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Characterization of UDP-arabinopyranose mutase genes in the Arabidopsis cell wall mutant mur5

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**Characterization of UDP-arabinopyranose mutase genes in the *Arabidopsis*
cell wall mutant *mur5***

University of Connecticut
Honors Thesis
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Spring 2014

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Abstract

The genome of *Arabidopsis thaliana* contains several coding regions for UDP-arabinopyranose mutases (UAMs) that are also known as reversibly glycosylated polypeptides (RGPs). The *mur5* cell wall mutant of *Arabidopsis* shows a 30% decrease in cell wall arabinose content, and a missense mutation in the *Reversibly Glycosylated Polypeptide 2* gene was recently proposed to cause this mutant phenotype. Through a traditional complementation analysis, *mur5* and a T-DNA insertion mutant in the *RGP2* gene were shown not to complement each other, indicating that the two genes are mutant alleles of the same locus. The *mur5* SNP located in *RGP2* caused a more severe arabinose deficiency than the gene disruption in the T-DNA insertion line, which could be explained by the operation of a subunit poisoning mechanism in the *mur5* mutant. Transcriptional analysis indicated that *RGP2* and its paralog *RGP1* were regulated independently of one another despite having identical functions and cell localization.

Introduction

The cell wall is an important part of the plant cell. It protects the plant from pathogens and provides the structure and rigidity needed for plants to stand upright. It is also the primary substrate in cellulosic biofuel production. The cell wall is composed of three major polysaccharide components: cellulose, hemicelluloses and pectins. Cellulose microfibrils confer rigidity on the cell wall. The β -(1 \rightarrow 4)-glycosidic bonds between the D-glucose monomers allow for significant hydrogen bonding, causing cellulose to be durable and water insoluble. Hemicelluloses are a diverse group of polysaccharides with β -(1 \rightarrow 4)-linked backbones in which most of the hydroxyl residues are arranged in an equatorial configuration. They include xyloglucans, xylans, (glucurono)arabinoxylans, mannans and glucomannans. Hemicelluloses are thought to crosslink the cellulose microfibrils, preventing lateral movement, and giving added strength to the cell wall (Park and Cosgrove, 2012). The pectin matrix is an amorphous gel in which the cellulose microfibrils and cross-linking glycans are embedded (Carpita and Gibeaut, 1993; McCann and Roberts, 1991). It is a highly heterogeneous mixture of polysaccharides mostly composed of negatively charged residues such as galacturonic acid. The function of the matrix is not yet fully understood, but it is believed to be important in regulation of wall porosity, charge density and microfibril spacing. Standard structural models of the cell wall have been developed explaining the complex network formed by these three polysaccharide components, but the accuracy of these models remains disputed. Cell wall biochemistry continues to be a significant topic of research.

About 10% of the plant genome is dedicated to building and maintaining the cell wall (Yong et al., 2005; Penning et al., 2009). While the *Arabidopsis* genome has been fully sequenced, the functions of the majority of genes within it, including those involved in cell wall biosynthesis, remain unclear. An important development in the field of cell wall biochemistry was the creation of the *mur* collection of cell wall mutants (Reiter et al., 1993, 1997). More than 5000 ethyl methanesulfonate (EMS) mutagenized *Arabidopsis* plants were analyzed for phenotypic abnormalities through chemical analysis of the cell wall material using gas chromatography (GC) of alditol acetates. The screening process identified 23 mutant lines encompassing 11 different loci, *mur1-11*. The cell wall mutants obtained through this screening process are categorized into three groups. The first group consists of mutant lines completely lacking a known

monosaccharide of the wild type cell wall, a second group shows a strong decrease in the relative amount of one monosaccharide, and the third group consists of plants which show a more complex change in cell wall composition, with alterations in the relative abundance of several monosaccharides (Reiter et al., 1997).

The *mur5* mutant, which falls into the second group of cell wall mutants, shows no easily distinguishable visual phenotype. By GC analysis of derivatized cell wall material, the mutant line shows a ~30% decrease in cell wall arabinose content compared to wild type Columbia (WT) during the early flowering stage of development, the time when the difference between arabinose content in *mur5* and WT is at its greatest (Nick Carpita, pers. comm., Reiter et al., 1997). The *mur5* low arabinose phenotype behaves as a single recessive Mendelian trait (Reiter et al., 1997). Through conventional genetic mapping, deep Next Generation sequencing and expression analysis, the Carpita lab at Purdue University found that *mur5* plants carry a single nucleotide polymorphism (SNP) in the *Reversibly Glycosylated Polypeptide 2 (RGP2)* gene. This SNP causes a missense mutation in *RGP2* and was suggested to cause the *mur5* phenotype (Dugard et al., unpublished).

RGP2 encodes a UDP-L-arabinopyranose mutase (UAM), which interconverts the furanose and pyranose forms of the L-arabinose (L-Ara) moiety in UDP-L-Ara (Konishi et al., 2007) (see Figure 1). This function is important in the cell because while UDP-L-Ara is more thermodynamically stable in its pyranose form (UDP-L-Arap), it is predominantly incorporated into polysaccharides, proteoglycans, and glycoproteins in its furanose form (UDP-L-Araf), (Konishi et al., 2007). *RGP2* is a member of the Reversibly Glycosylated Polypeptide (RGP) family of proteins, of which there are five in *Arabidopsis* (Girke et al., 2004). The RGPs are a cytosolically localized, plant specific family of proteins that were originally described for their ability to form reversible complexes with UDP-sugars in vitro (Dhugga et al., 1997). The biological function of this reverse glycosylation has yet to be described, but a number of RGPs have demonstrated UAM activity, such as *RGP1* and *RGP2* (Konishi et al., 2007). *RGP1*, which shares much sequence similarity to *RGP2*, is thought to be a result of a gene duplication event (Blanc et al., 2000). The protein products of these two RGP genes have a 93% identical amino acid sequence in *Arabidopsis* and are localized identically in the plant, specifically in actively growing tissue such as the apical meristem of young seedlings (Drakakaki et al., 2006). Both *RGP2* and *RGP1* associate with the cytosolic side of the Golgi membrane (Drakakaki et al.,

2006). RGP s have been observed to form heteroprotein complexes in numerous different plants, such as *Oryza sativa* (Langeveld et al., 2002; Drakakaki et al., 2006; De Pino et al., 2007). RGP1, RGP2 and RGP5 have been observed to associate with one another forming one of these hypothesized complexes (Rautengarten et al., 2011). It is believed that RGP UAMs are more active in heteroprotein complexes than in their monomeric form (Konishi et al., 2007). If these complexes are necessary for normal enzymatic function, normal UAM activity is expected to be dictated by the abundance of all component proteins.

The SNP located by the Carpita lab in the *RGP2* gene of *mur5* plants is a G→A transition, which causes the change of a cysteine residue to a tyrosine residue in the protein product (Dugard et al., unpublished results). With the use of qRT-PCR, an expression analysis was performed on *RGP1* and *RGP2*. The results suggested that *mur5* plants have a complete lack of transcription of *RGP2* at 30 days of growth after seed germination. It could not be concluded whether the *mur5* phenotype was caused by the SNP or by this lack of transcription of *RGP2*. The cell wall composition of *RGP2* overexpression lines was analyzed and indicated a rescue of the low arabinose phenotype; however, this result was not dispositive of whether *RGP2* was allelic to *mur5*. Because of the redundancy of UAM activity between RGP isoforms, phenotypic rescue by overexpression of any UAM gene, such as *RGP1*, is to be expected. The lack of transcription of *RGP2* in *mur5* plants was also difficult to explain, as there is no plausible reason why an SNP would cause this. Because *mur5* was mapped to a large interval through conventional genetic mapping, it is possible that a mutation affecting a different gene within that interval, for instance one that is a positive regulator of *RGP2*, is the cause of this lack of transcription. Prior to the work described in this thesis, no experiments had been performed to address this alternate possibility.

The Carpita lab also obtained results that suggested an involvement of *RGP2* in regulatory cross-talk with *RGP1*. When *RGP2* was upregulated in wild type Columbia, expression of *RGP1* was attenuated. In *mur5*, where *RGP2* transcripts could not be detected, *RGP1* was expressed at higher levels. This regulatory cross-talk was hypothesized to be a mechanism of coordinating nucleotide sugar interconversions amongst the RGP family of proteins mediated by the heteroprotein complexes that they may form. This regulatory influence was not observed for *RGP5*, however, which is believed to be a part of a RGP heteroprotein complex (Rautengarten et al., 2011).

