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Isolation of Arabidopsis thaliana plants homozygous for an insertional inactivation mutation within atPRP4.

Sydney Raitz
Southern Adventist University, sydneyraitz@southern.edu

Timothy D. Trott
Southern Adventist University, timtrott@southern.edu

Isolation of *Arabidopsis thaliana* plants homozygous for an insertional inactivation mutation within *atPRP4.*

Sydney Raitz

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Abstract

The AtPRP4 gene in *Arabidopsis thaliana* has been shown to function in several specific parts of the plant's cell wall. It is shown to be expressed in the seeds, radicles, roots, leaves, inflorescences, and embryos of *Arabidopsis thaliana*. These patterns have suggested unique functions for ATPRP4 in determining cell-type-specific wall structure during the development of a plant as well as contributing to defense reactions against physical damage to the plant and pathogen infection within the plant. In this study, a simple DNA prep was performed on the true leaves of *Arabidopsis thaliana*. Subsequent PCR reactions were performed using AtPRP4F/4R, AtPRP4F/Lba1, and AtPRP4F/4R/Lba1 primer combinations. The PCR products were analyzed on 1% (w/v) agarose gels in TAE, visualized by ethidium bromide staining, and imaged with a UVP EC3 imaging system. We expected to identify homozygous plants for a T-DNA insertional mutation. This work is poised to help develop a tool that will grant us the ability to examine the function of AtPRP4 inside specific cell walls as well as uncover any potential phenotypes associated with the loss of this protein found in *Arabidopsis thaliana*.

Introduction

The plant cell wall is composed of a variety of polysaccharides, proteins, and aromatic substances making it highly organized and structurally complex. Collectively, the structure of the cell wall functions as a major determinant of the plant's overall morphology (Carpita, 2001). These different components of the cell wall determine the features of individual cells found within the body of the plant. The wall plays important functional roles throughout the plant's life, including directing growth, regulating development, mediating reactions to environmental factors, and moderating pathogen and symbiont interactions. The plant cell wall develops in two major stages. First, the primary cell wall is assembled during cell division and initial growth. It has the capability of elongation to accommodate increases in cell volume during plant organ and tissue expansion. Following the elongation phase, the secondary cell wall is formed and provides mechanical support throughout the remainder of the plant's life (Borderies, 2003). Within the structure of the cell wall, unique proteins are present. These proteins bring about modifications to the cell wall that have been suggested to assist the plant during plant growth and development (Albenne, 2014).

Moreover, the proteins of plant cell walls are broken down into five protein classes; extensins, glycine-rich proteins (GRPs), solanaceous lectins, arabinogalactan proteins (AGPs), and proline-rich proteins (PRPs). Extensins are rich in the amino acids hydroxyproline and serine as well as some combinations of valine, tyrosine, lysine, and histidine. This protein is basic with isoelectric points of ~10 because of the high content of lysine. When it is deposited within the cell wall matrix, they have been shown to adopt a polyproline II helical structure and when observed using an electron microscope, they have a rodlike appearance. Extensins function in the plant's development wound repair, and defense against pathogen infections (Showalter, 1993). They also interact with transmembrane proteins and are possibly stabilized by cortical microtubules. The next class of protein is GRPs, which are characterized by the repetitive primary structure containing up to 70% glycine in an arrangement of short amino acid repeat units. GRP's are developmentally regulated with expression limited to specific cell types during particular periods of development and function within the vascular systems of a plant (Showalter, 1993). A third protein is Lectins. These proteins are carbohydrate-binding proteins or glycoproteins. They have a composition made up primarily of the amino acids hydroxyproline, arabinose, and carbohydrate. Lectins play a role in cell-to-cell interactions that includes sugar transport, stabilization for seed storage proteins, and controls cell division. Arabinogalactan proteins, AGPs are another important plant cell wall proteins. They are found to be very soluble and highly glycosylated. The last class of plant cell wall protein is Proline-Rich proteins. In soybeans, PRPs are in multiple parts of the plant's development, from germination to the early stages of nodulation (Showalter, 1993).

