gan *et al.,* 2016). I validated the results of this previous study and discovered that PME/I genes that are specific to *C. hirsuta*, and not found in *A. thaliana*, are specifically expressed during seed development. Moreover, I showed that three of these unique genes function as PME inhibitors and are required for wild-type levels of PME activity in *C. hirsuta* seeds. These findings, together with the genetic tools that I have generated, pave the way for future studies on the role of cell wall-remodeling genes in explosive seed dispersal in *C. hirsuta.*

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9-List of Abbreviations

ALC	ALCATRAZ
A. thaliana	*Arabidopsis thaliana*
amir	Artificial microRNA
bHLH	basic helix-loop-helix
B. napus	*Brassica napus*
B. rapa	*Brassica rapa*
B. juncea	*Brassica juncea*
B. campestris	*Brassica campestris*
C. hirsuta	*Cardamine hirsuta*
Col-0	Columbia-0
C. rubella	*Capsella rubella*
cDNA	Complementary DNA
DEG	Differentially expressed gene
enb	endocarp b
FUL	FRUITFULL
gDNA	Genomic DNA
GO	Gene ontology
HGA	Homogalacturonan

IND	INDEHISCENT
LD	Long day
miRNA	MicroRNA
MS	Murishuge Skoog
ox	Oxford
PCR	polymerase chain reaction
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
PG	polygalacturonases
qRT-PCR	Quantitative real time polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction
RNA	Ribonucleic acid
RNAseq	RNA sequencing
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
RPL	REPLUMLESS
SAM	shoot apical meristem
SEM	scanning electron microscopy
SHP1	SHATTERPROOF1

SHP2	SHATTERPROOF2
T_1	first transgenic generation
T_2	second transgenic generation
T_3	Third transgenic generation
T-DNA	Transferred DNA
WT	wild type
WMD	Web microRNA designer
CaMV *35S*	Cauliflower Mosaic Virus *35S* promoter
XGA	xylogalacturonan

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what not". I am deeply thankful to my mother who has support me step by step, in my whole life, with her love. I would never forget her tears, when she saw me in difficulties and she was always being worried that why she could not do anything just giving her emotional support and giving me sense of hope, which that was everything for me. Finally, I must express my very profound gratitude to my brother and sister and her daughter for supporting me spiritually throughout my life in general. My deepest thanks to them that they are always there for me. Without the support of my parents and my siblings, I could not reach this stage of my life. They always give their love to me specially when I fell down many times and I tried hard to stand up again and continue my life. Their love and their belief on me, was the only reason that I try to being brave, not to giving up, and standing on my feet again and again. This accomplishment would not have been possible without them.

I found this opportunity to express my feelings and to break my silence with my pen, because it was taught to Iranian women "Be quite!! Hush!! Girls don't scream; girls even shouldn't laugh laud". Living alone in a foreign country as a single Iranian girl, continuing my education despite many difficulties, reaching my goals and writing my PhD thesis during the hard time of my life, are also a start to the end of my silence. I am also thankful for you to take time and read this part of my thesis.

Farnaz Faezi
MPIPZ/University of Cologne
Cologne, January 2018

11-Erklärung

"Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen − , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von ***Prof. Dr. George Coupland*** betreut worden."

Köln, den 5 Januar 2018 Farnaz Faezi

12-Teilpublikationen

- Nikkhoo, M., Sayyahzadeh H., Rahimi-Mianji, Gh., Nikkhoo, M., **Faezi, F.**, and Khamesian, M. (2011). "Measurement of genetic parameters within and between breeder flocks of Arian broiler lines using randomly amplified polymorphic DNA (RAPD) markers". African Journal of Biotechnology Vol. *10 (36)*, pp. 6830-6837, 18 July, 2011 Available online at http://www.academicjournals.org/AJB ISSN 1684–5315 © 2011 Academic Journals

Weitere Pubklikationen

- **Faezi, F**., Rhimmian, H. "*Three improved method for isolating RNA from lime* (*citrus aurantifolia*) inoculated with *Citrus psorosis virus* (CPV)". The 19th Iranian Plant Protection Congress, 31 July-3August 2010.Proceeding of Plant Disease, 716.
- **Faezi, F**., Rhimmian, H. "Detection of *citrus psorosis virus* by RT-PCR". The 19th Iranian Plant Protection Congress, 31 July-3 August 2010. Proceeding of Plant Disease, 717.
- **Faezi, F**., Rhimmian, H., Ranjbar, Gh., Kazemitabar, S. "Reaction of *Citrus aurantifolia* to *Citrus Psorosis Virus* (CPV)". The 5th International Iran & Russia agriculture congress 8-9 October 2009, Saint Petersburg, Russia, 173.
- **Faezi, F.** "The estimation of growth regulator on tissue culture of *Sesamum indicum". The* 1st National Conference of Student Biology & Modern World. Gorgan University of Agricultural Sciences & Natural Resources 16-17 October 2008, 84.
- **Faezi, F**. Dezham, M. "The effect of growth regulator on tissue culture of *Sesamum indicum".* The 3d Agricultural & Natural Resources congress of Young researches club. I.A.U.FASA Branch. July 29-30, 2004.
- **Faezi, F**. "*Measurement of surface tension in the same density of liquids*". Congress of going toward The Nobel Prize in Poland, 2001.