This thesis seeks to investigate the hypothesis proposed by the Carpita lab that *MUR5* and *RGP2* are alleles of the same gene. Because of the lack of transcription of *RGP2*, a complementation or overexpression analysis fails to prove the hypothesized allelism. This lack of transcription has also not been proven to be caused by the *mur5* SNP. The missense mutation located in *RGP2* would be expected to cause the formation of a misshapen or non-functional protein rather than lead to a complete lack of transcription of the gene. Experiments were also designed to further test the hypothesized cross-talk between *RGP1* and *RGP2*. A more in depth analysis of how the *mur5* SNP causes changes in plant physiology was also performed. Understanding how the *mur5* mutation causes changes to the plant cell wall can provide insight into how the cell wall is being synthesized. A better understanding of the biosynthetic pathways of the cell wall and how to manipulate them may improve biofuel generation and agricultural production.

To confirm that *RGP2* and *MUR5* are alleles of the same gene, a complementation analysis was performed. The *mur5* mutant line was crossed with an *RGP2* knockout line from the Salk Institute which contains a large T-DNA insertion in the first intron of *RGP2* (SALK_148500C) (*salk1*). The cell wall monosaccharide composition of progeny plants was then analyzed. Because the *mur5* mutation is a single recessive Mendelian trait (Reiter et al., 1997), a wild-type cell wall composition in the F₁ generation would indicate that *mur5* and *salk1* complement each other and would not be allelic. In other words, the gene responsible for the arabinose deficiency in *mur5* would not be the mutant *RGP2* gene. The F₁ crosses would be heterozygous at both the *RGP2* and *MUR5* loci and lack the recessive low cell wall arabinose trait. If the arabinose deficiency is observed in the F₁ progeny, it would indicate that *RGP2* and *MUR5* are allelic and the mutant alleles each contribute to the recessive low cell wall arabinose trait.

A more detailed analysis of the difference in cell wall composition between *mur5* and *salk1* was performed by evaluating their cell wall monosaccharide composition of derivatized leaf material. Differences in phenotype are expected to provide clues to how an SNP and a large T-DNA insertion in the *RGP2* gene affect the plant cell differently. A complete knockout of *RGP2* in the *salk1* background may for instance cause a more severe arabinose deficiency if the missense mutation in *mur5* plants reduces but does not completely eliminate protein function. *Arabidopsis* root tissue was also analyzed to evaluate the difference in arabinose deficiency in

plant organs other than the leaves. Learning how plant organs are affected in the *mur5* and *salk1* mutants can provide insight into how each mutation disrupts normal cell function.

A feeding experiment was used to analyze the ability of *mur5* and *salk1* to rescue their respective low cell wall arabinose phenotypes through the incorporation of extracellular L-Ara into the cell wall. The cell accomplishes this through the salvage pathway, which is defined as the sequential action of L-arabinokinase and UDP-L-Ara pyrophosphorylase (Figure 2) (Burget et al., 2003). The *mur5* and *salk1* mutants were grown on arabinose plates of varying concentrations, and their cell wall composition was analyzed. This experiment was designed to better characterize the effect of the hypothesized non-functional RGP2 protein on plant physiology when L-Ara is not limiting. If the introduction of extracellular arabinose into the growth media increases the relative amount of arabinose in the cell wall, it would indicate that the salvage pathway is not interrupted by the respective mutant genes. If the introduction of extracellular arabinose in the growth media has no effect on relative cell wall arabinose levels, it would indicate that the salvage pathway is disrupted by mutations in the *RGP2* gene. While neither the *mur5* SNP nor *salk1* T-DNA are expected to disrupt the salvage pathway, observing a difference in their ability to incorporate extracellular L-Ara into the cell wall can give insight into how the *mur5* SNP and the *salk1* T-DNA insertion affect plant physiology differently.

Using RT-PCR, a detailed expression analysis was performed on the *RGP1* and *RGP2* genes in *mur5* and WT. Analysis of the relative expression levels of these two genes was designed to evaluate the hypothesized regulatory cross-talk between *RGP1* and *RGP2*. This experiment also serves to test the hypothesis that *RGP2* is not transcribed in the *mur5* background (Dugard et al., unpublished).

Results

mur5 x *salk1* Complementation Analysis:

A traditional complementation analysis was performed to test the hypothesized allelism between *MUR5* and *RGP2*. *mur5* was crossed with an *RGP2* knockout line, *salk1*, and the F₁ generation was analyzed for the low cell wall arabinose phenotype. The identity of the *mur5*, *salk1*, and F₁ progeny were confirmed using RT-PCR (Figure 3) in combination with molecular cloning and DNA sequence determination to confirm the *mur5* SNP. The *mur5* parental line was found to have a ~32% reduction in arabinose content compared to the WT control at 21 days, and a ~35.4% reduction at 28 days. An arabinose reduction of ~38% compared to WT was observed in *salk1* at 21 days and a ~28% reduction at 28 days. (Figure 4)

Compared to the parental *mur5* line, a slightly less severe arabinose deficiency was observed in the F₁ generation of the crosses, which had a heterozygous *mur5/salk1* background. Compared to WT, a 25% reduction in arabinose content was observed in the crosses with *mur5* as the seed bearing plant (*mur5* x *salk1*) at 22 days and a 35% reduction was observed at 33 days. A ~27% reduction in arabinose content was observed in *salk1* x *mur5* F₁s compared to WT at 26 days and a ~32% reduction was observed at 33 days (Figure 4). The arabinose deficiency observed in the F₁ progeny of the *mur5* x *salk1* and *salk1* x *mur5* crosses confirm the hypothesized allelism between *MUR5* and *RGP2*, as the two genes do not complement each other to rescue the recessive *mur5* phenotype.

Cell wall arabinose content of *mur5* and *salk1* plants:

The difference in mutant phenotype between *mur5* and *salk1* was further characterized through analysis of cell wall monosaccharide composition. A low cell wall arabinose mutant of a different locus, *mur4*, was used as a control. *mur4* is known to have a more severe arabinose deficiency than *mur5* due to a mutation in a gene encoding a UDP-D-xylose 4-epimerase that interconverts UDP-D-xylose and UDP-L-arabinose in the Golgi lumen. A ~36% reduction in arabinose content was observed in *mur5* compared to WT at 28 days. A ~28% reduction in

arabinose content was observed in *salk1* compared to WT and a ~46% reduction in arabinose content was observed in the *mur4* control compared to WT (Figure 5).

In the root samples, the lowest percent of cell wall arabinose was observed in *mur5* with a ~25% reduction compared to WT at 28 days, followed by *mur4* with a 10.4% reduction and *salk1* with a 9.1% reduction. In the 37 day samples, the lowest percent of cell wall arabinose was observed in *mur4* with a ~32% reduction compared to WT, followed by *mur5* with a ~23% reduction and *salk1* which had a 5% relative increase in cell wall arabinose compared to WT (Figure 6).

Arabinose Feeding Experiment:

An arabinose feeding experiment was conducted to evaluate the ability of *mur5* and *salk1* to incorporate extracellular arabinose through the salvage pathway. Plants were grown on arabinose plates of varying concentrations and were analyzed for cell wall monosaccharide composition. A less severe arabinose deficiency was observed in *salk1* compared to *mur5* at every concentration of extracellular arabinose. The highest arabinose content was observed in WT in all trials except at 60 mM arabinose, where a ~5% increase in arabinose content was observed in *mur4* compared to WT. Phenotypic rescue was not observed in either *mur5* or the *salk1* at any extracellular arabinose concentration. The *salk1* mutant reached an arabinose content plateau of ~26% at 30 mM, which was a 30% reduction compared to WT, while *mur5* plateaued at an arabinose content of ~20% at 15 mM, which was a 29% reduction compared to WT. WT had its highest relative arabinose content of 38% at 30 mM (Figure 7).

The difference in arabinose content between *mur5* and *salk1* lines grew as extracellular arabinose concentration increased. At 0 mM, there was a 9% difference in arabinose content between *mur5* and *salk1*, while at 60 mM there was a 23% disparity, *mur5* having the lower relative arabinose content.