Also, it has been observed that in higher plants, one of the five families of structural cell wall protein is Pro-rich proteins (PRPs). These proteins were first identified by their

accumulation in the cell wall responding to physical damage to the wall (Fowler 1999). They have also exhibited temporal regulation throughout the development of various plant organs. PRP gene expression is linked with the early stages of the formation of legume root nodules (Fowler, 1999). They have been suggested to function in determining cell-type-specific wall structure during the development of a plant as well as contributing to defense reactions against physical damage to the plant and pathogen infection within the plant. When contributing to defense reactions, PRPs are rapidly insolubilized within the cell wall when responding to physical damage, treatment of fungal elicitors, pathogen infections. This indicates that PRPs are involved in the defense reactions of plants (Fowler, 1999).

Within the Pro-line rich proteins, there are different classes. The PRP class of significance for this study is the AtPRP4 gene. The structure for this gene contains a signal peptide, a non-repetitive domain, and ends in a basic domain that has Pro-rich repeats within it. They have a high degree of amino acids. In the C terminal of AtPRP4, there are eight imperfect copies of the sequence PPPKIEHPPPVPVYK and six copies of the Cys-containing motif KKPCPP in the sequence of the protein. It is suggested this protein is a highly charged polypeptide. AtPRP4 may enable disulfide bond formation between the other PRPs and possibly other proteins that are found in the plant extracellular matrix (Fowler, 1999). The AtPRP4 gene has shown to be expressed in the seeds, radicles, roots, leaves, inflorescences, and embryos of *Arabidopsis thaliana*. It has been observed that it functions throughout the developmental stages of the plant and for normal seed development that it is important (Rabb, 2007).

This project aims to help develop a tool that will grant us the ability to examine the function of AtPRP4 inside specific cell walls found in *Arabidopsis thaliana*. As a result of identifying homozygous plants for this mutation, from there it will be permissible for us to

observe these plants for any potential phenotypes associated with the loss of this protein. Plants homozygous for a mutation within the AtPRP4 gene in *Arabidopsis thaliana* can be identified by PCR screen using DNA extracted from individual seedlings.

Methods and Materials

Seed lines used

All seed stocks were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Wild type plants used, Columbia-0 (CS70,000). Mutant stock (SALK_042425) was identified within the Salk Institute Genomic Analysis Laboratory (SIGnAL) collection.

Seeding Soil with Arabidopsis thaliana

A small amount of each type of seed was mixed with 4-5 ml of 0.1% cooled agarose in a 5 ml tube. The mixture needed to be diluted enough so that only a few seeds would be in each drop. By using a 1 ml pipette, the soil was seeded by placing one drop of 2-4 seeds per drop at a time where desired. After seeding, the seeds were set in soil by watering with a squeeze bottle. The tray was covered with saran wrap and then aluminum foil and stored at 4°C for. Following cold treatment, the aluminum foil was removed, and the saran wrap covered tray was placed in a growth chamber. After the first four true leaves emerged, seedlings were transplanted to individual pots for growth, genetic testing, and seed collection.

Growth Conditions

Seedlings were grown in 3” square (7cm wide x 7cm deep x 7cm high) pots. The light intensity for the plants was 100-150 μmol/m^2/s fluorescent. During the day, the temperature the plants were kept in was 22°C and 18°C at night. Tap water was used for the plants’ water source. The system for watering the plants was gentle top water using a squirt bottle when possible and then filling the tray with a small amount of water for the bottom of the pot to sit in. The plants were watered approximately every 7-10 days when the soil was completely dry to the depth of 1 cm of the surface. A general-purpose liquid fertilizer with micronutrients and the fertilizer strength was 150-200 ppm N was used. Every other irrigation, the plants were fertilized. Fafard 3B without perlite soil less mix was used.

