Analysis of Promotor Regions of Arabidopsis and Melaleuca *APETALA 1* Genes in Transgenic Plants

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Abstract

Because it grows quickly, and flowers early and frequently, *Melaleuca quiquenervia* has become a prominent invasive species in Southwest Florida Using knowledge regarding the expression of flowering genes in Melaleuca, it may be possible to alter flowering in other species. Flowering genes of interest are *APETALA1* (*AP1*) in the model species *Arabidopsis thaliana* and a homolog in Melaleuca: *MqAP1*. To compare activities of the regulatory regions of these genes, a construct had been made including the promoter region of the Arabidopsis *AP1* (*AtAP1*) upstream of the β-glucuronidase (GUS) reporter gene. This construct had been introduced into Arabidopsis through Agrobacterium transformation. Transgenic plants were selected on kanamycin-containing medium. Green seedlings were transferred to soil and DNA extracted from leaves. PCR was performed to confirm that the kanamycin-resistant plants were transgenic. To compare promoter activities between transgenic plants containing GUS fused to the *AtAP1* or the *MqAP1* promoters, the use of plants homozygous for one insertion of the constructs will be necessary. Transgenic plants were self-pollinated and seeds are being plated on kanamycin medium. Chi-square analysis is used to identify plants that have one insertion. Once the *MqAP1* gene is fully sequenced, the same procedure will be used to study expression from the *MqAP1* promoter.

Introduction

Melaleuca quinquenervia is an invasive species affecting South Florida, endemic to Australia. It flowers much earlier than other plants of its size, resulting in its rapid spread. Because it can flower in under 3 years after germination, it requires specific control measures, including controlled burns every two years, along with the targeted use of herbicide (Woodall 1983). The study of what enables this plant to flower so early could be highly beneficial to its control and to conservation efforts. In addition, this understanding may benefit the ability of the biotech sector to create crops that produce fruit faster, leading to wide agricultural applications.

The MADS box genes are a set of genes with conserved sequences, many of which have been shown to be involved in flowering in many species of plants. Specifically, some serve as homeotic genes in flower development. This means they control how certain parts of the flower form (Himi et al. 2001). Certain MADS box genes were shown to play a role flowering time. (Molinero-Rosales et al. 1999). The *APETALA 1 (AP1)* gene from the model species *Arabidopsis thaliana, AtAP1,* is an example of one of these genes. In Arabidopsis, *AP1* activates floral meristem identity genes in conjunction with *LEAFY* and *CALIFLOWER* (Han & Jiao 2015).

A MADS-box gene from *Melaleuca quinquenervia* with high homology to *AtAP1* has been recently identified in the laboratory. In order to analyze if expression of this gene contributes to the early and frequent flowering in Melaleuca, we want to compare its expression with that of the *AtAP1*.

Arabidopsis plants were transformed using *AtAP1pro-GUS,* which has the coding region of β-glucuronidase fused to a 1160 bp fragment of DNA known to be a working promoter for *AtAP1 (Ye et al. 2016)*. GUS, a reporter gene also called β-glucuronidase, enables the activity of the encoded enzyme and therefore location of expression of the gene to be quickly identified. Since the vector used contains a kanamycin resistance gene, transgenic plants were selected by germinating the seeds on media with kanamycin. To confirm that green seedlings from the kanamycin-containing plates are transgenic, we extracted DNA from them and performed PCR.

The use of transgenic plants will be essential to determine environmental effects on *MqAP1* expression, and differences these effects may have on *MqAP1* versus *AtAP1*. Analysis of GUS expression in plants transformed with *MqAP1* promoter-GUS and grown in different conditions will allow us to study the different factors that contribute to Melaleuca's unique flowering traits. To be able to compare GUS activity between transgenic plants we must have plants with the same number of copies of the transgene inserted in different genomic locations. We will use homozygous plants with a single insertion to analyze GUS expression. Plants that have a single insertion of the transgene are selected by a 3:1 segregation of resistance: sensitivity to kanamycin. These plants are allowed to self-pollinate and homozygous progeny plants producing 100% kanamycin resistant plants will be selected.

Methods

DNA extraction from transgenic plants

Arabidopsis flowers were exposed to *Agrobacterium tumefaciens* to generate transformed seeds. Seeds suspected to be transformed were plated on selective media containing kanamycin and timentin. Green seedlings growing on this medium were potted and had leaf samples collected for DNA extraction. Leaves were ground in an extraction buffer (100 mM Tris HCl, 100 mM NaCl, 20 mM EDTA, 1% sarkosyl) in order to lyse cell membranes. The DNA was purified with 3 phenol-chloroform extractions. Ethanol and NaCl were then added to precipitate the DNA from the aqueous solution. The DNA was dried, resuspended in water, and treated with RNAse. The phenol-chloroform extractions, ethanol precipitation, and resuspension were then repeated.