Semi-quantitative Expression Analysis of *RGP1* and *RGP2*:

An expression analysis was performed to test the hypothesized lack of transcription of *RGP2* in the *mur5* background, and to analyze the extent of regulatory cross-talk between *RGP1* and *RGP2*. Equal amounts of *mur5* and WT-derived RNA were mixed together and reverse transcribed (Figure 8). The cDNA mixture was amplified with *RGP2*-specific primers which produced PCR fragments containing the *mur5* SNP in their centers (Figure 9). The *mur5* and WT-derived PCR fragments could then be differentiated by digestion with the Tsp45I restriction endonuclease because the *mur5* SNP causes the disappearance of a Tsp45I restriction site in the *RGP2* gene. Visualization of the digested *mur5*/WT mixture of PCR products shows that the WT-derived *RGP2* fragments are cleaved into 319 & 233 bp fragments (Fig. 10, lanes 2, 3, and 5), whereas the *mur5*-derived *RGP2* fragments remain at 552 bp (Fig. 10, lanes 2, 3 and 4). By comparing the relative band intensities of *mur5* and WT-derived *RGP2* fragments (Fig. 10, lanes 2 and 3) this result suggests that *mur5* expresses *RGP2* at higher levels than WT. A serial dilution was made to better indicate the difference in *RGP2* expression between *mur5* and WT. Taking into consideration the sum of the WT-derived fragments, the WT bands in the 2:5 dilution is similar in combined band intensities to the 1:20 dilution of the *mur5*-derived band (Figure 11).

An expression analysis of *RGP1* was also performed. *mur5* and WT RNA samples were separately reverse transcribed. The resulting cDNA was then PCR amplified with *RGP1* and *RGP2*-specific primers at 31, 29, and 27 cycles. Following agarose gel electrophoresis, the EF1 α and *RGP1* bands between the *mur5* and WT-derived samples had relatively similar band intensities within respective cycle numbers. Assuming that the 31 cycle samples met saturation, a relative increase in *RGP2* expression was observed in the 29 and 27 cycle *mur5* samples compared to WT at the respective cycles. Expression levels of *RGP2* were higher in *mur5* plants than in WT, while expression levels of *RGP1* were similar in *mur5* and WT plants (Figure 12).

Discussion

mur5 x salk1 Complementation Analysis:

No rescue of the low cell wall arabinose phenotype was observed in the F₁ generation of the *mur5 x salk1* and *salk1 x mur5* crosses (Figure 4). Because the *mur5* phenotype is a single recessive Mendelian trait (Reiter et al., 1997), this lack of complementation between *mur5* and *salk1* indicates that *MUR5* is allelic to *RGP2*.

Cell wall arabinose content of *mur5* and *salk1* plants:

The *salk1* mutant had a less severe cell wall arabinose deficiency than *mur5* at 28 days after germination (Figure 5). The *salk1* T-DNA insertion causes a complete knockout of the *RGP2* gene while the *mur5* missense mutation causes the translation of a mutant RGP2 protein. A complete knockout of *RGP2* in the *salk1* background was hypothesized to cause a more severe arabinose deficiency because the missense mutation in *mur5* plants could reduce but not completely eliminate protein function in RGP2. This was not observed, however, as the translation of the mutant RGP2 in *mur5* caused a more severe arabinose deficiency compared to *salk1*. This mutant RGP2 could be inhibiting the function of other UAMs in the cell. A 90% arabinose deficiency was observed in a double knockout of *RGP1* and *RGP2* (Rautengarten et al., 2011) and suggested that these two proteins account for most of the cell UAM function in *Arabidopsis*. RGP1 and RGP2 are thought to associate in heteroprotein complexes (Rautengarten et al., 2011), which suggests that the mutant RGP2 could be inhibiting RGP1 allosterically through subunit poisoning. This potential subunit poisoning would decrease the UAM activity of RGP1 and the proposed RGP heteroprotein complex. Because *RGP2* is not expressed in *salk1*, RGP1 would not be inhibited by a mutant form of RGP2.

A separate hypothesis is that because the mutant RGP2 in *mur5* is a non-functional protein, it may still bind UDP-L-Arap but fail to interconvert it to its furanose form. This could

decrease substrate availability for other UAMs such as RGP1. This hypothesis is addressed below in the context of the arabinose feeding experiment.

Arabinose Feeding Experiment:

An increase in cell wall arabinose was observed in *mur5* and *salk1* as the concentration of extracellular arabinose increased, indicating that the salvage pathway is intact in both mutant lines. The *mur4* mutant shows phenotypic rescue at 60 mM extracellular arabinose. This is because *mur4* contains a missense mutation in a gene encoding a UDP-D-xylose 4-epimerase, which interconverts UDP-D-xylose and UDP-L-Arap in the Golgi lumen. The mutation in this gene reduces the amount of UDP-L-Arap that is produced through its de novo pathway. UDP-L-Arap was limiting for UAM activity in *mur4*, so introduction of extracellular L-Ara through the salvage pathway rescued the low cell wall arabinose phenotype (Burget 2003). No phenotypic rescue was observed in *mur5* or *salk1* at any extracellular arabinose concentration.

salk1 had a less severe arabinose deficiency compared to *mur5* at every concentration of extracellular arabinose. The difference in arabinose deficiency between *mur5* and *salk1* grew with an increase in concentration of extracellular arabinose. At 0 mM *salk1* had a 9% higher arabinose content compared to *mur5*, at 15 mM *salk1* had an 11% higher arabinose content compared to *mur5* and at 30 mM *salk1* had a 32% higher arabinose content. These results suggest that UAM activity is lower in *mur5* than in *salk1* plants. This data supports the RGP2 subunit poisoning hypothesis because the uninhibited *salk1* RGP1 could process UDP-L-Arap faster than the *mur5* RGP1 which would be inhibited by the mutant RGP2 subunit. As the concentration of UDP-L-Arap increases, the disparity between *salk1* and *mur5* enzymatic efficiency became more pronounced. This result also sheds some light on the possibility that the *mur5* mutant RGP2 inhibits RGP1 by decreasing substrate availability. If this were the case, the difference in arabinose content between *salk1* and *mur5* would be expected to decrease as extracellular arabinose increases and UDP-L-Arap becomes less of a limiting factor.

Semi-quantitative Expression Analysis of *RGP1* and *RGP2*:

RGP2 was expressed at higher levels in the *mur5* background than in WT. This suggests that the low arabinose phenotype in *mur5* is not caused by a lack of expression of *RGP2* but more likely by translation of a non-functional or inhibitory RGP2 protein. Since *RGP2* expression was increased in *mur5* compared to WT, it is expected that *RGP1* expression would be attenuated (Dugard et al., unpublished). This was not observed, however, as *RGP1* was expressed at similar levels in *mur5* and WT. These results contradict the hypothesis that *RGP1* and *RGP2* are involved in regulatory cross-talk as the expression of each gene does not appear to influence the other. The increase in expression of *RGP2* in the *mur5* background suggests that the cell is sensing that RGP2 UAM activity is disrupted and upregulates its expression in compensation. Further experiments on the characterization of the hypothesized RGP heteroprotein complexes would be useful, as well as expression analyses that investigate additional UAM genes in *Arabidopsis*. The lack of expression of *RGP2* in *mur5* plants in the Carpita lab could be due to an environmental factor. Since growth conditions were different between the Reiter lab and the Carpita lab, this possibility cannot be disregarded. Expression analysis of *RGP2* in *mur5* plant grown under different growth conditions may help to address this hypothesis.

Materials and Methods

Plant Growth and Propagation:

Plants of all lines were planted in the following manner. Pots were filled $\frac{3}{4}$ with Miracle-Gro moisture control potting mix and ~10 cm of Fafard super fine germinating mix professional grade on top. Soil was then soaked with room temperature water. Seeds were mixed into 5 mL of 0.1% agar and dripped on top of soaked soil with a 10 mL micropipettor. Pots were then covered with saran wrap to keep moisture in and left covered for 4 days. Following removal of saran wrap, pots were watered from below whenever the soil was dry. Plants were misted from above with a hose when needed. As seedlings matured, individual plants were removed to prevent crowding stress before analysis.

Arabidopsis plants of all lines were grown in the lab growth room. Plants were grown in constant 70% humidity at 18°C during an 8 h dark period and 23°C during a 16 h light period with a light intensity of approximately $120 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a light temperature of 4100 K. Plants on phytagel growth plates were grown vertically to allow the roots to grow along the length of the plates for future sampling.

Plant Growth Plates:

For a final volume of 500 mL, MS basal salts mixture was dissolved to half the standard concentration (2.22 g/L) in 475 mL double distilled water. The solution was then titrated with HCl or KOH to pH 5.7. BactoAgar (BD) was then added to 0.8% for the arabinose feeding experiment, or 2 g/L of phytagel (Sigma-Aldrich) for the vertical root growth plates. The media was then autoclaved for 20 minutes and allowed to cool to ~30°C. Under a laminar flow hood, 500 μL Gamborg's vitamin solution and 25 mL of 20% (w/v) sucrose was then added through a 0.2 μm filter (Corning). For arabinose feeding plates, four separate growth media were made, each containing a different concentration of L-Ara, 0 mM, 15 mM, 30 mM and 60 mM. The L-Ara was also added to the growth media through the 0.2 μm filter following the autoclaving step.