Simple DNA Prep

Once four true leaves had emerged, one small leaf was removed and used for individual DNA preparations. Leaves were transferred to individual microcentrifuge tubes, 350 ul of extraction buffer was added to the tube, and ground with a blue plastic pestle. The extraction buffer was prepared with 0.2 M Tris-HCl pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS, and ddH2O to final volume. Then, the tube was centrifuged at a high speed in a microcentrifuge for 5 minutes. Without aspirating debris, 350 ul of the supernatant was transferred into a clean microcentrifuge tube that contained 350 ul of isopropanol. The tube was mixed by inversion and allowed to stand at room temperature for 30-60 minutes. Afterward, it was centrifuged for 10 minutes. The liquid was poured off and the pellet was dried by inversion on a paper towel for 30-60 minutes. Once dried, 50 ul TE was added and the DNA was resuspended by vortexing and shaking test tubes 4-5 times within 30 minutes.

PCR Setup and Primers

PCR reactions were performed for AtPRP4F/4R, AtPRP4F/Lba1, and AtPRP4F/4R/Lba1 primer combinations.

LBa1 primer: 5' TGGTTCACGTAGTGGGCCATCG 3'

LBb1 primer: 5' GCGTGGACCGCTTGCTGCAACT 3'

PRP4F primer: ATGAGGATCTTACCCGAACCTCGAGGTTC (binds to the beginning of the *atprp4* coding sequence beginning with the start codon)

PPR4R primer: TCAAGGGTGTGGAGGCAACGGAGGCCATTT (binds to the end of the *atprp4* coding sequence ending with the stop codon)

The PCR amplifications were performed in a mixture containing 0.5 µl of 10 µMoles of each primer, 12.5 µl One-Taq Quick-Load 2X Master Mix (BIOLABS - M0486S) in Standard Buffer, and 1 µl of template DNA. The final volume was adjusted to 10.5 µl using distilled sterile water.

The targeted regions were amplified using a BIO-RAD C1000 Touch™ Thermal Cycler with the profiles: 94ºC for 4 minutes, followed by 40 cycles of 30 seconds at 94ºC, 30 seconds at 55ºC, 1 minute and 30 seconds at 68ºC , ended with 5 minutes at 68ºC. Then later adjusted to 94ºC for 4 minutes, followed by 40 cycles of 30 seconds at 94ºC, 30 seconds at 50ºC, 1 minute and 30 seconds at 68ºC , ended with 5 minutes at 68ºC. A second adjustment was made to 94ºC for 4 minutes, followed by 40 cycles of 30 seconds at 94ºC, 30 seconds at 45ºC, 1 minute and 30 seconds at 68ºC , and ended with 5 minutes at 68ºC.

Electrophoresis, Ethidium Bromide Staining, Imaging

PCR products were analyzed on 1% (w/v) agarose gels in TAE. Products were visualized by ethidium bromide staining and imaged with a UVP EC3 imaging system. Plants containing the T-DNA insertion will be identified by the production of a 694bp PCR product generated from the PRP4F/Lba1 primer set. Heterozygous plants, containing one wild type version of atPRP4 and one T-DNA insertion within the second atPRP4 allele, should generate PCR products with both the PRP4F/4R and PRP4F/Lba1 primer sets. Homozygous mutant plants will be identified by the presence of a product from AtPRP4F/Lba1 primers while not producing a product from AtPRP4F/4R primer combinations. The AtPPR4F/4R primer generates a PCR 1,439 bp fragment. A 694 bp fragment is expected when using the AtPRP4F/Lba1 primer sets on DNA containing the T-DNA insertion mutation.

Results and Discussion

We expected to identify homozygous plants with the AtPRP4 gene mutation present in the T-DNA insertion (Figure 1) by analyzing PCR products in an electrophoresis gel (Figure 2).