PCR amplification

To perform PCR, a forward primer was created complementary to the *AtAP1* promoter, and a reverse primer was created complementary to the GUS coding region. These primers were combined with genomic DNA, PCR master mix, and Taq polymerase. This mixture was subjected to an initial denaturation of the double stranded DNA at 94°C for 5 minutes, then to 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, followed by 10 minutes at 72°C. The amplified DNA was analyzed by agarose gel electrophoresis.

Figure 1. Diagram of the GUS expression construct. The arrowheads show location of PCR primers used to confirm plants that showed resistance to kanamycin were transgenic for *AtAP1* promoter and GUS coding region.

Selection of single insertion homozygous individuals

Plants confirmed to have the insertion by PCR were grown to maturity and allowed to self-pollinate. The seeds collected from these plants were planted on plates of agar containing kanamycin. Seedlings on the plates were allowed to grow until they were big enough to distinguish between resistant and sensitive seedlings. A plant containing one insertion is expected to have a 3:1 ratio of offspring with the insertion to without the insertion. Plants that do not contain the insertion are white and small on kanamycin media. After counting, χ^2 analysis was done to determine if the progenies met the expected ratio (χ^2<3.841). A few seedlings from a plant confirmed to be heterozygous for one insertion were moved into potting soil and allowed to grow to maturation and be self pollinated. Cross pollination was prevented by surrounding the plant with a plastic tube. Seeds collected from these plants will be transferred to kanamycin media once again. Homozygous individuals will have progeny that are all green on these plates.

Results

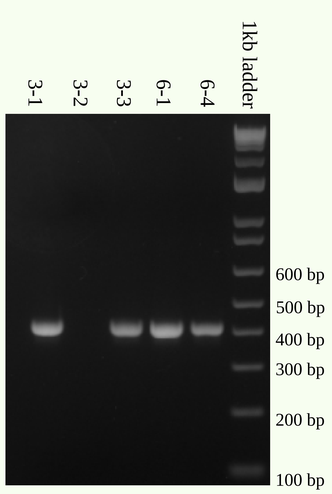
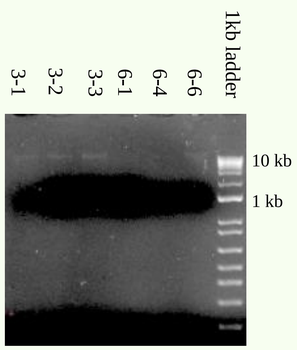


Figure 2 (left). Imaging of gel electrophoresis results for PCR of DNA extracted from plants 3-1, 3-2, 3-3, 6-1, and 6-4. The fragment is 414 bp and is amplified at the expected size.

Figure 3 (right). Gel electrophoresis of genomic DNA from plants 3-1, 3-2, 3-3, 6-1, 6-4, and 6-6. DNA from 6-1 and 6-4 are present in very small amounts, but enough that they were able to replicated by PCR. 3-2 is present here but isn't present in PCR.



Plant	Green	White	χ^2	PCR
AP1 2-1	188	15	33.578	
AP1 2-2	50	0	16.66667	
AP1 3-1	110	32	0.460094	+
AP1 3-2	115	29	1.814815	-
AP1 3-3	166	61	0.424376	+
AP1 3-4	172	26	14.87542	
AP1 3-5	371	25	73.75084	
AP1 3-6	238	7	64.06667	
AP1 4-1	0	41	123	
AP1 5-1	109	3	29.7619	
AP1 5-2	130	9	25.44125	
AP1 5-3	179	10	39.1552	
AP1 6-7	73	2	19.95111	
AP1 6-1	291	88	0.641161	+
AP1 6-2	192	41	6.811159	
AP1 6-3	124	109	58.95422	
AP1 6-4	243	78	0.084112	+
AP1 6-5	115	7	24.14208	
AP1 6-6	59	0	19.66667	

Figure 3 (right). Chart with chi square values for different plants. Experimental values closer to the expected ratio of 3:1 of kanamycin resistant to kanamycin sensitive plants result in a lower χ^2 value. If χ^2 < 3.841, we fail to reject the hypothesis that the plants are heterozygous for a single insertion of the transgene.

Discussion

- Plants have been confirmed to contain a single insertion of AtAP1proGUS; however, more work is required to create an insertion for MqAP1. Furthermore, the GUS assay still needs to be conducted on these plants.
- This procedure has so far demonstrated that 4 plants are transgenic for a single heterozygous *AtAP1* insertion. These plants come from no less than 2 independent transformation events. It is not currently confirmed whether related plants (i.e. 3-1, 3-2, and 3-3; and 6-1, and 6-4) have the insertion in the same location
- Plants have been detected that grow on the kanamycin in the correct ratio of 3:1 but don't show DNA in the selective PCR. This may be because the Insertion was not completely inserted into the PGA42 vector.
- More work needs to be conducted on the flowering mechanisms of *Melaleuca quinquenervia*. This body of work may help scientists assess how the distribution of *Melaleuca* will change as climate change progresses. Furthermore, the application of these mechanisms may help in agriculture sector to get more fruits from plants earlier, and by the conservation sector to assess how to effectively control melaleuca where it is invasive.

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