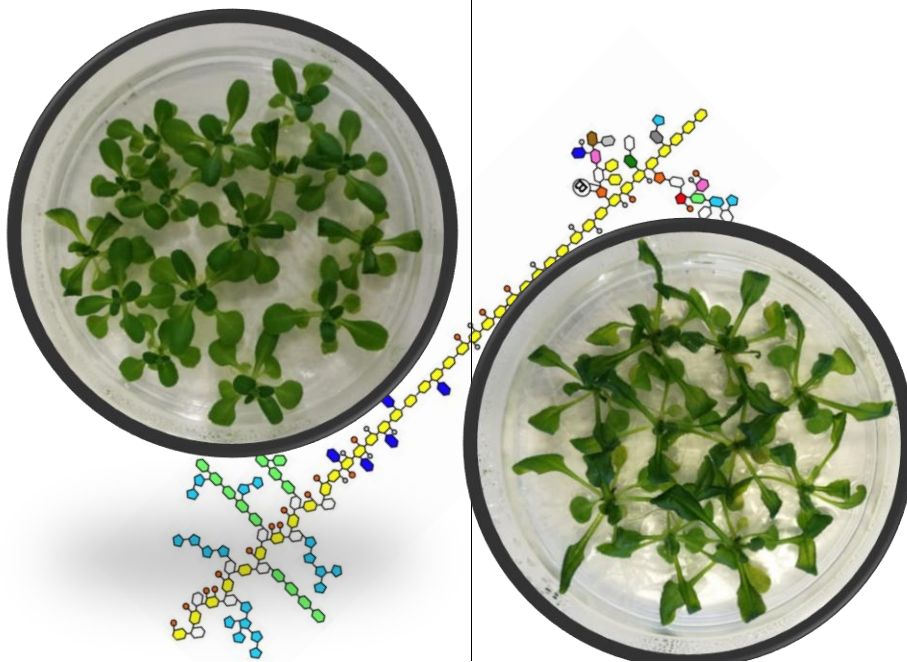


# Effect of Calcium ( $\text{Ca}^{2+}$ ) on Pectin and Pectin Methyl Esterases (PME) in Normal and Hyperhydric Leaves of *Arabidopsis* Cultured *in Vitro*



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## Abstract

Tissue culture is a powerful and indispensable tool and has been used for vegetative propagation, plant breeding, and protecting plant material from pathogens in agriculture and horticulture. However, the culture might lead to water accumulation in the plant apoplast, which is named hyperhydricity (HH). HH is a physiological disorder in tissue culture, and has serious effects on commercial micropropagation by reducing the quality and multiplication rate. HH plants are not able to adjust water balance and accumulation in cells and accumulate water in the apoplast. *Arabidopsis thaliana* leaves contain cellulose, hemicelluloses and pectins, which are three major types of polysaccharides of the cell wall. Pectins have an impact on the plant tissue integrity and rigidity, so they have an important role on the growth and development of plants. Functions of pectin are manifold, such as ion transport, control of wall porosity and hydration. Pectins are the major polysaccharide of the cell walls, approximately 50% in *Arabidopsis* leaves, rich in galacturonic acid (GalA) and homogalacturonan (HG) is one of the main elements of polysaccharides. Homogalacturonans (HGs) the most abundant pectic polymer in plant cell walls and they are highly methyl-esterified within the cell walls. Pectin methyl-esterases (PMEs) catalyze the removal of methyl-groups from the HG backbone. PME activity of the cell wall is controlled by the degree of methyl-esterification of pectins and the action of PMEs in the cell wall might affect the properties of the cell wall and there are connections between pectin domains by crosslinking each other through Calcium ( $\text{Ca}^{2+}$ ) bonds. Pectin Methyl-esterases (PMEs) modify these connections by regulating the crosslinks of pectin to  $\text{Ca}^{2+}$  ions. Calcium is a crucial plant nutrient that is necessary for strengthening and maintaining rigidity of the cell wall structure, which might affect HH, membrane function and signaling responses. In addition, the mechanical properties of pectin might be changed by cross-linking with  $\text{Ca}^{2+}$  ions. Calcium binds to PMEs during the process of plant cell wall formation. In this thesis, We investigated the effect of calcium on pectins and PMEs in normal and HH *Arabidopsis* seedlings of two different genotypes (Col-0 and less-pectin *gae 6-1* mutant) cultured *in vitro*. We measured total pectin and PME activity with optimised protocols for both measurement. We found that by adding 5.98 mM  $\text{Ca}^{2+}$  to the 0.4% Gr (Gr) medium reduced the symptoms of HH for both *gae 6-1* and Col-0 with parallel increase of both GalA and PME activity when compared to 0.4% Gr alone. Our results suggest that exogenous applied  $\text{Ca}^{2+}$  have an effect on pectin levels and PMEs that may help to reduce HH.

Key words; Hyperhydricity, Pectins, PMEs, Calcium, *in vitro*, *Arabidopsis thaliana*.

## Introduction

### Hyperhydricity

Recently, tissue culture has been used for vegetative propagation, plant breeding, and protecting plant material from pathogens in agriculture and horticulture (van den Dries, Gianni, Czerednik, Krens, & de Klerk, 2013). This powerful and indispensable tool is performed on a plate or dishes containing media with agar, macro- and micro- nutrients, vitamins, carbon sources, phytohormones, and water. However, the culture might lead to water accumulation in the plant apoplast, which is named hyperhydricity (HH) due to extreme and unnatural conditions (Debergh et al., 1992; Debergh et al., 1981; Rojas et al., 2010; van den Dries et al., 2013).

HH is a physiological disorder in tissue culture, and has serious effects on commercial micropropagation by reducing the quality and multiplication rate. The first report about HH was published in 1977 on *Prunus* leaves and described as a succulent appearance with lack of development (Quoirin and Lepoivre, 1977). Hyperhydric tissues have a high water content that replaces the air in the apoplast (Gribble et al., 1998; van den Dries et al., 2013) and leads to reduced cellulose and lignin content (George., 1996) with swelled cells (Canny and Huang, 2006). Common symptoms of HH shoots are thick, brittle, curled, and translucent leaves (Gaspar et al., 1995; Saher et al., 2005). In addition, poorly developed epicuticular wax layer, a reduced number of palisade cells, and large intercellular spaces in the mesophyll (Olmos and Hellin, 1998; Picolli et al., 2001; Jausoro et al., 2010), less lignin content (Kevers et al., 1987), stomatal malfunction (Apostolo and Llorente, 2000) on wide variety of plant species (van den Dries et al., 2013) can be observed. Moreover, anatomical, morphological, molecular, and biochemical studies have been conducted related to the symptoms, but still the mechanism remains unclear (de Klerk et al., 2017).

Gelling agent (Debergh et al. 1981), relative humidity (RH)(Hakkaart and Versluijs., 1983), type of explants, and the medium composition have effect on development of HH (de Klerk et al., 2017). In addition, cytokinins (Kadota and Niimi, 2003) and ethylene accumulation (Park et al., 2004) were reported as another factors for inducing HH in plants.

Gelrite (Gr) is, a type of substitute gelling agent of agar, used indispensably with some advantages such as clearer appearance and consistent product quality (Quiala et al., 2014), whereas it might cause an increase in HH in tissue culture conditions (Franck et al., 2004).

HH plants are not able to adjust water balance and accumulation in cells (Rojas-Martinez et al., 2010) and accumulate water in the apoplast (Gribble et al. 1996, 1998) which is

defined as the cell wall continuum and the intercellular spaces in a plant (Evert, 2006). Therefore, disrupted gas exchange within plant tissues might cause physiological disorders (Gribble et al., 2003) due to slow gas diffusion in water (Jackson, 1985). Some articles reported that non-HH plants have water filling 15 % of the apoplast, but HH plants have water filling up almost 85 % of the apoplast (van den Dries et al., 2012) and HH Arabidopsis seedlings contained an even higher water accumulation in the apoplast compare to non-HH seedlings (Van den Dries et al. 2013) . Finally, it was suggested that transpiration plays an important role in removing apoplastic water (de Klerk et al., 2017), in this way avoiding HH or restoring the normal phenotype.

## **Role of Pectin, Calcium and PMEs on Plants**

### **Pectins**

Pectins as polysaccharides present in the primary cell walls and in the middle lamella of higher plants are found in non-grass monocotyledonous and dicotyledonous plants and are synthesised in the Golgi vesicles (Mohnen, 2008; Xiao and Anderson, 2013). In addition, they comprise around one-third of the dry weight of dicotyledonous cell walls (Pérez et al., 2000).

Pectins have an impact on the plant tissue integrity and rigidity, so they have important role on the growth and development of plants (Sila et al., 2009; Voragen et al., 2009). Functions of pectin are manifold, such as ion transport, control of wall porosity and hydration (Willats et al., 2001), adjusting wall extensibility by influencing the alignment of Cellulose Microfibrils (CMFs) (Majda and Robert, 2018). All these functions depend on pectin structure and concentration in cell walls (Gawkowska al., 2018).