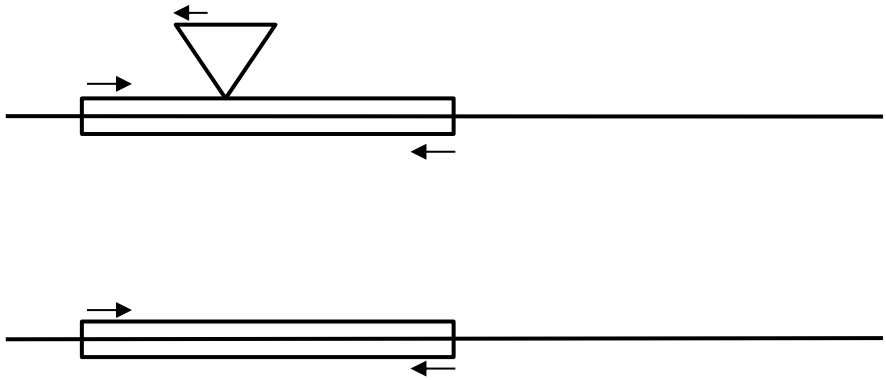

Figure 1. An illustration of the wild type AtPRP4 gene and the location of the T-DNA insertion within the AtPRP4 mutant line. The mutant has a T-DNA insertion within the PRP4 gene coding region. The presence of the T-DNA insertion will be identified by the production of a PCR product using the F and Lba1 primers. The wild type AtPRP4 gene can be amplified using the F and R primers.

