DETERMINE PHYTOENE DESATURASE GENE COPY NUMBER IN HYDRILLA VERTICILLATA

by

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IN HYDRILLA VERTICILLATA

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ABSTRACT

Hydrilla verticillata is an invasive aquatic weed in the United States (U.S.) that has recently developed resistance to the herbicide fluridone. In this study, we utilized genome walking and quantitative real-time PCR to investigate the phytoene desaturase (PDS) gene copy number of hydrilla samples with different ploidy levels. We asked 1) if copy number simply corresponds to the ploidy level, and 2) if there is increased PDS copy number in resistant populations due to gene duplication. Using qPCR and microsatellite loci to compare PDS copy number between diploid, triploid and tetraploid samples, we found that diploid hydrilla from Africa showed higher PDS copy number than triploid populations from the U.S. The results also indicated that there was no significant difference in PDS gene copy numbers between the fluridone-resistant and -susceptible triploid populations. Our study suggests that PDS amplification may not be a mechanism responsible for fluridone resistance in hydrilla.

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TABLE OF CONTENTS

ABSTRACT 3

ACKNOWLEDGEMENT 4

INTRODUCTION 6

MATERIALS AND METHODS 8

1. Karyotyping for determining ploidy level of standard sample 8
2. DNA extraction of the standard sample 8
3. Genome walking-based method for determining PDS copy number 8
4. Quantitative real-time PCR for determining PDS gene copy number 10

RESULTS 11

1. Ploidy level of standard sample 11
3. Quantitative real-time PCR 12

DISCUSSION 12

TABLES & FIGURES 17

REFERENCES 20

INTRODUCTION

Invasive plant species have become a serious problem in the United States, costing billions of dollars annually in control efforts and damaging native ecosystems (Pimentel et al. 2005). Due to their high level of endemism and isolation, freshwater ecosystems are especially vulnerable to ecological damage by invasive aquatic plants (Richter et al. 1997; Dudgeon et al. 2006). Besides threats to freshwater biodiversity, exotic aquatic plants also cause substantial economic loss for agriculture, forestry, fisheries, power production, water utilities, and international trade (Lovell 2006). Such extensive environmental and economic impacts call for actions by federal agents to develop and conduct procedures for weed management.

Herbicide is considered the most effective technique in aquatic weed management (Puri 2007a). However, intensive and repeated treatments of weeds with the same herbicide has led to the evolutionary development of herbicide resistance in many weed populations (Jasieniuk et al., 1996). Since the first serious case of herbicide resistance was discovered in 1968 in common groundsel (*Senecio vulgaris*) (Ryan 1970), herbicide resistance has increased substantially worldwide and become a major threat to this management practice (LeBaron et al., 1991).

Herbicide resistance can be categorized into two types: target site resistance and non-target site resistance (Powles and Yu 2010). In the former type, herbicides enter the plant and inhibit specific enzymes that are essential for important metabolic pathways in the plant. Resistant plants possess genetic mutations that alter these enzymes. Such a modified enzyme still binds to its substrate and remains functional in the metabolic pathways, yet its conformational change may prevent interaction with and inhibition by the herbicide (Gressel 2009). Target-site resistance can also occur via overexpression of the targeted enzyme, which can overcome a normally lethal dose of the herbicide. Non-target-site herbicide resistance encompasses any mechanism that is not directly associated with the target site, but inhibits a lethal dose of herbicide from reaching its target site. Examples are impeded herbicide infiltration into the plant, reduced rate of herbicide translocation, and enhanced rate of herbicide metabolism (Powles and Yu 2010).

Hydrilla verticillata is an invasive aquatic species that was first discovered in the United States in 1960 in Florida (Blackburn et al. 1969). Since then two biotypes of hydrilla, one a female dioecious (plants having female flowers only) and the other monoecious (plants having male and female flowers on the same plant), have been found in the U.S. Genetic analyses suggested evidence for two separate hydrilla introductions: the dioecious biotype was introduced from Sri Lanka into Florida, and the monoecious biotype in the U.S. originated from South Korea (Madeira et al. 1997). While dioecious hydrilla adapts better to warmer climates, the monoecious form shows optimal growth in cooler climates (Ames et al. 1986; Steward and Van 1987). Together these biotypes have spread throughout the U.S. rapidly and infested water bodies in approximately 21 states (Robert 2008).