Pectins represent a highly heterogenous group of polymers consisting of homogalacturonan and rhamnogalacturonans I and II. Both conts control cell wall porosity and ionic status, traits which are implicated in the development of intercellular spaces (ICs).

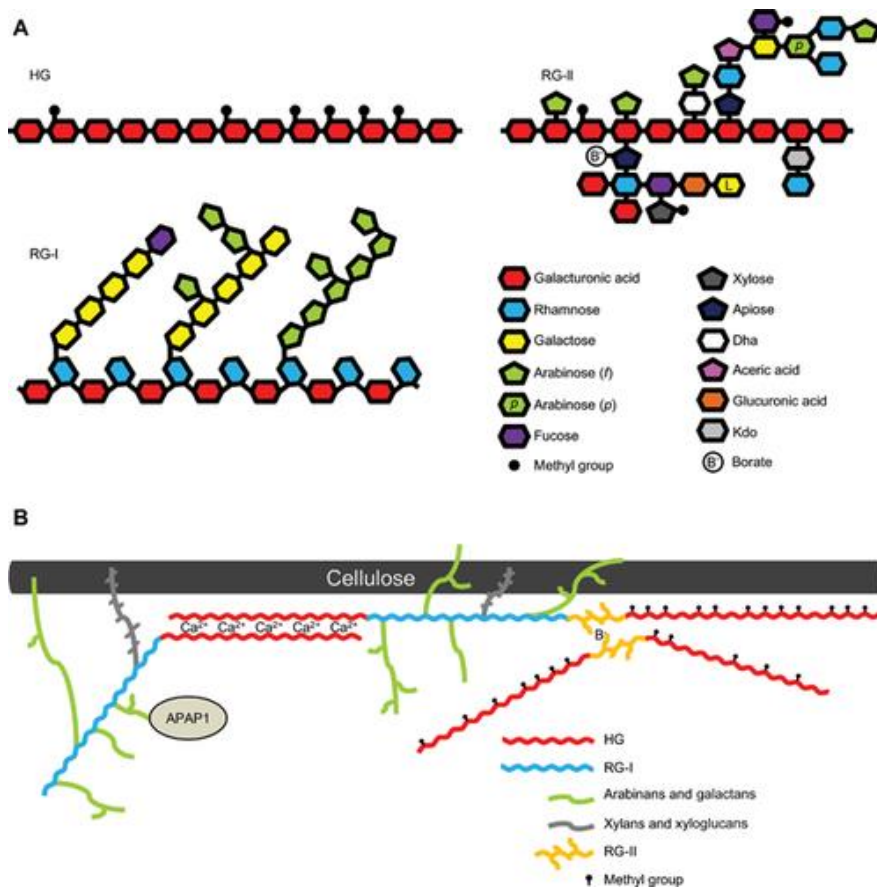
Pectins are distinguished by four main elements making them highly heterogeneous polysaccharides: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II) and xylogalacturonan (XGA) (Majda and Robert, 2018).

Majda and Robert, 2018, mentioned that HG, which is a linear polymer of  $\alpha$ -1,4-linked-D-galacturonic acid (Wolf et al. 2009), frequently has highly methylesterified galacturonic acid residues (Majda & Robert, 2018). Galacturonic acid (GalA) is found in all pectins (Wolf et al. 2009) as the backbone and many pectins with a galacturonic acid backbone may have acquired different sidechains such as xylose residues and such a xylogalacturonan (XGA) can be located in small amounts in cell walls (Zandleven et al., 2007).

RG-I is a complex pectin with a backbone consisting of repeating (1,2)- $\alpha$ -L-rhamnose-(1,4)- $\alpha$ -Dgalacturonic acid disaccharide units. Rhamnose residues in RG-1 might be substituted with galactans, arabinans, arabinogalactans (Atmodjo et al. 2013), and fucose (Nakamura et al. 2002; Anderson et al. 2012) (Figure 1), while the structure of RG-II is well-conserved and highly complex with a HG backbone having more than 12 distinct monosaccharides present across up to six sidechains (Figure 1; Saffer, 2018). The presence of borate esters in between RG-II-specific sugar residues is a common characteristic of RG-II (Majda and Robert, 2018).

**Figure 1.** Pectins and their organization in the cell wall, retrieved with the legend from Saffer, 2018.

(A) Cartoons of pectin structures. HG is a polymer of galacturonic acid that can be modified by methylesterification. RG-I has a rhamnose and galacturonic acid backbone, and various sidechains rich in arabinose and galactose. RG-II is a complex substituted HG with six sidechains that include at least 12 different monosaccharides, and forms a borate (B<sup>-</sup>)-mediated dimer. Arabinose in the cell wall can be in either the furanose (f) or pyranose (p) form. Most galactose in the cell wall is D-galactose, but RG-II also contains a single L-galactose residue. (B) An illustration of the interactions and organization of pectins in the cell wall. Pectin domains are likely covalently-linked through their backbones. Borate-mediated dimerization of RG-II and calcium-mediated interactions between de-methylesterified HG molecules further crosslink pectins. RG-I has arabinan and galactan and possibly some xylan and xyloglucan sidechains that can interact with cellulose, and pectin backbones can also be in close association with cellulose. Many RG-I molecules might be connected to the arabinogalactan protein APAP1. Molecules are not drawn to scale or presented in proportion to their actual abundance in the cell wall.



## Calcium, Pectins and Pectin Methylesterases (PMEs)

Calcium is a crucial plant nutrient that is necessary for strengthening and maintaining rigidity of the cell wall structure, which might affect HH, membrane function and signalling responses. In addition, the mechanical properties of pectin might be changed by cross-linking with Ca<sup>2+</sup> ions (Bidhendi and Geitmann, 2016). Calcium binds to PME during the process of plant cell wall formation (Burstrom, 1968; White and Broadley, 2003; Hepler,

2005). Moreover, yield and quality reduction are important negative economic effects in many plant species associated with calcium deficiency (White and Broadley., 2003).

The activity of cell wall PME is controlled by the degree of methyl-esterification of pectins. It is known that homogalacturonans are highly methyl-esterified when present within the cell walls and later de-esterified by the action of PMEs in the cell wall which can affect the properties of the cell wall. It is reported that there are connections between pectin domains by crosslinking each other through calcium and boron bonds. Pectin Methyl-esterases (PMEs) modify these connections by regulating the crosslinks of pectin to  $\text{Ca}^{2+}$  ions. There are two possible accepted hypotheses reported on the mechanism of PME action: Acting either randomly or linearly on the pectin chains. Moreover, HG pectins are highly methylated (Micheli, 2001; Wolf et al., 2009). And those are de-esterified by PMEs in the cell wall. De-methylation of pectin might occur linearly or randomly manner. When the PME acts linearly (blockwise de-esterification) on GalA residues, methoxyl group removal, resulting in a continuous region of de-esterified pectin. In addition, linearly demethylation of GalA residues (more than nine) allows strands of pectin to be linked more efficiently via  $\text{Ca}^{2+}$  bonds, leading to gelation and enhanced stiffness (Willats et al., 2001a; Wolf and Greiner, 2012; Bidhendi and Geitmann, 2016). When the PME acts randomly (non-blockwise de-esterification),  $\text{Ca}^{2+}$  bridging is less effective on a pectic domain or even may not bind together by  $\text{Ca}^{2+}$  bridges. Therefore, this might result in cell wall softening (Willats et al., 2001b; Wakabayashi et al., 2003; Arancibia and Motsenbocker, 2006).

### **Pectin and PMEs on HH**

Only a limited number of articles have been published about the effect of pectins on HH e.g. in carnations (Saher et al., 2005) and apples (Marga et al., 1995). Pectin content in hyperhydric carnation leaves was found to be lower than in normal leaves while PME activity was high which correlated with pectin content modifications (Saher et al., 2005).

There are few reports on the effect of calcium on HH, one of them was a study on regenerated shoots of *Lavandula angustifolia* Mill. Based on this paper, when the  $\text{Ca}^{2+}$  content of the culture medium was increased to 1320 mg/L, HH was decreased (Machado et al., 2014). Calcium is important for the cell wall as an integral component and for maintaining membrane integrity (Burstrom, 1968; Marschner 1995; Machado et al. 2010; Singha et al., 1990; Bairu et al., 2009; George et al., 2008).

### **Pectin and Arabidopsis thaliana**

Arabidopsis leaves consist of cellulose, hemicellulose and pectins, and these are the major type of polysaccharides. Pectin content in Arabidopsis leaves was reported as approximately 50% of the leaf cell walls (Zabackis et al., 1995; Harholt et al., 2010), and that amount consisted of galacturonic acid (GalA), arabinose (Ara), rhamnose (Rha), xylose (Xyl), Galactose (Gal), and Fucose (Fuc) (Bethke et al., 2016). In addition, around 65% of all pectins in Arabidopsis leaf consisted of Homogalacturonan (HG), which is a linear homopolymer of (1,4)- $\alpha$ -linked GalA residues (Zabackis et al., 1995; Mohnen, 2008).