After the growth media was well mixed, 150x25 mm plates were poured to ~0.5-1 cm and allowed to solidify in the laminar flow hood overnight with plate lids on.

Prior to plating, seeds were sterilized in separate 50 mL centrifuge tubes. Around 20-30 seeds of the respective line were added to each tube and sterilized with 95% ethanol for 1 minute. The ethanol was poured off and the seeds were then incubated for 15 minutes in a 30% bleach, 0.05% Tween 20 mixture. The bleach mixture was poured off and the seeds were then washed six times with double distilled water under the laminar flow hood. After the seeds were washed and the water poured off, the seeds were resuspended in 10 mL of 0.1% agar. The resuspended seeds were then poured on the solidified growth plates and left to dry overnight with the plate lids rested diagonally on top of the plates. The resuspended seeds were spread uniformly on the arabinose feeding growth plates; ~20 seeds were spread in a single line along one side of the phytigel root growth plates.

DNA Extraction:

0.4 g of leaf material from rosettes was harvested from individual plants. Leaf material was then ground up in a clean autoclaved mortar and pestle in liquid nitrogen until a fine light green powder was obtained. Ground leaf material was then transferred to a 1.5 mL microcentrifuge tube cooled in liquid nitrogen. Samples were kept in liquid nitrogen until the addition of the 2% CTAB lysis buffer (2% CTAB (Cetyl Trimethyl Ammonium Bromide), 1.4 M NaCl, 20 mM EDTA @ pH 8.0, 100mM Tris HCl @ pH 8.0, 2% polyvinylpyrrolidone, Ultrapure Water, 1% β -mercaptoethanol added just before using). 600 μ L of lysis buffer was added to each sample and was vortexed. Samples were then incubated in a 65° C water bath for 30 minutes. After incubation, 600 μ L of chloroform was added and samples were vortexed. The samples were then centrifuged at 13,000 x g for 2 minutes. The upper phase of the sample was then transferred to a new microcentrifuge tube. The process was then repeated starting at addition of 600 μ L of chloroform. 450 μ L of isopropanol was then added to the newly transferred upper phase and the samples were vortexed. This was followed by a 30 minute incubation at -20° C. Samples were then centrifuged at 13,000 x g for 15 minutes. The supernatant was then discarded and the pellet was washed with 600 μ L of 70% ethanol and vortexed. The samples were then centrifuged at

13,000 x g for 5 minutes and the supernatant was discarded. The pellet was then dissolved in 90 μ L of UltraPure water by incubating at room temperature for 5 minutes. The dissolved sample was then transferred to a new microcentrifuge tube and quantitated via spectrophotometry.

RNA Extraction and Reverse Transcription PCR (RT-PCR):

0.2 g of leaf material was ground in liquid nitrogen as described above and transferred to a microcentrifuge tube. 1000 μ L of Trizol (Life Technologies) reagent was added and samples were vortexed. Samples were then aliquoted into two samples. An additional 500 μ L Trizol was added to each sample and were centrifuged at 13,000 x g for 5 minutes at 4° C. The supernatant was then transferred to a new microcentrifuge tube. 200 μ L chloroform was then added. Samples were then shaken vigorously for 15 seconds and let sit at room temperature for 3 minutes. Tubes were then centrifuged at 13,000 x g for 15 minutes at 4° C. The upper aqueous phase was then transferred to a new microcentrifuge tube including no interphase or lower phase. 500 μ L of isopropanol was then added and samples were incubated at room temperature for 10 minutes. Tubes were then centrifuged at 13,000 x g for 10 minutes at 4° C. The supernatant was then poured out and 1 mL of 75% ethanol was added and tubes were vortexed. Samples were then centrifuged at 13,000 x g for 5 minutes at 4° C. The supernatant was then poured out and the pellet was air dried for 10 minutes at room temperature with tube lid open. The pellet was then dissolved in 20 μ L of UltraPure water by gently mixing with a pipette. The samples were quantitated via agarose gel electrophoresis and spectrophotometry.

cDNA was synthesized using the Superscript III reverse transcriptase as outlined in the protocol by Life Technologies with a Bio-Rad C-1000 Thermal Cycler.

Polymerase Chain Reaction:

PCR was done using *Taq* polymerase (New England Biolabs) with the protocol outlined by the manufacturer with a Bio-Rad C-1000 Thermal Cycler. Cycle conditions were as follows. Samples were heated up to 95° C for 30 seconds, cooled to 55° C for 30 seconds, then ramped up to 72° C for 1 minute. These conditions cycled for 34 cycles unless otherwise noted. Following the cycle phase, the samples were kept at 72° C for 5 minutes and then cooled to 4° C until the tubes were collected.

Preparation of Alcohol-Insoluble Material for Analysis of Arabidopsis Cell Wall Material:

About 50-100 mg of fresh leaf or root material was cut off from the sample plant and placed in a 1.5 mL microcentrifuge tube. 1 mL of 70% ethanol was added no later than 20 minutes after material collection to dissolve low molecular weight compounds including lipids. Samples were incubated in a 70° C water bath for ~2 hours, and the ethanol was removed. The ethanol incubation was repeated overnight. Root samples taken from phytagel plates were washed in boiling water for ~5 minutes to remove all residual phytagel (Sigma Aldrich) which could cause rhamnose contamination. Following removal of ethanol or water, 1 mL of acetone was added. Samples were then incubated at room temperature for 5 minutes. The acetone was removed and the incubation was repeated with another 1 mL of acetone. The acetone was removed and samples were allowed to air dry overnight with microcentrifuge tube lids open. The sample cell wall material was then autoclaved for 1 hour in 250 μ L 2 M TFA with 100 μ g myo-inositol as an internal standard. The liquid of the samples was transferred to large screw cap vials with Teflon-lined lids. 100 μ L of 9 M NH_3 was added to each vial, and the samples were vortexed. 1 mL of 2% (w/v) NaBH_4 in DMSO was added to each tube and tubes were vortexed. The samples were then incubated at 40° C for at least 30 minutes. 250 μ L of acetic acid was added and samples were vortexed. 250 μ L of 1-methylimidazole and 4 mL acetic anhydride were added to each tube. Samples were vortexed and then incubated at room temperature for 10 minutes. 8 mL of distilled water was added to each tube. The tubes were capped and mixed by inversion. The tubes were uncapped and the samples were allowed to cool to 30° C. 2 mL of CH_2Cl_2 was added to

extract the alditol acetates. The tubes were capped and mixed by inverting them 40 times. The tubes were then incubated for 2 hours or overnight at 4° C. The CH₂Cl₂ phase was recovered with long 9 mL Pasteur pipettes to small disposable test tubes. The test tubes were then incubated in a 55° C water bath for ~30 minutes to evaporate the CH₂Cl₂. 210 µL of CH₂Cl₂ and 2 mL of distilled water were added, and the tubes were then capped and vortexed vigorously for 15 seconds each. The lower phases of the samples were subsequently transferred to GC vials and stored at -20° C until they were run on the gas chromatograph. If less than 210 µL of CH₂Cl₂ was transferred, additional CH₂Cl₂ was added.

Gas Chromatography of Alditol Acetates:

Derivatized samples were run on an HP 5890 Series II+ Gas Chromatograph. Run conditions were essentially as described in Reiter et al. (1997)

Molecular Cloning to Sequencing:

Molecular cloning was performed using the TOPO TA Cloning Kit (Invitrogen) as per manufacturer's instructions. Recombinant plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen). Samples were sequenced by the UConn Biotechnology center using an 8-Capillary 3500 Genetic Analyzer by Applied Biosystems.

mur5 x *salk1* Complementation Analysis:

The identity of the parental lines and progeny of the crosses (F₁) was confirmed by RNA extraction of leaf material followed by RT-PCR. The primer sets used for PCR were a *mur5* confirmatory set consisting of left and right flanking primers on either side of the T-DNA insertion site (Figure 13). Another primer set, which was confirmatory for *salk1*, consisted of a T-DNA border primer which hybridizes to the 3' region of the T-DNA insertion, and a right

flanking primer (Figure 14). By evaluating DNA fragment sizes through agarose gel electrophoresis of these PCR products, the identity of the plants could be confirmed. The placement of the *mur5* SNP in the *RGP2* gene was also confirmed in the *mur5* and F₁ progeny plants with the use of molecular cloning and DNA sequence determination.