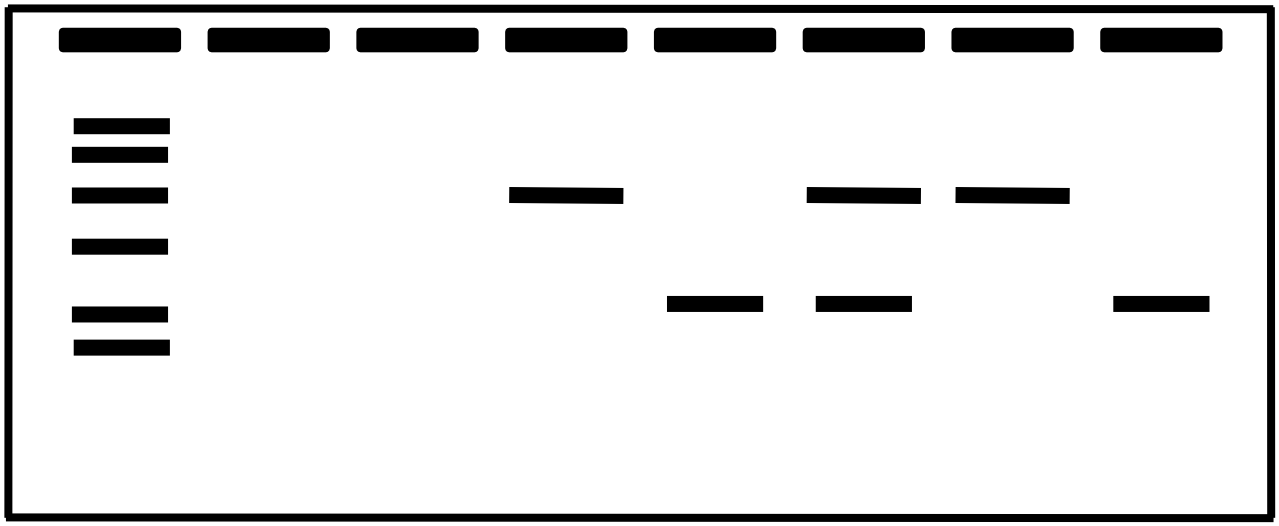

Figure 2. Illustration of electrophoresis gel containing PCR products for Arabidopsis with the gene-specific AtPRP4F primer, AtPRP4R primer, and Lba1 primer of the AtPRP4 gene. The number of base pairs is shown above the band in each lane. The first lane is the standard, followed by a no primer control lane and a no template control lane. In lanes 1, 2, and 3 all three primers were used. Lane 1 (-/-) is homozygous absent with only the wild type allele observed. Lane 2 (+/+) is homozygous present with only the T-DNA insertion observed and not the wildtype allele. Lane 3 (+/-) is heterozygous with both the wild type allele and the T-DNA insertion observed. Lane 4 contains the AtPPR4F/4R primer set. Lane 5 contains the PRP4F/Lba1 primer set.

To identify the AtPRP4 mutant gene, electrophoresis was performed for each of the 14 *Arabidopsis thaliana* plants (**Figure 3**) with the three different PCR primer combinations, AtPRP4F/AtPRP4R, AtPRP4F/AtPRP4Lba1, and AtPRP4F/AtPRP4R/AtPRP4Lba1. Less than half of the 14 plants exhibited the expected AtPRP4F/AtPRP4R primer set. Only five of the *Arabidopsis thaliana* plants: A1, A3, A4, B1, and C2 showed bands for the AtPRP4F/AtPRP4R primer set. However, there were no bands for the AtPRP4F/AtPRP4Lba1 or the AtPRP4F/AtPRP4R/AtPRP4Lba1 primer set presented on the gels of these five plants. For the remaining nine plants, none of the PCR primer sets revealed visible bands on any of the six electrophoresis gels. Notably, the standard lane in each gel appeared relatively clear. In addition, a thick smear composed of primers that were not utilized were observed at the bottom of the gel in a majority of the lanes for each electrophoresis conducted (**Figure 4**).

Figure 3. The *Arabidopsis thaliana* plants were stored in the growth chamber in two layered trays. The plants were labeled by groups (A, B, and C) and each plants were numbered when put into their individual pots within the group. As shown above there were 4 plants in groups A and B. In group C, there were 6 plants.