13-Lebenslauf

CURRICULUM VITAE

Family Name: **FAEZI**

Name: **FARNAZ**

Address: Max-Planck-Institute für Züchtungsforschung
Carl Von Linne weg 10, 50829, Köln, Germany

Telephone: +49 221 5062-140/153, +491772600743

Email: Farnaz@mpipz.mpg.de
mailto:farnaz.faezi.biotech@gmail.com

Place of birth: Shiraz (Iran)

Date of birth: Sep, 19th, 1983

Marital status: Single

Nationality: Iranian (Persian)

Educational Background:

Ph.D. Student

University of Cologne, Faculty of Mathematics and Natural sciences,
Department of Comparative development and Genetics, (Director: Miltos Tsiantis),
Genetic basis of phenotypic evaluation, (Group leader: Angela Hay),
MPIPZ- Max Planck Institute for Plant Breeding researches, Köln, Germany.

Date of working on PhD project: 2014 June-2018 March

Dissertation: "*The role of pectin methylesterase activity in explosive seed dispersal in Cardamine hirsuta"*

Supervisors: Pro. Dr. George Coupland, Dr. Angela Hay

Master Degree

The Graduate Center: Department of Plant Biotechnology Mazandaran University, Sari, Iran.

Date of Graduation: Sep, 1st.2007- Feb, 3d, 2010

Dissertation: "*Detection of citrus psorosis virus by RT-PCR in citrus*"

Supervisors: Pro. H. Rahimian and Dr. GH. Ranjbar

Advisor: Dr. S. Kamal Kazemitabar

Bachelor Degree

The Undergraduate Center: Islamic Azad University, Fasa Branch, Iran.

Degree, Major: B.Sc Degree in Agronomy & Plant Breeding

Dissertation*:* Plant tissue culture,

Date of Graduation: Sep, 1St, 2001- 21St July, 2006

Work Experience:

- BAC recombineering, MPIPZ, (October 2013-May 2014).
- University instructor at Islamic Azad University, Kazeroon, Iran, (2010-2012).
- Practicum in Crops Greenhouse cultivation in Technical and Vocational Training Organization, (2012).
- Practicum in Research Center for Agriculture and Natural Resources of Fars Province, Zarghan, Iran, (2006).
- Working on my professors researches in the university while I was undergraduate student.

Achievements / Awards:

- DAAD (Deutscher Akademischer Austauschdienst) scholarship award (April 2013 - Sep. 2017).
- The second rank master's student in the field of Plant Biotechnology (2010).
- Graduated with distinction *(Top 5)*, bachelor's student in the field of Plant Breeding & Agronomy (2005).
- Best paper awards of the 3rd Agricultural & Natural Resources congress of Young researchers, I.A.U.FASA Branch. July 29-30, 2004.
- Achieved the honour rank in the 3 rd high school congress of Physic of Fars province, Iran (2000).

- Top acceptable paper in the 9th international competition of `The First Step to Nobel Prize in Physics" in the Polish Academy of Sciences (2000-2001).

Professional Memberships:

- Environmental health organization of Fars (from Jan 2001)
- Young Researchers club (from Jan 2005)
- Iranian Biotechnology Society of Iran (from April 2007)
- Biosaifty Society of Iran (from April 2007)
- Agricultural & Natural Resources Engineering organization (from May 2010)

Languages:

- Persian (Mother Language)
- English (Fluent)
  1. IELTS certificate from International English language testing system , University of Cambridge ESOL Examination (2012).
  2. IELTS certificate from Navid Pars Educational Group, Shiraz, Iran (2011).
  3. IELTS certificate from English language from BAHAR-Language-Institute (B.L.I), Shiraz, Iran (2010).
- German (Intermediate)
  1. Six month (624 Trainingseinheiten) intensive Deutch language course at Carl Duisberg Centrum, Köln, Germany (April 2013- Sep. 2013).
  2. Deutch course certificate from University of Cologne, Germany (Oct. 2014-Feb.2015).
  3. Deutch Schnellkurs Amt für Weiterbildung-Volkshochschule, Köln, Germany (Jan.2017-April 2017).