Nucleotide sugars are necessary for carbohydrate biosynthesis (Seifert, 2004) and most of them are synthesized from UDP-glucose, e.g. UDP-glucuronic acid (UDP-GlcA). From this precursor, UDP-D-glucuronic acid the key building block of pectins, UDP-D-galacturonic acid, is produced as the monomeric precursor of pectin, through the action of the enzyme glucuronate 4-epimerases (GAEs) (Bethke et al., 2016).

The Arabidopsis genome has six GAE encoding genes and one of them is GAE6, which is evolutionarily older than other GAE family members (Usadel et al., 2004), and strongly expressed in many plant tissues (Bethke et al., 2016). Therefore, we used a less-pectin-mutant, which has a mutation in GAE6 gene as described in Bethke et al. (2016).

*Arabidopsis thaliana* is a plant sensitive to HH. It is known as a model plant, growing fast and already many mutants are available mutant. Because of this,, it is a good choice to study the underlying mechanism of HH. Earlier in the lab of WUR Plant Breeding it was found that applying exogenous calcium to the media could help to overcome HH. In this study, we investigated the effect of adding calcium on pectin content of the seedlings under HH-inducing conditions and studied the PME activity.

## Research Problem and Objectives

Plants cultured in vitro conditions struggle with an accumulation of water in the leaves so apoplastic air in these leaves reduces, this situation result in Hyperhydricity (HH), which is a physiological and morphological disorder in *in vitro* conditions which result in loss of the plant's ability to grow normally (Hazarika et al., 2008). The mechanisms involved in its development are poorly understood. Pectins and PMEs have significant effect on the cell wall compaction and stiffening and adhesion due to interaction with  $\text{Ca}^{2+}$  ions as mentioned in the introduction part of this study. Therefore, many researchers studied on the effect of these compounds (e.g. calcium ions) to prevent this phenomenon (Singha et al., 1990;

Bairu et al., 2009; Machado et al., 2014) In this thesis, the effect of exogenous calcium on pectins and PMEs, and their effect on HH were investigated.

Pectin level of Arabidopsis seedlings grown in in vitro might be varied due to  $\text{Ca}^{2+}$  level in the medium. In addition, plant enzymes have effect on pectin content in plants. For example, PMEs may crosslink with  $\text{Ca}^{2+}$ . Therefore, the aim of the thesis work is to investigate the effect of calcium on pectins and PMEs in normal and HH Arabidopsis seedlings of two different genotypes cultured in vitro. In this thesis, it is considered that exogenous applied  $\text{Ca}^{2+}$  might have effect on pectin levels and PMEs that may help to reduce HH.

## Research Questions

### Research Question 1 (about calcium ions);

What is the effect of adding different concentrations of calcium on the development of HH in in vitro grown *gae 6-1* mutant and *col-0* Arabidopsis seedlings?

#### Subquestions:

- Is there any difference between the *gae 6-1* mutant and *Col-0* seedlings in HH?
- Which calcium concentration is the best to alleviate HH for Arabidopsis?

### Research Question 2 (about pectin);

Does calcium have any different effect on total pectin content in Arabidopsis seedlings (*Col-0* and *gae 6-1*) under HH inducing growth conditions?

#### Subquestions:

- What is the GalA level in calcium treated Arabidopsis seedlings (*Col-0* and *gae 6-1*)?
- What is the level of monosaccharides in *gae 6-1* mutant and *Col-0* in calcium treated seedlings under HH inducing growth conditions?
- Is the total pectin level of HH seedlings less when the PME activity is high?
- Is there any difference between the calcium concentrations given in the media and the amount of total pectin?

### Research Question 3 (about PME);

What is the PME activity of calcium treated Arabidopsis seedlings (Col-0 and *gae 6-1*) under HH inducing growth conditions?

#### Sub Questions:

- What is the relation between PME activity and HH?
- Is there any effect of the calcium concentration on PME activity?
- Is there any relation between pectin and PME activity?
- Is the total pectin level of HH seedlings less when the PME activity is high?

### Research Question 4 (about the gene);

Is there any difference in the expression level of GAE in the *gae 6-1* (less-pectin) mutant when the seedlings grown in in vitro condition on different media?

#### Sub Questions:

- What is the expression of the gene in seedlings grown on agar or on Gr?
- Is there any difference at the expression level when  $\text{Ca}^{2+}$  (5.98 mM) is applied to the medium?

## Materials and methods

### Plant material and tissue culture conditions

Two different *Arabidopsis thaliana* lines, the wild type (Col-0) and the *Arabidopsis thaliana* less-pectin-mutant (*gae 6-1*), were used in the experiments. Col-0 and the mutants were germinated in vitro and subsequently transferred to and grown in the greenhouse; then seeds were harvested since there were not enough mutant seeds available at first to use in the different treatments. Seeds were sterilized with 70% (v/v) ethanol for one minute and 2% (w/v) sodium hypochlorite for 15 minutes. To rinse them, distilled water was used subsequently three times for 10 minutes. Half-strength MS basal salt mixture with vitamins (Murashige and Skoog, 1962) was prepared with 1.5% (w/v) sucrose supplemented and solidified with 0.7% (w/v) Microagar (all from Duchefa Biochemie, Haarlem, The Netherlands) in high-sided Petri dishes. Seed stratification was in the dark for 3 days at 4 °C and germination in a growth chamber with 16 h light/8 h dark (30  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , Philips TL33) at 21°C to all seeds. 7-days-old pectin mutant (*gae6-1*) and wild type (Col-0) *Arabidopsis thaliana* seedlings were transferred and cultured in high-sided Petri dishes with 10 seedlings per dish solidified with 0.2% (w/v), 0.4% (w/v) Gr (Duchefa Biochemie) or with 0.7% (w/v) Micro-agar. To 0.4% (w/v) Gr 2.99 mg/L  $\text{Ca}^{2+}$ , 5.98 mg/L  $\text{Ca}^{2+}$  or 8.97 mg/L  $\text{Ca}^{2+}$  were added. Each treatment was replicated three times; all dishes were sealed with parafilm.

Plants were harvested 14 days after transfer to the treatment media. The leaf samples were weighed, 50 mg was taken for total pectin measurement, 100 mg for PME analysis per treatment.

### Total pectins determination

Total pectins of the seedlings was determined based on the protocol as described by Pettolino et al. (2012). Ex vitro grown Col-0 plants were used for optimisation. After this, the optimised protocol was applied on seedlings from the different treatments.

### 1-Preparation of alcohol-insoluble residues (AIR)

Tissue samples in liquid nitrogen were ground using Retsch TissueLyser II (QIAGEN, Chadstone Centre, VIC, Australia) for 1 minute at 30 Hz and then put the tissue samples in liquid nitrogen and ground for another 1 minute at 30 Hz. TissueLyser blocks were pre-frozen by storing then at -80°C for 2 h. Cells were well disrupted in order to get the cell wall preparations as clean as possible. After grinding tissue samples in 2 ml eppendorf tubes, 1.5 ml of 80% (v/v) ethanol was added to tubes and extracted for 30 minute on ice. Right after this, tubes were centrifuged 5 minutes at 10,000g at room temperature and the supernatant was discarded. This was repeated at least three times with changes of ethanol. The pelleted material was subsequently extracted with first 1.5 ml of acetone

and then 1.5 ml of methanol at room temperature, both centrifuged at 5 minutes at 10,000g at room temperature after which the supernatant was discarded. After this, the starch content was checked by iodine/KI staining for presence or absence. Whenever starch was present, an  $\alpha$ -amylase digestion treatment was done on all samples. Before the  $\alpha$ -amylase treatment, samples were at least 2 days air-dried and the weight was recorded.

## **2- Alpha-Amylase Digestion**

Air-dried samples were soaked to find the best washing conditions with different amount (0.2 ml, 0.5 ml, 1 ml and 2 ml) of 10 mM Tris-maleate buffer (pH 6.9) for 30 minutes. The best washing conditions were found to be with 1 ml buffer. Then the starch granules were gelatinized by placing samples in boiling water for at least 5 minutes, later samples were equilibrated to 40 °C and the  $\alpha$ -amylase solution (2 U/mg of carbohydrate) was added to the samples and the mix was incubated for 1 h at 40 °C. This incubation was continued by adding another one-half the amount of  $\alpha$ -amylase added previously and incubating 30 minutes further at 40 °C. After incubation, four volumes of cold absolute ethanol were added to precipitate remaining polysaccharides (e.g. pectins) overnight at – 20 °C. Samples were centrifuged at 1,500g at room temperature for five minutes the following day, and the supernatant was removed; then the peller was washed further three times with adding cold absolute ethanol at room temperature, 5 minutes, 1,500g centrifugation. Last, samples were air-dried for at least two days under in the fume hood.

## **3- Saeman hydrolysis**

63  $\mu$ l of 7.34 M sulfuric acid was added to each air-dried sample in Eppendorf tubes, then kept at 30 °C for one hour and vortexed intermittently. At this point, sample colour was seen as a dark brown to black colour. To dilute the acid to 1 M, 690  $\mu$ l of distilled water was added. After that, samples were placed in a thermomixer (Eppendorf® Thermomixer Compact, 5384000020, USA) at 100 °C for 3 h to further hydrolysis.