Plant cell wall monosaccharide composition was analyzed at 22-26 and 28-33 days of age after germination, depending on the stage of plant growth. Plant lines were potted on the same day but germination time was variable depending on plant background.

Cell wall arabinose content of *mur5* and *salk1* plants:

Leaf material from 18 individual plants was analyzed from each line at 21 and 28 days of age. Plants were grown in pots in the lab growth room under growth conditions noted below. Root material was analyzed from 8 plants of each line at 28 and 37 days after germination. Plants were grown on phytigel plates to prevent sugar contamination from fungi or bacteria. Plates were oriented vertically in the lab growth room to allow roots to grow along the length of the growth plates.

Arabinose Feeding Experiment:

Plants were grown on agarose plates containing arabinose at concentrations of 0, 15, 30 and 60 mM, respectively. At 28 days post-germination, the rosettes were removed from their root base and analyzed for cell wall monosaccharide composition. (Burget et al., 2003)

Semi-quantitative Expression Analysis of *RGP1* and *RGP2*:

Bulk RNA was extracted from *Arabidopsis* leaf material from *mur5* and WT plants. Utilizing RNA quantitation via agarose gel electrophoresis and spectrophotometry, equal amounts of RNA were mixed together from a *mur5* RNA prep and a WT RNA prep. These *mur5*/WT mixed RNA

samples were then reverse transcribed to cDNA. The cDNA was PCR amplified using an *RGP2* specific-primer set (Figure 15). The *RGP2* PCR fragment was designed to contain the *mur5*-derived *RGP2* SNP locus in its center. The SNP in the *mur5 RGP2* gene causes the disappearance of a Tsp45I restriction site in the third exon of *RGP2*. Digestion with the Tsp45I restriction endonuclease, as per manufacturer's instruction (New England Biolabs), allows for differentiation between the *mur5* and WT-derived PCR fragments by cleaving the WT-derived cDNA into 319 & 233 bp fragments. With the use of agarose gel electrophoresis, the band intensities of *mur5* and WT-derived *RGP2* fragments in the mixed samples were then compared to analyze relative expression levels.

To compare relative *RGP1* and *RGP2* expression, separate *mur5* and WT RNA preps of similar concentration were used. Agarose gel electrophoresis and spectrophotometry were utilized to quantitate RNA and obtain identical concentrations in all samples. The utilization of restriction enzymes to distinguish between *mur5* and WT-derived *RGP1* fragments was not feasible in this experiment because *RGP1* does not contain an SNP in *mur5*. Therefore samples were processed separately instead of mixing RNA. Following reverse transcription, three different primer sets were used for PCR for each sample: EF1 α , *RGP1* (Figure 16) and *RGP2* (Figure 15). These samples were PCR amplified at 31 cycles, 29 cycles and 27 cycles, respectively. By comparing agarose gel electrophoresis band intensities of PCR reactions having not met saturation, relative expression levels can be compared. Comparing PCR reactions that have met saturation do not provide accurate results. Because there is an equal amount of deoxynucleotide triphosphates (dNTPs) in each reaction, which is the substrate for polymerization, PCR reactions that have met saturation should have identical band intensities. Other factors can cause variable conditions in PCR reactions, such as the formation of primer dimers and non-target PCR products. Comparing PCR reactions that have not met saturation lessens the affect that these factors have on an expression analysis.

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Figures

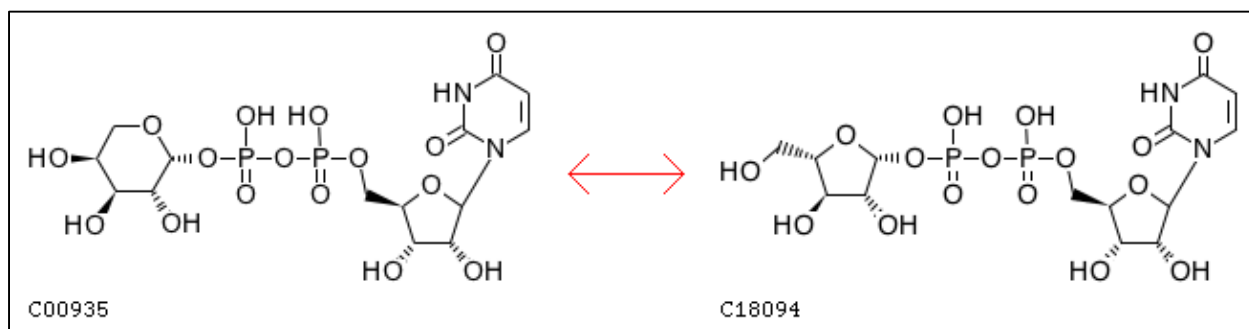


Figure 1: Interconversion of UDP-L-arabinopyranose and UDP-L-arabinofuranose

Source: http://www.genome.jp/dbget-bin/www_bget?rn:R09009

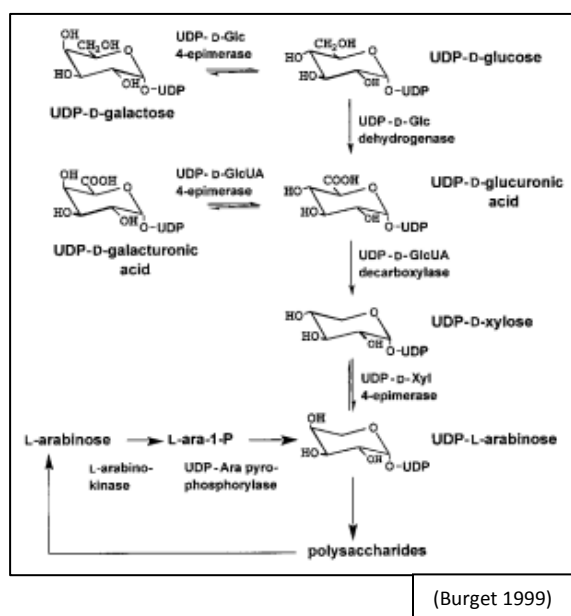


Figure 2: The *Arabidopsis* UDP-L-Ara de novo and salvage pathway.