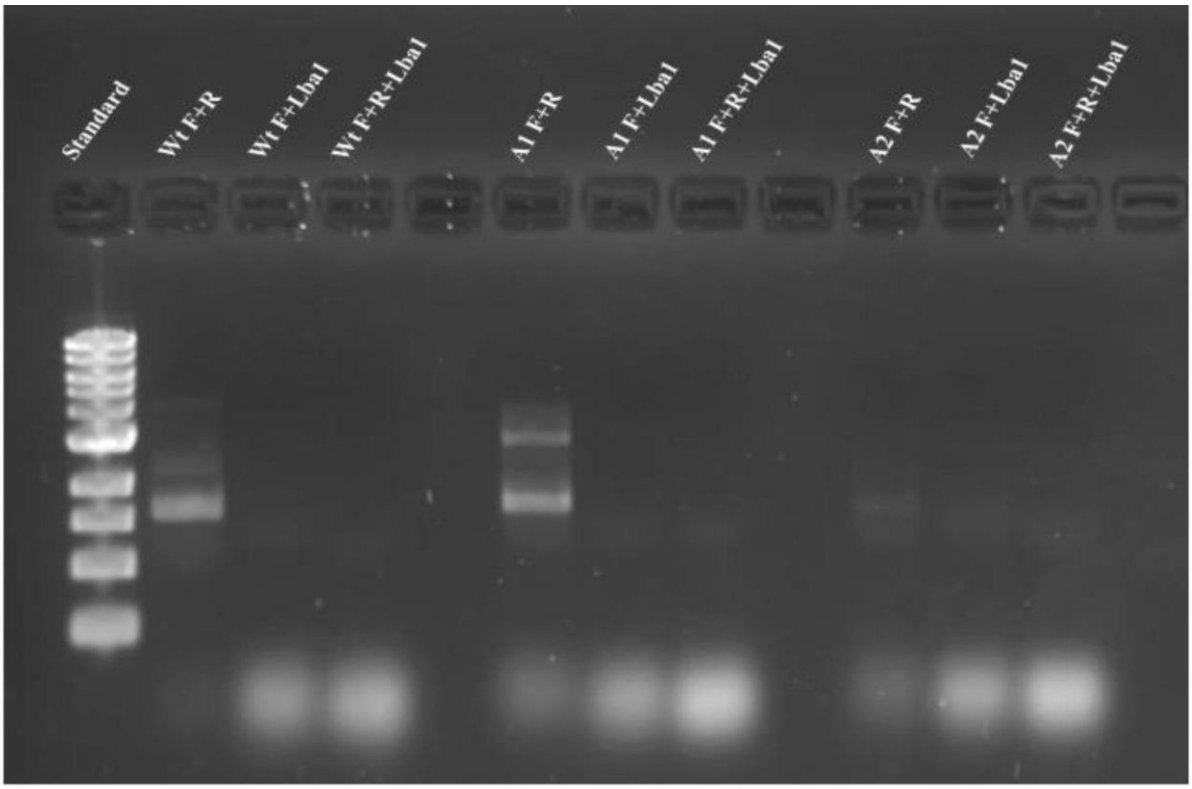

Figure 4. This is an image of the electrophoresis gel containing PCR products of *Arabidopsis thaliana* and the first lane contains the standard that was used. Three bands appeared on the lane containing PCR product of the wild type DNA with forward and reverse primers (Wt. F+R). Two bands appeared on the lane containing PCR product, DNA with forward and reverse primers from plant 1 in group A (A1 F+R).

As summarized in Table 1, less than half of the 14 plants exhibited the expected AtPRP4F/AtPRP4R primer set. The results obtained from the --six electrophoresis gels performed using the 14 *Arabidopsis thaliana* plant's DNA with the three different PCR primer combinations were inconclusive. Therefore, the expected results were not obtained, and the AtPRP4 mutant gene was not identified.

Plant Number

	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4	C5	C6
4F + 4R	+	–	+	+	+	–	–	–	–	+	–	–	–	–
4R + Lb1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4F + 4R + Lb1	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 1. This is a visual representation of which primer set presented bands on the gel (+) and which primer set did not show any bands (–) for each plant.

With the aim of establishing better results, the PCR annealing temperature was adjusted, new primer sets were made and one last electrophoresis was performed. DNA from plant A1, A4, and B1 were loaded into the gel with the newly made primers. These adjustments showed no significant change to the results for the A4 lanes. In contrast, A1 and B1 F+R lanes did not show any bands like observed in the gel prior to these adjustments being made. Due to the insufficient data, the answer to what the functional role AtPRP4 protein plays within plant cell walls remains unclear.

The results might suggest that the *Arabidopsis thaliana* plant DNA used contained contaminants. Since the method used for DNA preparation was very simple and quick, it did not yield very clean DNA Thus, it would negatively affect the PCR. As previously described, the PCR conditions used were inadequate and lead to the primers inability to bind to the sequence of DNA necessary for the expected results.

The results of this research has enabled the opportunity for further research to be carried out. For the purpose of continuing the search for the AtPRP4 mutant gene, all 13 surviving plants had been dried out in large plastic bags for seed collection. Each individual plant's seeds were stored in a labeled microcentrifuge tube for future work. With this in mind, future studies should make modifications to the PCR conditions, like adjusting the annealing temperature of the primers to optimize primer binding. Also, using a new commercial DNA extraction kit, which yields cleaner DNA. Subsequently, the seeds acquired from this project's *Arabidopsis thaliana* plants can further investigate and identify homozygous plants with the AtPRP4 gene mutation. Thus, the goal is to establish a AtPRP4 null homozygous seed line and to identify any potential phenotypes that are associated with the loss of this protein. Ultimately, defining the function of this protein in *Arabidopsis thaliana.*

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