## **4- Galacturonic acid and monosaccharide sugar measurement**

After hydrolysis, samples were diluted as 100  $\mu$ l and 50  $\mu$ l samples to 1 ml with MQ. Based on preliminary HPLC results, GalA could not be detected possibly due to a high dilution factor. To obtain GalA in HPLC, later dilutions were done 1 to 1, 100  $\mu$ l of sample plus 100  $\mu$ l of MQ. Then, the GalA was detected with monosaccharide sugars.

## **Optimisation and Determination of PME activity assay**

The PME activity assay was modified for *Arabidopsis thaliana* from Mueller et al. (2013). The principle of the method is that PME activity is determined by oxidation of methanol released from methylesterified pectins to formaldehyde via alcohol oxidase and then NAD<sup>+</sup> is reduced to NADH by formaldehyde dehydrogenase. The formation of NADH is then

followed spectrophotometrically and the estimation of the PME activity is calculated in the protein extract.

### **1- Extraction of PME**

Tissue samples, 100 mg, in liquid nitrogen were ground using Retsch TissueLyser II (QIAGEN, Chadstone Centre, VIC, Australia) for 1 minute at 30 Hz and then put the tissue samples in liquid nitrogen and ground for another 1 minute at 30 Hz.. TissueLyser blocks were pre-frozen by storing them at -80 °C for 2 h. Protein extraction buffer was prepared with 100 mM Tris-HCl (pH 7.5), 500 mM NaCl and 1x protease inhibitor cocktail (Sigma-Aldrich, P9599) and the buffer was added to 2 ml eppendorf tubes as 2x (100 mg tissue with 200 µl buffer), 5x, 10x and 20x of the fresh weight (w/v) and the tissue was allowed to thaw in the buffer. Each volume of buffer was used several times to determine the proper amount of the buffer. After vortexing 10 seconds, extracts were rotated (Snijders, rotary-mixer 34526) in 2 ml eppendorf tubes at 4 °C for 30 minutes and centrifuged at 11,500 x g at 4 °C for 20 minutes. The supernatant was immediately used for the PME activity assay.

### **2- PME activity assay**

To determine the optimal sample amount, 10 µl and 50 µl extracts were tried and the 10 µl sample was found to be the best with 0.4 mM NAD<sup>+</sup> in 100 mM NaOH instead of 100 mM phosphate buffer (pH 7.5). While the NAD<sup>+</sup> (Sigma-Aldrich, N8410) solution was prepared in 100 mM NaOH, 0.1 U/µl Alcohol oxidase (Sigma-Aldrich, A2404), 0.5 U/µl Formaldehyde dehydrogenase (Sigma-Aldrich, F1879), PME from orange peel (Sigma-Aldrich, P5400) were all prepared in phosphate buffer. All solutions were adjusted to pH 7.5 for PME enzyme activity assay. The master mix solution consisted of 20 µl of 0.5 % (w/v) pectin (Sigma-Aldrich, P9135), 2 µl alcohol oxidase solution, 2 µl formaldehyde dehydrogenase solution and 156 µl NAD<sup>+</sup> solution for per microplate well. Protein extraction buffer only was used as the negative control, and commercial PME was used as a positive control, with a solution of commercially available 7.8 mU PME (Sigma-Aldrich, P5400) in protein extraction buffer. Master mix (180 µl) first and then 10 µl pectin solution were added to each well and mixed by pipetting up and down without bubbles. An example of a pipetting scheme can be seen in the table below.

Soluble Protein Extracts	Negative Control	Positive Control	Background
10 $\mu$ l sample, 180 $\mu$ l Master mix, 10 $\mu$ l pectin solution	10 $\mu$ l protein extraction buffer, 180 $\mu$ l Master mix, 10 $\mu$ l pectin solution	10 $\mu$ l commercial PME in protein extraction buffer, 180 $\mu$ l Master mix, 10 $\mu$ l pectin solution	180 $\mu$ l Master mix

A 96-Microwell plate was put immediately into the microplate reader measuring absorption at 340 nm over the duration of 15 minutes at room temperature. The change in absorption per unit time over the linear part of the reaction was calculated for each well, and used to calculate the increase in concentration of NADH. As extinction coefficient  $\epsilon_{340}$  for NADH (6,220 M<sup>-1</sup>cm<sup>-1</sup>) was used to calculate the NADH concentration.

### RNA Isolation

Tissue samples, approximately 100 mg, in liquid nitrogen were ground using Retsch TissueLyser II (QIAGEN, Chadstone Centre, VIC, Australia) for 1 minute at 30 Hz and then put the tissue samples in liquid nitrogen and ground for another 1 minute at 30 Hz. TissueLyser blocks were pre-frozen by storing them at -80 °C for 2 h. mRNA isolation was performed using the plant RNeasy® kit (QIAGEN) based on the manufacturer's instructions. After mRNA isolation, the total mRNA quantification and the quality checking was done using NanoDrop ND-100 spectrophotometer (Isogen).

### cDNA Synthesis

The iScript™ cDNA synthesis kit (Bio-Rad Laboratories, USA) was used for Complementary DNA (cDNA) synthesis from 1  $\mu$ g of the extracted RNA based on manufacturer's manual. Before the following step, qPCR reaction, the cDNA was diluted.

### Gae6 Transcript quantification and gene expression

Primers used for the amplification of the *Gae6* were chosen based on Bethke et al. (2016). (Supplementary data Table 1.). CFX96 Real-Time PCR (Bio-Rad Laboratories, USA) was used for Quantitative PCR by using SYBR green (QIAGEN). The manufacturer's instructions was taken into account for PCR cycles.. Gene expression was measured relative to the housekeeping gene *Arabidopsis Actin2*.

### Statistical Analysis

The experiment design was completely randomized and each plot was represented by one petri dish with 10 seedlings. Statistical differences between *gae 6-1* and Col-0

samples were calculated with one-tailed unpaired Fisher's protected least significance test. P-values <0.05 were considered significant. The data are presented as means  $\pm$  SE. The normality of the data was analyzed with the SPSS software (IBM SPSS 24).

## Results

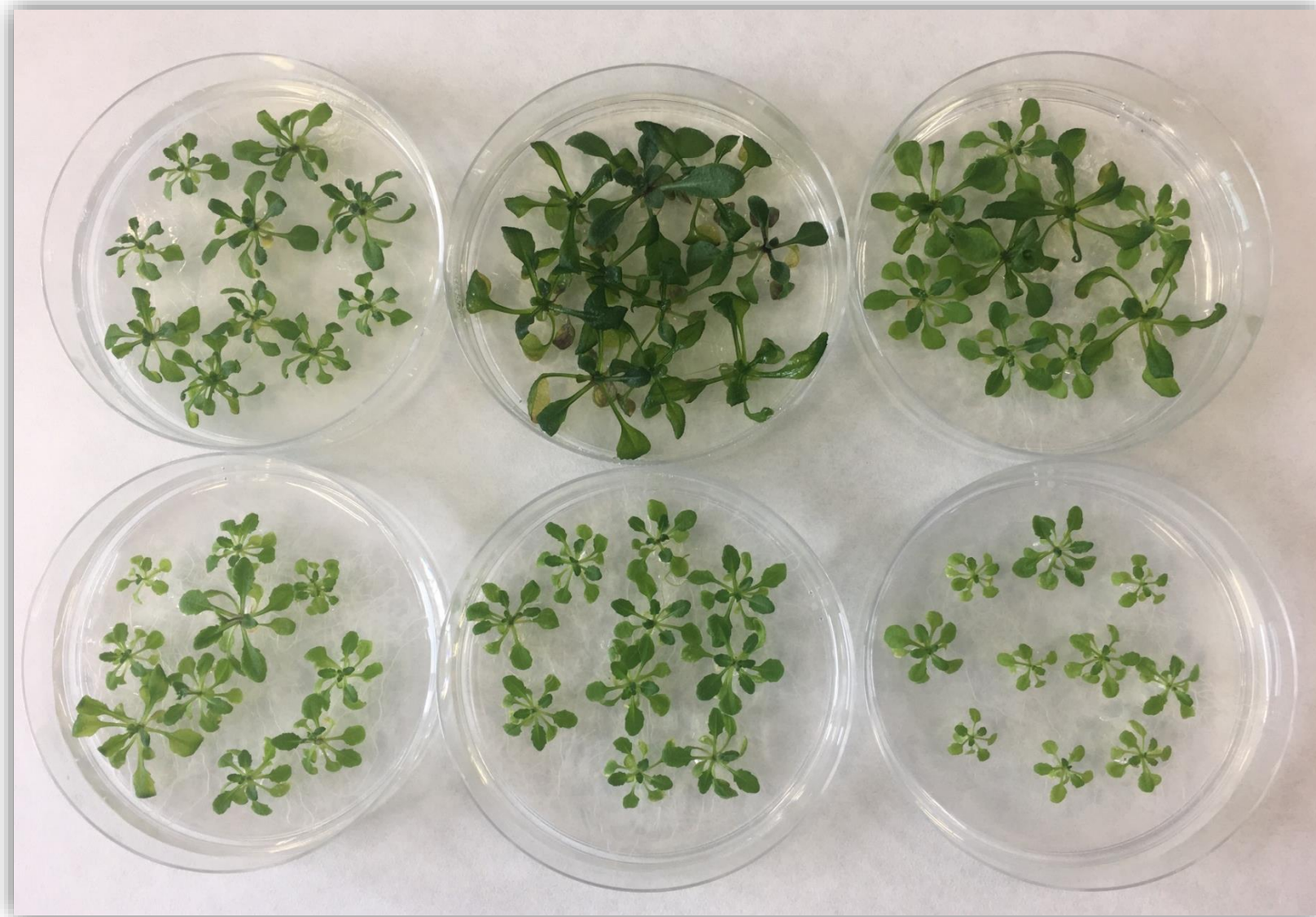
### HH Symptom

When hyperhydricity (HH) occurs, the seedlings have symptoms as translucent, wrinkled and thick leaves, and elongated petioles. Gr was used to induce HH and the Col-0 seedlings grown on 0.2% (w/v) Gr and 0.4% (w/v; this will be left out from here on) Gr showed HH symptoms as expected (Fig. 3).