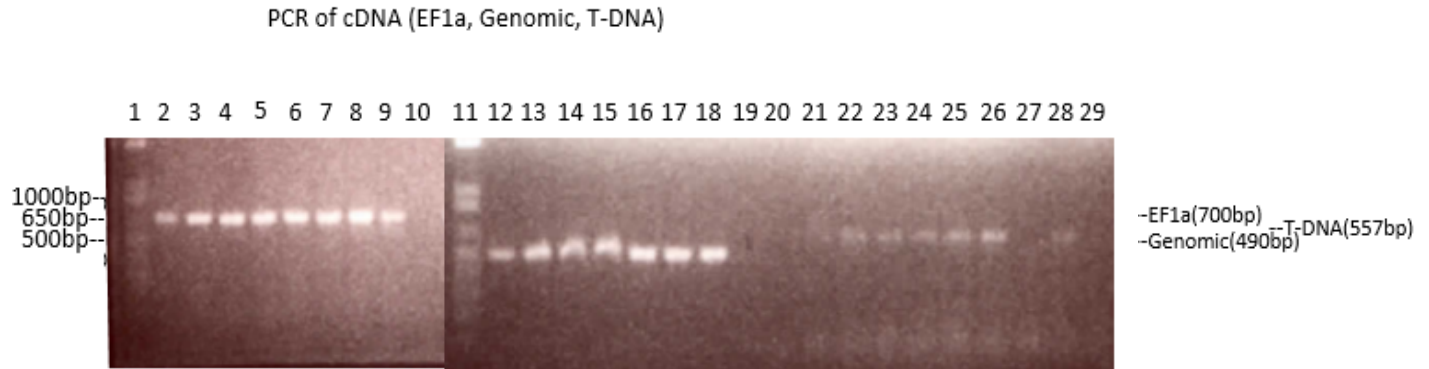


Figure 3: PCR of cDNA extracted from F_1 progeny of *mur5 x salk1*, *salk1 x mur5* crosses as well as *mur5*, *salk1* and WT controls. Three sets of primers were used. The EF1 α housekeeping gene primer set was used to measure extent of cDNA amplification and as a positive control. A genomic primer set which flanks the *salk1* DNA insertion site, which shows a band size of 490bp if not interrupted by the T-DNA. The third primer set consists of a border primer which hybridized to the 3' region of the T-DNA in combination with a right genomic flanking primer. A 557bp band will be observed if the T-DNA is present in the sample, otherwise no band will be observed. Lane 1: 1 kb plus ladder, Lane 2: *salk1 x mur5* 1 (EF1 α), Lane 3: *salk1 x mur5* 2 (EF1 α), Lane 4: *salk1 x mur5* 3 (EF1 α), Lane 5: *mur5 x salk1* 1 (EF1 α), Lane 6: *mur5 x salk1* 2 (EF1 α), Lane 7: *mur5 x salk1* 3 (EF1 α), Lane 8: *mur5* (EF1 α), Lane 9: *salk1* (EF1 α), Lane 10: WT (EF1 α), Lane 11: 1 kb plus ladder, Lane 12: *salk1 x mur5* 1 (Gen), Lane 13: *salk1 x mur5* 2 (Gen), Lane 14: *salk1 x mur5* 3 (Gen), Lane 15: *mur5 x salk1* 1 (Gen), Lane 16: *mur5 x salk1* 2 (Gen), Lane 17: *mur5 x salk1* 3 (Gen), Lane 18: *mur5* (Gen), Lane 19: *salk1* (Gen), Lane 20: WT (Gen), Lane 21: *salk1 x mur5* 1 (T-DNA), Lane 22: *salk1 x mur5* 2 (T-DNA), Lane 23: *salk1 x mur5* 3 (T-DNA), Lane 24: *mur5 x salk1* 1 (T-DNA), Lane 25: *mur5 x salk1* 2 (T-DNA), Lane 26: *mur5 x salk1* 3 (T-DNA), Lane 27: *mur5* (T-DNA), Lane 28: *salk1* (T-DNA), Lane 29: WT (T-DNA)

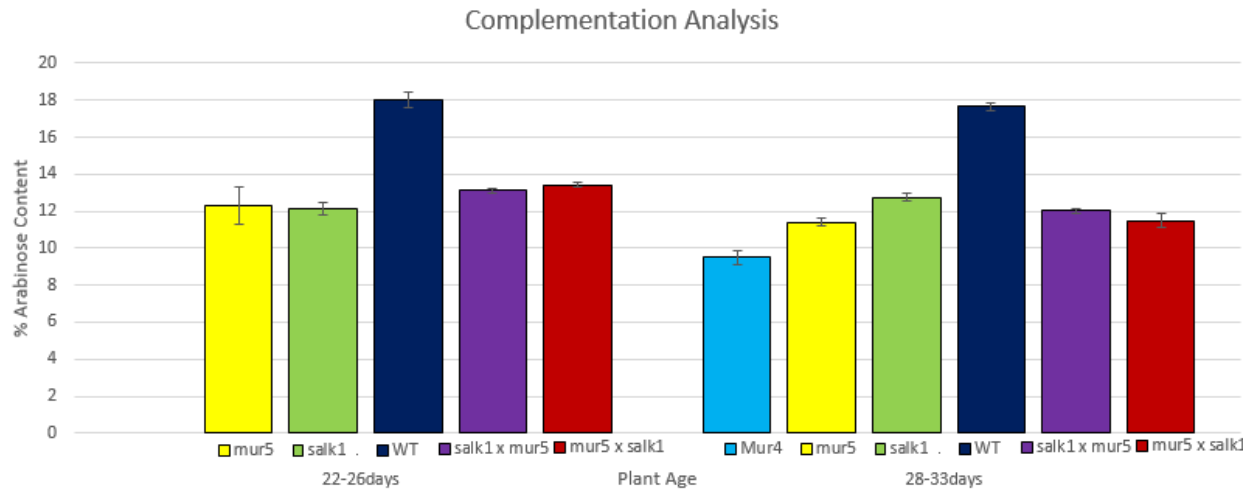


Figure 4: Histogram of relative cell wall arabinose content in the *mur4*, *mur5*, *salk1*, WT, *salk1 x mur5* F₁ progeny and *mur5 x salk1* F₁ progeny plant leaf material. All lines but *mur4* were analyzed at two ages, between 22-26 days after germination depending on timing of the young rosette stage and between 28-33 days after germination, depending on timing of the early flowering stage.

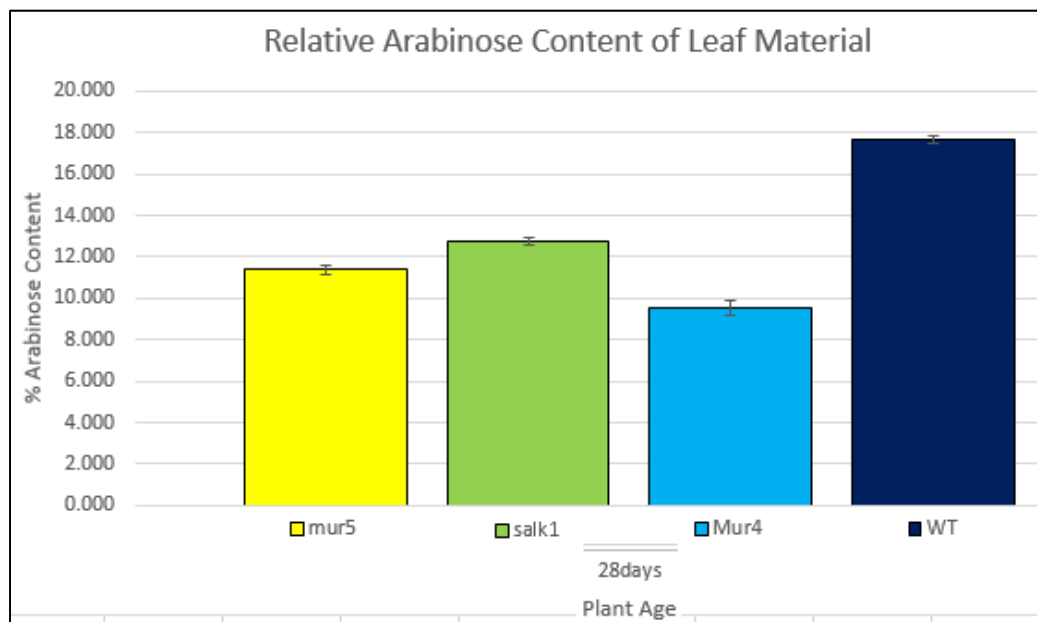


Figure 5: Histogram of relative cell wall arabinose content in *mur5*, *salk1*, *mur4* and WT plant leaf material. Leaf material was collected from plants 28 days after germination.

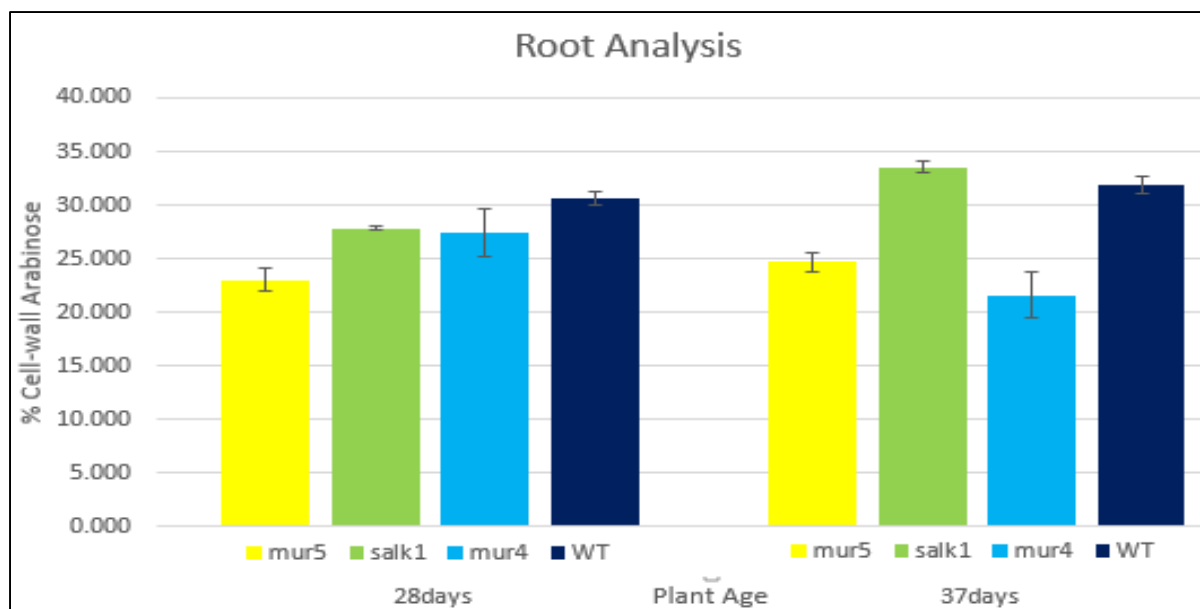


Figure 6: Histogram of relative cell wall arabinose content in *mur5*, *salk1*, *mur4* and WT plant root material. Root material was collected from plants 28 and 37 days after germination.