The *gae 6-1* mutant was chosen as less-pectin-mutant with reduced GalA content. The HH symptoms of this mutant can be seen in Figure 2 and were as expected. The *gae 6-1* (less-pectin-mutant) in Agar showed HH in leaves as opposed to the Col-0 and HH is highly induced on 0.2% Gr and 0.4% Gr. By adding calcium to the 0.4% Gr medium reduced the symptoms of HH for both *gae 6-1* and Col-0 when compared to 0.4% Gr alone.

Based on the phenotypes of the seedlings for both genotypes, the most promising Ca<sup>2+</sup> concentration was found to be 5.98 mM. In addition, it was noticed that some seedlings were without any HH symptoms in the Ca<sup>2+</sup> treatment medium for both *gae 6-1* and Col-0, however they grew in smaller size than their control seedlings (Agar) with shorter petioles, smaller leaves (Fig.2 and 3).

**Figure 2.** Treatments for *gae 6-1* mutant



The picture shows 14-days-old gae 6-1 mutant seedlings after transfers to the treatment dishes. Orders from top-left dishes to right; Agar, 0.2% Gr, 0.4% Gr. Orders from bottom-left dishes to right; 0.4% Gr + 2.99mM Ca<sup>2+</sup>, 0.4%

0.2% Gelrite

Gr + 5.98mM Ca<sup>2+</sup> and

0.4% Gelrite

0.4% Gr + 8.97mM Ca<sup>2+</sup>

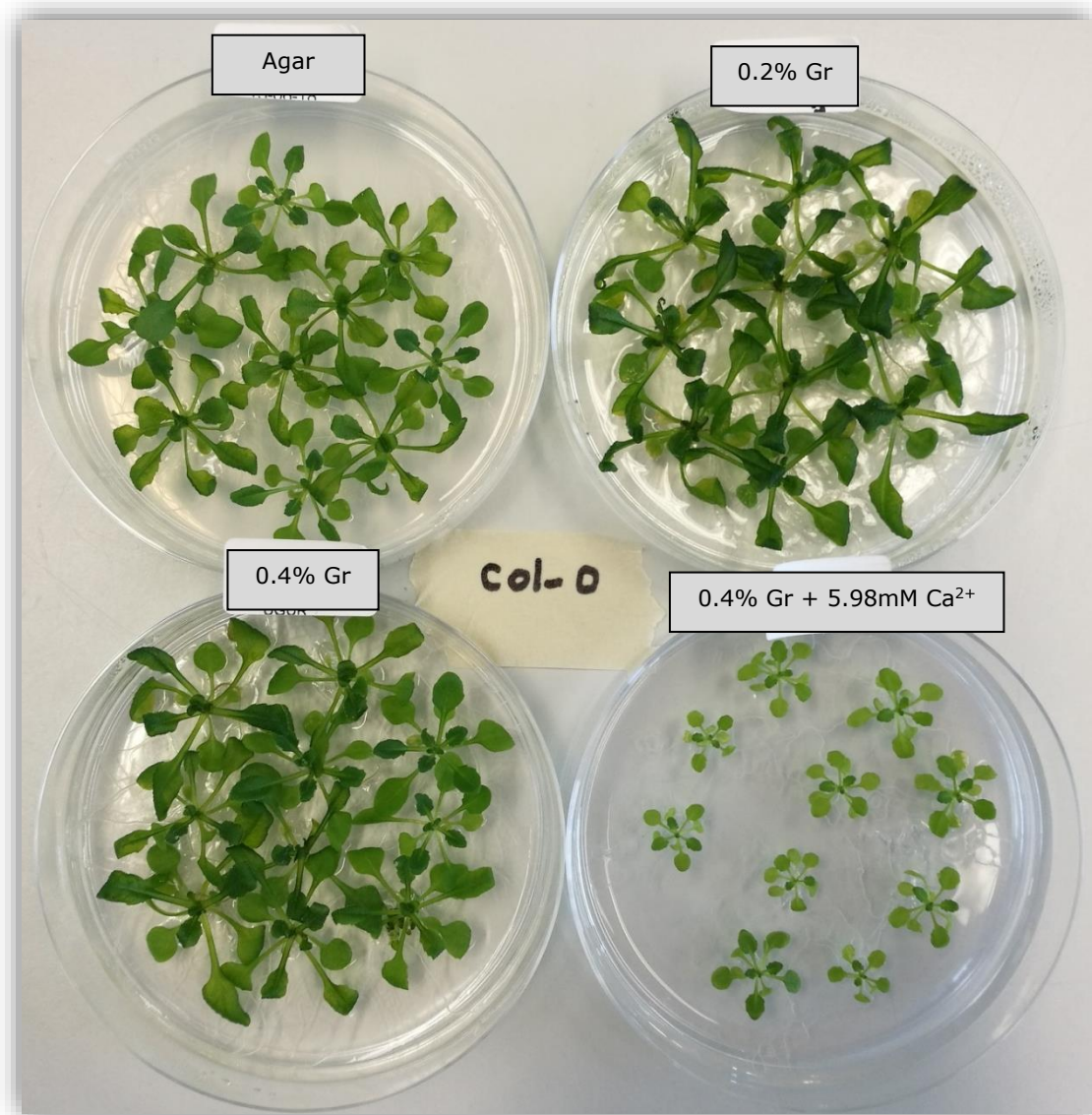
0.4% Gelrite + 2.99mM Ca(NO<sub>3</sub>)<sub>2</sub>

0.4% Gelrite + 5.98mM Ca(NO<sub>3</sub>)<sub>2</sub>

0.4% Gelrite + 8.97mM Ca(NO<sub>3</sub>)<sub>2</sub>

**Figure 3.** Treatments for Col-0

The picture shows 14-days-old Col-0 seedlings after transfers to the treatment dishes. Orders from top-left dishes to right; Agar, 0.2% Gr (Gr). Orders from bottom-left dishes to right; 0.4% Gr (Gelrite) and 0.4% Gr (Gelrite) + 5.98mM  $\text{Ca}(\text{NO}_3)_2$



## GalA levels after Ca<sup>2+</sup> treatments on *gae 6-1* and Col-0

GalA is a major component of pectin. Arabidopsis leaf pectins consist of approximately 65% as homogalacturonan (HG), which is a linear homopolymer of (1,4)- $\alpha$ -linked GalA residues. To approximate pectin content of *gae 6-1* and Col-0 walls, the total GalA concentration was measured using HPLC assay (Pettolino et al. (2012)). The measurement of the GalA for both *gae 6-1* and Col-0 was done as described in the material and method section and the results are given in Figure 4 as mg GalA g<sup>-1</sup> AIR (Alcohol-Insoluble Residues).

Total GalA was reduced in 0.2% and 0.4% Gr seedlings on both *gae 6-1* and Col-0 but was indistinguishable from Col-0 in *gae 6-1* agar and both *gae 6-1* and Col-0 on Ca<sup>2+</sup> treated seedlings. In *gae 6-1* agar media, GalA content in cell wall was reduced by 15% whereas in 0.2% and 0.4% Gr media reduced about 37-38% compared to Col-0. In wild type (Col-0) 0.4% Gr media GalA content was decreased 30% compared to Col-0 in Control agar media. Exogenously applied Ca<sup>2+</sup> on *gae 6-1* and Col-0 seedlings in 0.4% Gr increased the GalA concentration from 22-30% when compare to 0.4% Gr alone. This suggested that *gae 6-1* seedlings have reduced pectin and adding calcium in the media increased pectin concentration (GalA) which demonstrated cross-link between and pectin.

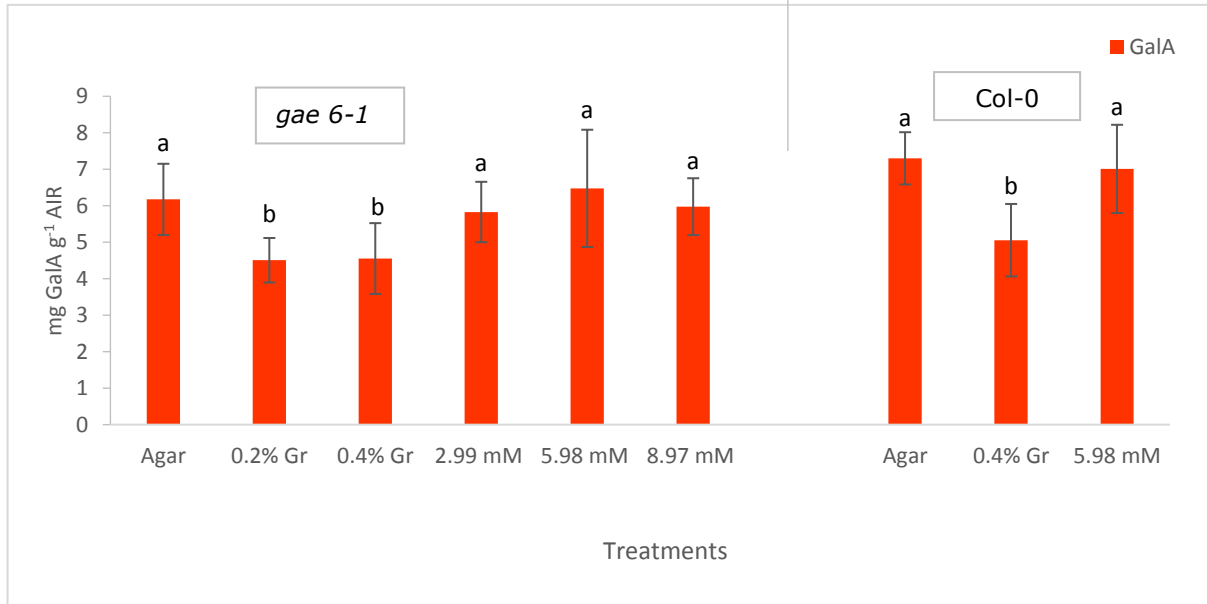
There was a difference in pectin levels as represented by the amount of GalA between Gr and agar with the latter being the highest for both phenotypes. The *gae6-1* mutant was treated with different Ca<sup>2+</sup> concentrations and GalA levels were restored to control level, but no significant differences were observed on the Gr between the different Ca<sup>2+</sup> treatments.

When Ca<sup>2+</sup> concentration increased to 5.98 mM or more, the GalA content in both Col-0 and *gae 6-1* had a significant increase compared to 0.4% Gr medium.

In summary, there were significant differences between 0.4% Gr with calcium treated seedlings on 0.4% Gr on the galacturonic acid level for both mutant and Col-0 seedlings.

**Figure 4.**

The graph shows the effect of different media with different calcium concentration on levels of GalA (mg GalA g<sup>-1</sup> AIR) in cell wall material isolated from the *gae 6-1* (left) and WT (Col-0; right). The different media are 1/2MS15 with 0.7% (w/v) Agar or with 0.2% (w/v) Gelrite (Gr), ; 0.4% GR and 0.4% Gelrite with 2.99 mM, 5.98 mM and 8.97 mM of Calcium Nitrate. Cell wall material (AIR) was prepared and quantified as described in the M&M section. Bars show the average ± SE. GalA, galacturonic acid.



### Sugar levels after Ca<sup>2+</sup> treatments in *gae 6-1* and Col-0

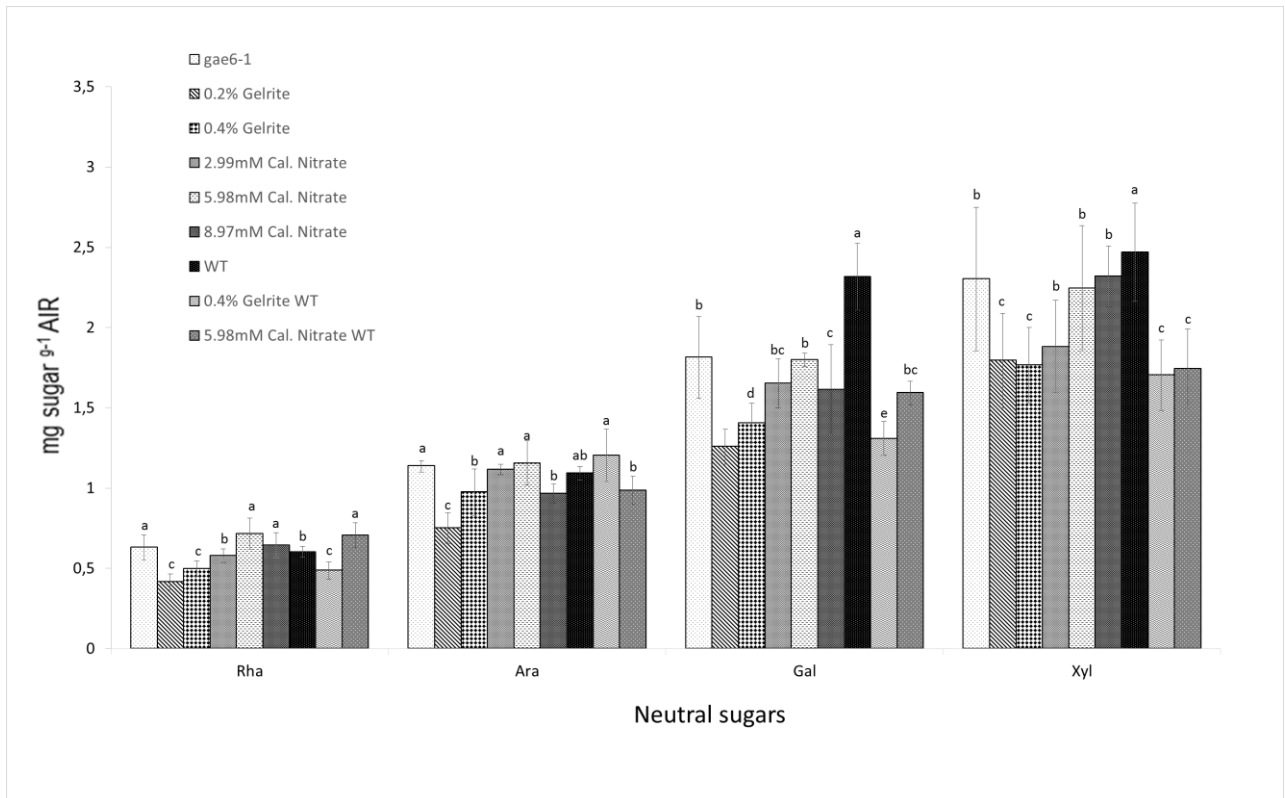
The levels of four neutral monosaccharides were measured as described in the material and method section to investigate in more detail the cell wall composition in leaves of *gae6-1* and Col-0 on HH inducing medium with different Ca<sup>2+</sup>treatments.

Rha = Rhamnose (Rha), Arabinose (Ara), Galactose (Gal) and Xylose (Xyl) contents are shown as pectin fractions of control and the treatment seedlings of *gae 6-1* mutant and Col-0 (Fig. 5).

We found that there was significant difference between Col-0 and *gae 6-1* mutant on Rha, Gal and Xyl levels when compare to both Agar and 0.4% Gr mediums. Only Rha level showed significant increase by adding Ca both Col-0 and *gae 6-1* mutant when compered to 0.4% Gr medium.

**Figure 5.**

The graph shows the levels of neutral sugars as mg sugar g<sup>-1</sup>AIR extracted from leaves of *gae6-1* and Col-0 on different media with different calcium concentrations added. Cell wall material (AIR) was prepared and quantified as described in the M&M section. Col-0 and *gae 6-1* on ½ Ms Agar; 0.2% Gr, 0.2% Gelrite; 0.4% Gr, 0.4% Gelrite; 2.99 mM, 5.98 mM and 8.97 mM of Calcium Nitrate concentrations. Values represent average ± SE. Letters indicate significant differences between treatments (Fisher's LSD, *P* < 0.05). Rha = rhamnose, Ara = arabinose, Gal = galactose, Xyl = xylose.



### PME activity assay

It is thought that the activity of PME in cell walls might affect the degree of esterification of pectins. HG, as the most abundant pectin, is found in the cell wall and many other pectins are substituted HG with a galacturonic acid backbone. Moreover, demethylesterified HG backbones are able to crosslink with Ca<sup>2+</sup> and this results in more rigid or weaker cell wall. Because the methylesterification state of pectin is controlled by pectin methylesterases (PMEs), we wanted to compare PME activity and pectins in hyperhydric and normal Arabidopsis seedlings. The PME activity was measured for *gae-1* and Col-0 leaves on HH inducing medium with Ca<sup>2+</sup> treatments as described in the materials and methods section. The PME activity is shown in Figure 6 and low PME activity was detected in *gae 6-1* leaves compared to Col-0 leaves for all treatments.

PME activity of hyperhydric leaves for Col-0 was significantly lower in 0.2% Gr and 0.4% Gr medium compared to Agar medium. However, the PME activity of hyperhydric leaves for *gae 6-1* was lower than the activity in leaves from Agar medium seedlings but only for the 0.4% Gr medium the difference was significant.