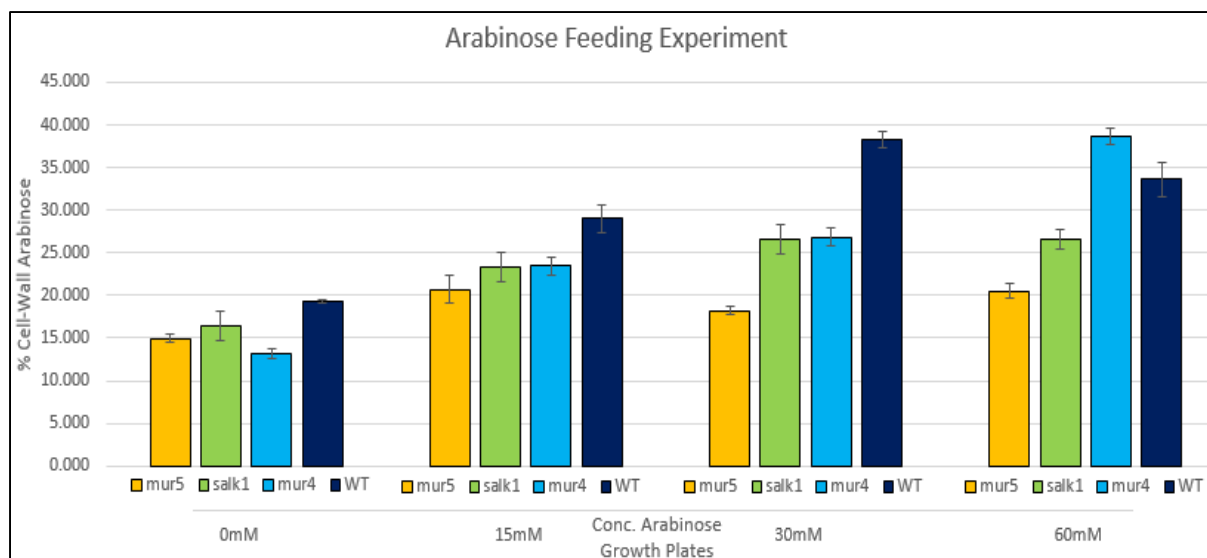


Figure 7: Histogram of relative cell wall arabinose content in *mur5*, *salk1*, *mur4* and WT plant leaf material of plants grown on growth plates containing 0, 15, 30, 60mM arabinose. Leaf material was analyzed from plants 28 days after germination.

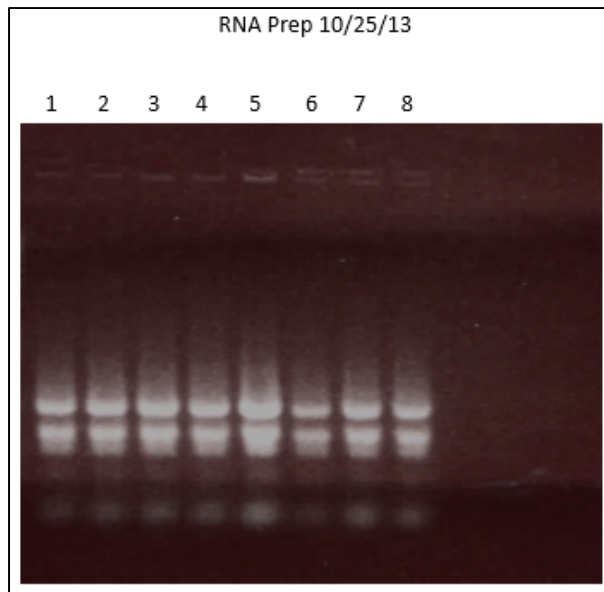


Figure 8: RNA Prep from bulk Leaf material from two pots (*mur5* & WT). Bulk leaf material from each pot split into 4 different RNA preps. Lane 1: *mur5* (M1), Lane 2: *mur5* (M2), Lane 3: *mur5* (M3), Lane 4: *mur5* (M4), Lane 5: WT (W1), Lane 6: WT (W2), Lane 7: WT (W3), Lane 8: WT (W4)

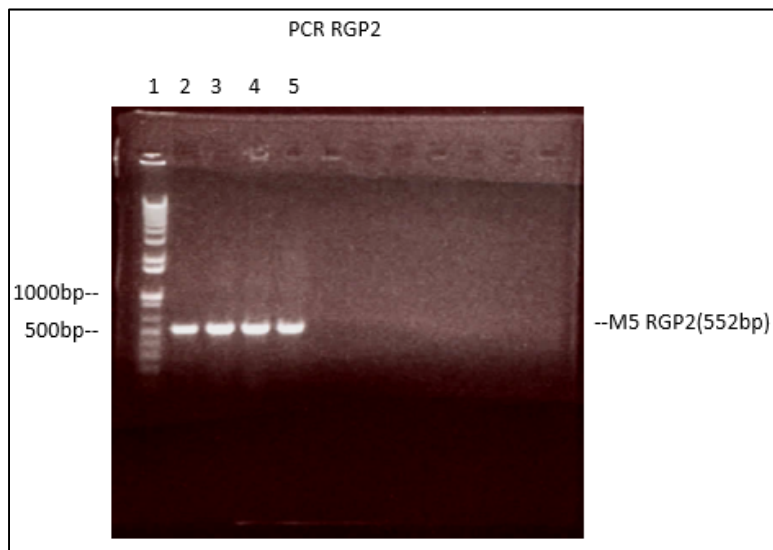


Figure 9: PCR of cDNA derived from mixed *mur5* & WT RNA samples. *RGP2*-specific primers were used with the *mur5* *RGP2* SNP locus in the center of the fragment. Lane 1: 1 kb plus ladder, Lane 2: *mur5*/WT (M1/W3), Lane 3: *mur5*/WT (M2/W4), Lane 4: *mur5* (M4), Lane 5: WT (W2)

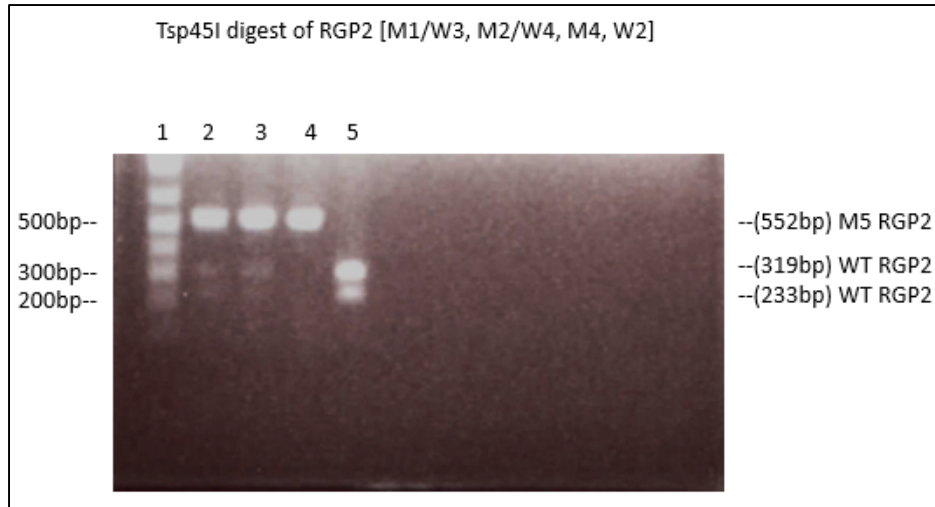


Figure 10: Tsp45I digest of *mur5*/WT mixed *RGP2* fragments. PCR products from the previous figure were digested with Tsp45I. WT *RGP2* fragments, in lanes 2, 3 and 5, are cleaved into 319 & 233bp fragments. *mur5*-derived *RGP2* fragment remain uncleaved because of the lack of a Tsp45I site. Lane 1: 1 kb plus ladder, Lane 2: *mur5*/WT (M1/W3), Lane 3: *mur5*/WT (M2/W4), Lane 4: *mur5* (M4), Lane 5: WT (W2)