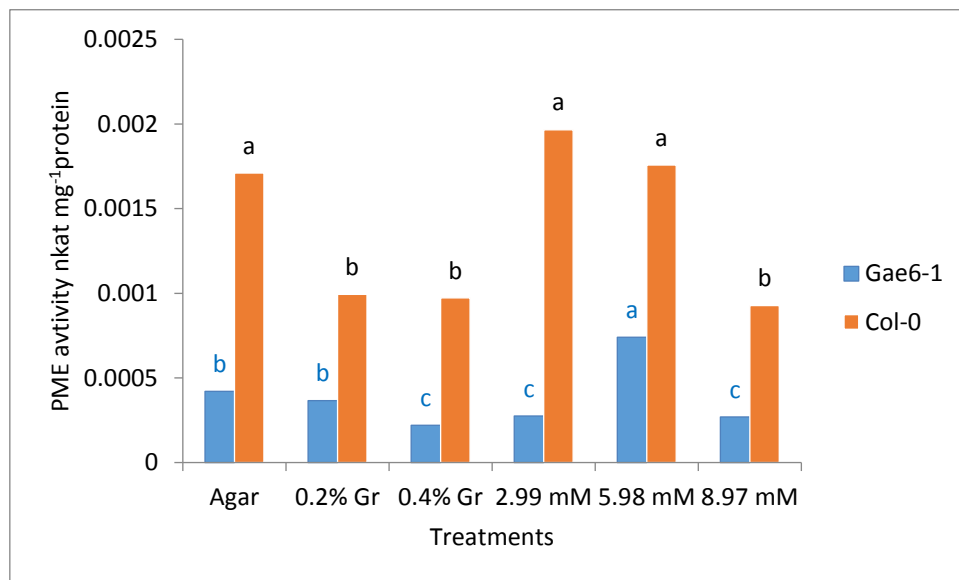
When we have a look at the effect of  $\text{Ca}^{2+}$  in HH included medium (0.4% Gr), the PME activity of the Col-0 was restored up to normal levels or even higher, however for the highest concentration of 8.97 mM  $\text{Ca}^{2+}$  medium, the PME activity was again significantly lower compared to the Agar control. The PME activity of *gae 6-1* seedlings showed significant decrease for 2.99 mM  $\text{Ca}^{2+}$  and 8.97 mM Ca mediums compared to Agar medium, however they were similar to the 0.4% Gr control. By contrast, significantly higher PME activity was obtained on 5.98 mM  $\text{Ca}^{2+}$  medium. While the PME activity was reduced in Col-0 by adding the highest concentration of exogenous Ca, this pattern was not found in less-pectin-mutant.

Looking at both Col-0 and *gae 6-1* on HH inducing medium (0.4% Gr), both GalA content and the PME activity significantly decreased compared to the Agar medium.

Both PME activity and GalA level of Col-0 did not show significant difference on 5.98 mM  $\text{Ca}^{2+}$  medium compared to Agar medium. There were significant difference between *gae 6-1* and Col-0 for the Agar treatment and when adding calcium to Gr media compared to Gr alone.

Figure 6.

The graph shows PME activity as nkat mg<sup>-1</sup>protein (y axis) extracted from leaves of *gae6-1* and Col-0 on different calcium concentration treatments. Nkat represents nmol/second/mg tissue. PME activity was measured as explained in materials and methods part. X axis shows Agar = ½ Ms Agar; 0.2% Gr = 0.2% Gelrite; 0.4% Gr = 0.4% Gelrite; 2.99 mM, 5.98 mM and 8.97 mM are Calcium Nitrate concentrations. Y axis shows nkat mg<sup>-1</sup>protein. Values represent average ± SE. Blue (for *gae6-1*) and black (for Col-0) letters indicate significant differences in between treatments (*Fisher's LSD, P < 0.05*).



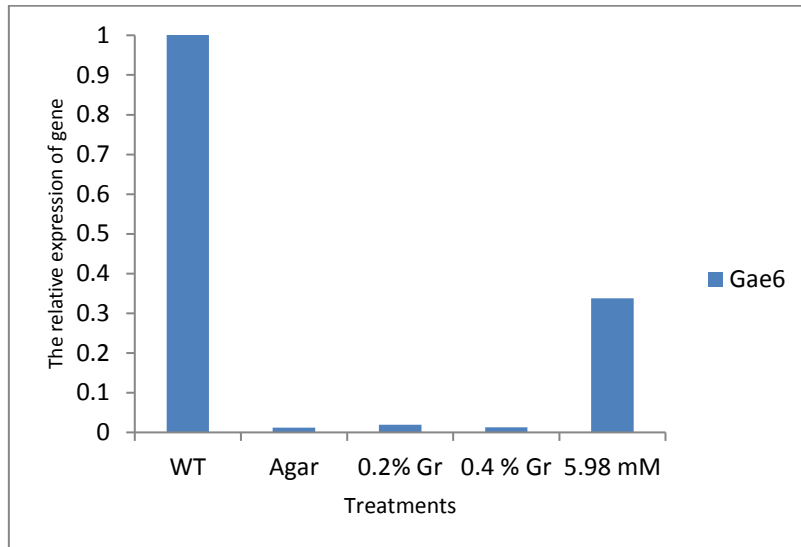
### Gae6 Expression

Since we showed here that the mutant Arabidopsis (*gae 6-1*) had brittle leaves and a lower galacturonic acid content, we investigated whether the *gae6-1* mutant had indeed less expression of the GAE6-1 gene than Col-0 and to show whether any change in expression level could be observed by adding 5.98 mM Ca<sup>2+</sup>. The expression of GAE6 in leaves of wild-type Col-o and mutant (*gae 6-1*) plants is shown in Figure 7.

The expression of the gene in the mutant was found to be as expected, it was low in Agar, 0.2% Gr and 0.4% Gr mediums. However, mutant seedlings treated with Ca<sup>2+</sup> showed an upregulation of expression compared to the others but the expression of gene did not reach to standard Col-0 Agar level. This might indicate that the gene is not completely knocked-out because it could be stimulated by adding Ca<sup>2+</sup>.

**Figure 7.**

The graph shows the relative expression of the gene *GAE6* in the mutant (*gae 6-1*) and WT (Col-0) in different treatments. WT = Col-0 in Agar; The other bars represent the mutant; Agar= ½ Ms Agar; 0.2% Gr = 0.2% Gelrite; 0.4% Gr = 0.4% Gelrite; 5.98 mM = 0.4% Gr with 5.98 mM of Calcium Nitrate



## Discussion and Conclusion

Most researches targeting HH are based on visual assessments and morphological abnormalities and malformation of HH plants (Debergh et al. 1981; Rojas-Martinez et al. 2010; Gaspar et al. 1995; Saher et al. 2005; Gribble et al. 2003; Ziv 1991), however there have been only a few reports on the underlying mechanisms of HH. The first assessment about the severity of HH was reported by Van den Dries et al. (2013) by measurement of apoplastic water and apoplastic air volumes in hyperhydric plant leaf tissues. *Gae 6-1* mutant was chosen as reported less pectin level regarding as GalA. It was expected that the mutant shows HH leaves due to less pectin. Therefore, we wanted to test the effect of  $Ca^{2+}$  on HH regarding to pectin level by adding exogenous Ca under inducing HH conditions and PME activity.. In the present study, *Arabidopsis* seedlings ( Col-0 and mutant *gae 6-1*) were shown to be tolerant to HH after exogenous  $Ca^{2+}$  was applied. Mediums 0.2 % Gr and 0.4% Gr were used and 0.4% was chosen as reference since 0.4% Gr is more rigid and than 0.2% Gr medium (Huang et. Al., 1995). When  $Ca^{2+}$  was applied on 0.4% gel, the HH symptoms decreased compared to Gr alone (Fig. 2 and 3). This result suggested that low pectin results in HH, even on agar, and that exogenously applied calcium influenced HH, both in Col-0 as well as in the low pectin mutant, *gae6-1*. We also wanted to consider testing a high pectin line (*pmr6*), too and obtained seeds from TAIR but that they did not germinate although we tried to germinate several times.

Saher et al. (2005) did a study on PME and pectins in normal and hyperhydric shoots of carnation cultured *in vitro* and the total pectins of hyperhydric leaves of all varieties were reported as a significant decrease in comparison with controls. In this study, we optimised and used a method to measure both pectin levels (as GalA quantity) and PME activity on wild type (Col-0) and the less-pectin-mutant (*gae 6-1*) *Arabidopsis thaliana* seedlings, cultured on media supplemented with different Ca<sup>2+</sup> concentrations *in vitro*. Although the *gae 6-1* mutant showed HH on Agar already, we found no difference on pectin levels (as GalA quantity) for the same mutant. However, we found that in HH seedlings of both Col-0 and *gae6-1*, the GalA content was significantly increased when adding 5.98 mM Ca<sup>2+</sup> to the HH inducing medium (0.4% Gr) compared to the control (0.4 % Gr), and they even reached levels similar to the control Agar medium (Fig. 4). The GalA content of *gae 6-1* on control medium (agar) was found less than Col-0 but not to be significantly different from the Col-0 GalA content on the same medium whereas *gae 6-1* mutant was reported as less-pectin-mutant. However, it is reported by Bethke et al. (2016), GalA content of *gae 6-1* was found significantly less than Col-0. This contradiction might be explained that our experiment was conducted *in vitro* conditions. To investigate further on GalA, we looked at the gene supposedly involved in pectin biosynthesis and measured GAE6 gene expression in the mutant plant and found that the expression of GAE was extremely low compared to Col-0, as expected (Fig.7). However, Exogenously applied Ca induces changed the gene expression being between 17-fold to 28-fold higher in *gae6-1* seedlings by adding 5.98 mM Ca<sup>2+</sup>. This meant that the mutation of the gene doesn't seem to interfere with pectin levels as we measure them. As mentioned earlier, these results on the mutant agreed with those of Bethke et al. (2016), where the *Arabidopsis* seedlings were not grown under *in vitro* conditions but under *in vivo* conditions. In retrospect, the mutant *gae6-1* proved to be not suitable for our study on the relationship between pectin levels and calcium. Still, the mutant showed a HH phenotype on Agar and had low gene expression that could be upregulated by addition of calcium. It would be interesting to find out the relationship between the GAE6 gene and pectin synthesis.

Based on our findings, when Ca<sup>2+</sup> concentration increased to 5.98 mM or more, the GalA content in both Col-0 and *gae 6-1* had a significant increase compared to 0.4% Gr medium. We might say that replacement of GalA content regarding to Agar medium level obtained thanks to increased Ca<sup>2+</sup> concentration and this results in non-HH seedlings.

We expected that when the PME activity is high, pectin content is low or the other way around. Both GalA content and PME activity showed similar increase and decrease trends on 0.4% Gr and  $\text{Ca}^{2+}$  5.98 mM media compared to agar. The reason for this may be that PMEs have different modes of action (Saher et al., 2005; Micheli, 2001) depending on the pH (Denes et al., 2000). PMEs might act linearly (blockwise) or randomly (non-blockwise) along the chain of pectins such as HG. The linear acting of PMEs on HGs promotes formation of  $\text{Ca}^{2+}$ -mediated cross-linking (via de-methylation of pectins), by generating free carboxyl groups that are able to bind  $\text{Ca}^{2+}$ , which could result in creating a pectate gel so it contributes to cell wall stiffening (Saher et al., 2005; Pelloux et al., 2007; Wolf and Greiner., 2012; Freitas et al., 2012). Therefore, we might conclude that PMEs acted linearly and that this resulted of non-HH seedlings in our experiment.

HG pectins are highlyly methylated (Micheli, 2001; Wolf et al., 2009). and those are de-esterified by PMEs in the cell wall. De-esterification of HGs by PMEs results in changing the mechanical properties of pectin like de-esterification enables cross-linking by positively charged  $\text{Ca}^{2+}$  ions (Bidhendi and Geitmann, 2016). However, it was reported as an paradox that the de-esterification might be resulted in both decreased and increased on cell wall stiffness *in vivo* (Palin and Geitmann, 2012; Bidhendi and Geitmann, 2016). This contradiction might be explained by different actions of PMEs. De-methylation of pectin might occur linearly or randomly manner. Moreover. some paramaters like pH, cation concentration and the initial stage of methylesterification are thought to influence the mode of HG de-esterification (Osorio et al., 2008; Bidhendi and Geitmann, 2016).

PMEs demethyl-esterify pectins and they create carboxyl groups between pH values of 6 to 7 (Ruan et al., 1995; Domingos and Huber, 1999). Moreover, PMEs can interact with calcium in the cell wall network (Demarty et al., 1984; Ralet et al., 2001; Bosch and Hepler, 2005). Pectin binds with Ca and the total Ca<sup>2+</sup>: pectin-bound  $\text{Ca}^{2+}$  ratio depending on the  $\text{Ca}^{2+}$  concentration and pH (Tibbits et al., 1998). According to Wolf and Greiner (2012), when PMEs act randomly on HGs, the pH is reduced locally to provide an acidic environment within the cell wall so the action of endogalacturonases is promoted and cell wall-loosening is the result. In addition, When the PME acts linearly (blockwise de-esterification) on GalA residues, methoxyl group removal, resulting in a continuous region of de-esterified pectin. In addition, linearly demethylation of GalA residues (more than nine) allows strands of pectin to be linked more efficiently via  $\text{Ca}^{2+}$  bonds, leading to gelation and enhanced stiffness (Willats et al., 2001a; Wolf and Greiner, 2012; Bidhendi and Geitmann, 2016).

When the PME acts randomly (non-blockwise de-esterification), Ca<sup>2+</sup> bridging is less effective on a pectic domain or even may not bind together by Ca<sup>2+</sup> bridges. Therefore, this might result in cell wall softening (Willats et al., 2001b; Wakabayashi et al., 2003; Arancibia and Motsenbocker, 2006).

While the PME activity of *gae 6-1* showed significant increase on 5.98mM Ca<sup>2+</sup> medium, GalA level did not show significant difference compare to Agar medium. Louvet et al (2006) suggested that cell wall stiffening and loosening can be explained either associated with different PMEs or by differences in apoplastic pH and the availability of cations on different microenvironment conditions. In our experiment, pH of the medium was adjusted to 5.8 for all treatments. Although PME activity is high in some Ca<sup>2+</sup> treatments, we might obtain similar pectin levels with parallel PME activity and non-HH seedlings due to the acidic pH of the medium. According to Wolf and Greiner (2012), when PMEs act randomly on HGs, pH is reduced locally to acidic environment within the cell wall so the action of endogalacturonases is promoted and cell wall-loosening is contributed. In our experiment, pH was adjusted to 5.8 to all treatments.

We found that in Col-0 seedlings under HH induced medium (0.4% Gr), the PME activity is significantly reduced compared to Agar. However, the activity increased to the control level (Col-0) by adding Ca<sup>2+</sup> (2.99 and 5.98 mM).

The PME activity of *gae 6-1* on 0.4% Gr medium is found significantly lower than Agarmedium, this difference might be explained because of the mutant type Arabidopsis. According to Banjongsinsiri (2003), all plant PMEs have their optimum pH range between 7 to 9 and the activity is affected by the presence of salts, temperature and various inhibitors as well as pH (Rexova-Benkova and Markovic, 1976). PME inhibitor (PMEI) has inhibitory effect on PMEs and the PMEI related genes reported on Arabidopsis (Balestrieri et al., 1990; Wolf et al., 2003). Moreover, high concentration of cations, pectic acid, polygalacturonic acid, phenolic compounds show inhibitory effect of PMEs as well as fruit PMEs due to high sugar concentrations (Banjongsinsiri,2003). These effects of cations and salts might explain the significantly lower PME activity on high 8.97 mM Ca<sup>2+</sup> medium for both Col-0 and *gae 6-1*.

We measured the expression of the GAE6 gene to verify whether the expression is less compare to Col-0. We found that there was a decrease in the expression level of GAE6 gene in the *gae 6-1* mutant as we expected the paper Bethke et al. (2016) when the seedlings grow in in vitro condition on different media (Fig. 7). The expression of the gene was found at extremely low level on both Agar and Gr mediums. However, interestingly, the expression of the gene was upregulated by addition of Ca<sup>2+</sup> (Fig. 7). We might conclude that the mutant is not completely knocked-out or a wrong mutant was sent for our

experiment by the supplier company. In other words, GalA levels of *gae6-1* and Col-0 were found to be close to each other in Agar medium although the *gae6-1* had extremely low GAE expression levels. By contrast, the expression in *gae6-1* increased by adding 5.98 mM  $\text{Ca}^{2+}$  and the GalA level of *gae6-1* on 5.98 mM  $\text{Ca}^{2+}$  medium did not show a significant difference from Col-0 on Agar. Comparing the PME activity and *GAE6* expression of mutant seedlings, we found a positive effect of  $\text{Ca}^{2+}$  (5.98 mM) on both. It might be said that the mutant does not effect on GalA level, but it can affect the other pectins or there might be independent effects of  $\text{Ca}^{2+}$  or PME activity responds to pectin levels. While the mutant type did not affect pectin levels as monitored by measuring GalA, it suprisingly affected PME activity. It might be said that GalA level reached the level of the Agar medium level by adding  $\text{Ca}^{2+}$  in HH inducing medium. We conclude that there is a relationship between pectin,  $\text{Ca}^{2+}$  and HH whereas we cannot say if there is a direct relationship between PME activity and HH.

## Supplementary Data

Supplemental Table 1. Primers used in this study

Primer Name	Sequence ( 5'-3')
<i>Arabidopsis thaliana</i> ACTIN2-F	TCCCAGTGTTGTTGGTAGGCCA
<i>Arabidopsis thaliana</i> ACTIN2-R	ACCAATCGTGATGACTTGCCCA
GAE6 RT1-F	ATGCCCTGTCGGCGACGGC
GAE6 RT1-R	CGTACTCCGGCTTGAGCGGC
GAE6 RT2-F	ACAGACCAACCAGCGAGTCT
GAE6 RT2-R	CTGAGCTTGCCTCGCTTCT

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