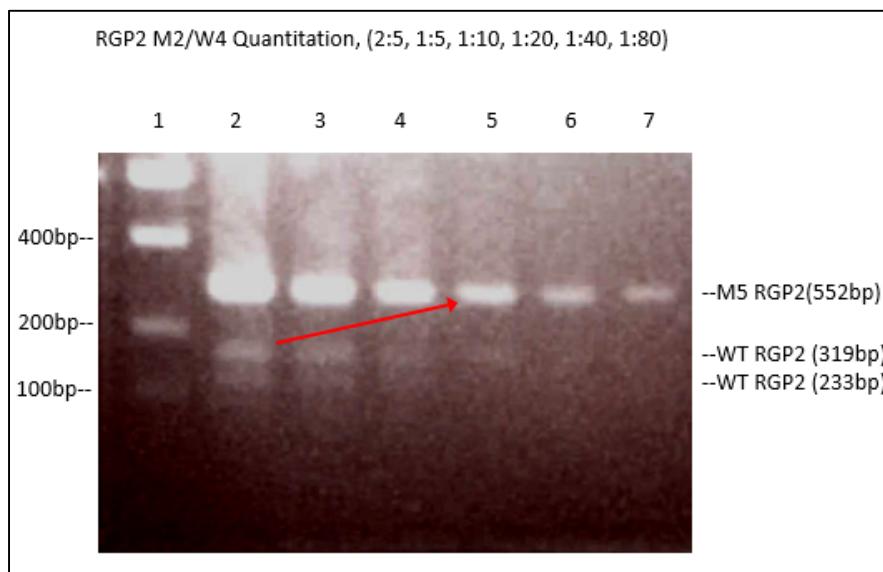


Figure 11: A serial dilution was made to better visualize the difference in band intensities between *mur5* and WT *RGP2* expression. Taking into consideration the sum of the WT-derived fragments, the WT bands in the 2:5 dilution is similar in combined band intensities to the 1:20 dilution of the *mur5*-derived band. Lane 1: Low DNA mass ladder, Lane 2: *mur5*/WT (M2/W4)

(2:5), Lane 3: *mur5*/WT (M2/W4) (1:5), Lane 4: *mur5*/WT (M2/W4) (1:10), Lane 5: *mur5*/WT (M2/W4) (1:20), Lane 6: *mur5*/WT (M2/W4) (1:40), Lane 7: *mur5*/WT (M2/W4) (1:80).

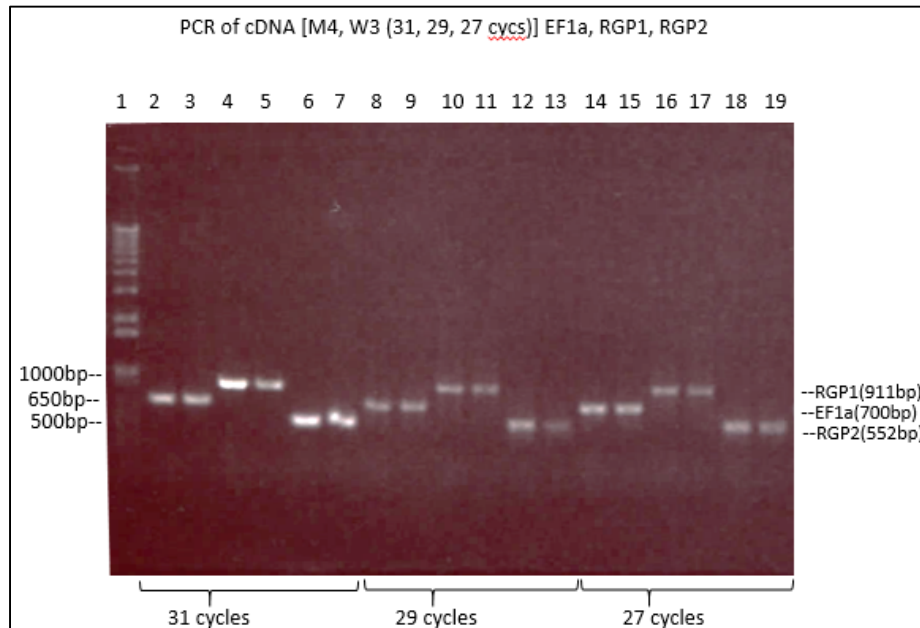


Figure 12: M4 and W3 cDNA used for PCR. Three different primer sets used for each: EF1 α (middle bands), *RGP1* (top bands) and *RGP2* (lower bands). Samples amplified at 31 cycles, 29 cycles and 27 cycles. EF1 α bands between M4 & W3 had relatively similar band intensities within respective cycle numbers. *RGP1* fragments also had similar band intensities within respective cycles. Assuming that the 31 cycle samples met saturation, the 29 and 27 cycle samples indicate a relative increase in *RGP2* expression in *mur5* samples compared to WT of the respective cycle number. Lane 1: 1 kb plus ladder, Lane 2: *mur5* (M4) EF1 α (31cy), Lane 3: WT (W3) EF1 α (31cy), Lane 4: *mur5* (M4) *RGP1* (31cy), Lane 5: WT (W3) *RGP1* (31cy), Lane 6: *mur5* (M4) *RGP2* (31cy), Lane 7: WT (W3) *RGP2* (31cy), Lane 8: *mur5* (M4) EF1 α (29cy), Lane 9: WT (W3) EF1 α (29cy), Lane 10: *mur5* (M4) *RGP1* (29cy), Lane 11: WT (W3) *RGP1* (29cy), Lane 12: *mur5* (M4) *RGP2* (29cy), Lane 13: WT (W3) *RGP2* (29cy), Lane 14: *mur5* (M4) EF1 α (27cy), Lane 15: WT (W3) EF1 α (27cy), Lane 16: *mur5* (M4) *RGP1* (27cy), Lane 17: WT (W3) *RGP1* (27cy), Lane 18: *mur5* (M4) *RGP2* (27cy), Lane 19: WT (W3) *RGP2* (27cy)

Genomic Primer Set

- Left Flanking(LP): Forward primer: 5'-TCTCTCATTTTCGAAACCATGG-3'
- Right Flanking(RP): Reverse primer: 5'-GGGCATCGTAGTCAGGGATG-3'
- Expected amplification product from WT/*mur5* genomic DNA: 1185 bp
- Expected amplification product from WT/*mur5* cDNA: 490bp



Figure 13: Primer set used for amplification of *RGP2* in *mur5* and *salk1*. The flanking primers are placed on either side of the T-DNA insertion site. Amplification of the *salk1* knockout line results in a large fragment that does not form in significant amounts under the conditions for PCR.

T-DNA Primer Set

- Border Primer: Forward primer: 5'-ATTTTGCCGATTTTCGGAACCAC-3'
- Right Flanking: Reverse primer: 5'-GGGCATCGTAGTCAGGGATG-3'
- Expected amplification product from *salk1* cDNA: 557bp



Figure 14: Primer set used for amplification of *RGP2* in *mur5* and *salk1*. Amplification on the *salk1* knockout line will result in a 557bp fragment, no fragment will be produced for amplification in other lines.

RGP2 Primer Set

- RGP2: Forward primer: 5'-CATCCCTGACTACGATGCCC-3'
- Reverse primer: 5'-TGCTCTCAAGCTTTGCCACT-3'
- Expected amplification product from RGP2 genomic DNA: 747 bp
- Expected amplification product from RGP2 cDNA: 552 bp
- Expected fragments upon cleavage of cDNA (wild type sequence) with Tsp45I: 319 bp; 233 bp

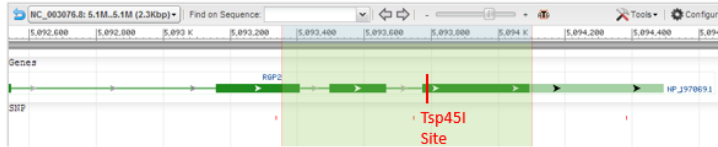


Figure 15: Primer set used for amplification of *RGP2*.

RGP1 Primer Set

- RGP1: Forward primer: 5'-CGAAGACCATTGCTGTCCCT-3'
- Reverse primer: 5'-CTGCTCAAGCTTTAGTGGGTG-3'
- Expected amplification product from RGP1 genomic DNA: 1679 bp
- Expected amplification product from RGP1 cDNA: 911 bp

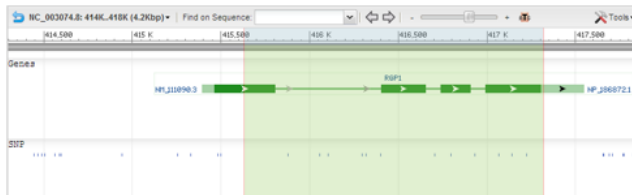


Figure 16: Primer set used for amplification of *RGP1*.