Hydrilla is considered "the perfect aquatic weed", for it possesses multiple characteristics of a competitive and effective colonizer. The submersed plant has a highly specialized growth habit that allows it to grow in an environment with just 1% of sunlight, low nutrients, and a wide range of water chemistry conditions. Its rapid growth rate and several asexual, vegetative reproduction mechanisms, including fragmentation, stolons, tubers, and turions, helps hydrilla quickly grow to high density, effectively compete for resources and easily disperse to new water bodies via humans, water fowl, and water movement (Langeland 1996, Robert 2008). These attributes allow hydrilla to outcompete and displace native species, block irrigation canals, increase sedimentation and flooding, impair water supplies, and disrupt the natural balance of the native fishery communities (Langeland 1996, Lovell et al. 2006). For these reasons, hydrilla is considered the most economically damaging aquatic weed in the United States (Robert 2008).

What makes hydrilla even more damaging is its herbicide resistance to fluridone, the systemic herbicide that has been intensively used for large-scale hydrilla management. Fluridone is a non-competitive inhibitor of the enzyme phytoene desaturase (PDS), a chloroplast protein encoded by the nuclear gene PDS (Bartley et al. 1991). It catalyzes the conversion of a colorless precursor phytoene into the colored carotenoid zeta-carotene, which is a critical, rate-limiting step in carotenoid biosynthesis in the isoterpenoid pathway (Chamovitz et al. 1993). Carotenoids play an important role in protecting the thylakoid membrane from light-dependent oxidative damage induced by high-light and high-temperature conditions by quenching the excess excitation energy (Davison et al. 2002). Fluridone acts to inhibit PDS enzyme activity, which deactivates carotenoid synthesis in developing tissue, leading to the destruction of chlorophyll molecules and eventually plant death (Benoit & Les 2013, Puri et al. 2006). With this mechanism, fluridone had offered an efficient solution to control hydrilla at a large scale without harming native vegetation until the first fluridone resistant hydrilla biotype was observed 16 years ago in Florida (Puri et al. 2006). Today, this resistant biotype has become dominant in many water bodies in Florida.

Only the female dioecious biotype of hydrilla is found in the lakes of Florida, and it can reproduce via asexual means in the absence of male flowers. Asexual reproduction places plants under strong uniparental constrains and renders the evolution of herbicide resistance unlikely. Therefore, the discovery of fluridone resistance in dioecious populations in Florida was unanticipated (Michel et al. 2004). In vitro assays by Michel et al. 2004 reported that three-point mutations at the arginine 304 codon of the PDS gene, which are serine (AGT), cysteine (TGT) and histidine (CAT), confer three levels of fluridone resistance: low, intermediate and high resistance respectively. Another study by Puri et al. 2007b suggested that the levels of fluridone resistance, however, exist in a continuous range rather than three distinctive variants. Besides mutations on the PDS gene, gene duplication could be a possible explanation for the variations in its adaptation to the herbicide. Resistant hydrilla are found to exist as diploids ($2n = 2x = 16$), triploids ($2n = 3x = 24$) and tetraploids ($2n = 4x = 32$) (Puri et al. 2007b). The PDS gene can either get amplified along with genome duplication via polyploidy, or the locus could be

duplicated elsewhere in the genome. Such events can potentially lead to increased frequency of the resistance alleles as well as amplification of PDS gene expression to a level that may overcome the effects of fluridone (Puri et al. 2007b, Jasieniuk et al. 1996).

In this study we asked two questions: 1) whether the number of PDS genes corresponds to the ploidy levels in hydrilla and 2) whether PDS may have been amplified in resistant hydrilla. Using quantitative real-time PCR (qPCR) along with microsatellite information, we sought to compare the PDS gene copy number between diploid, triploid and tetraploid hydrilla. We were also interested in determining whether there was a difference in PDS copy number between the resistant triploid population from Florida and the susceptible triploid population from Texas. This difference could indicate PDS amplification as a potential contributor to the observed continuum of resistance. In addition, we applied a genome walking-based technique to sequence the flanking region of the PDS gene to determine if the PDS gene may have been duplicated elsewhere in the genome.

MATERIALS AND METHODS

1. Karyotyping for determining ploidy level of standard sample

We used karyotyping to identify the ploidy level of a fresh hydrilla sample from Texas. This standard sample with known ploidy level serves to create a standard qPCR curve for one of the three ploidy levels observed in hydrilla and to confirm the ploidy level of Texas samples.

We collected hydrilla root tips between 7am and 9am when cell proliferation occurs at a high rate to obtain the most metaphase cells. Collected root tips were soaked in Carnoy's fixative at 60°C for 15 minutes, then transferred to and stored in 70% EtOH. A 1-millimeter long root tip section was placed on a slide and slightly lacerated, then treated with hydrochloric acid for 5 minutes, in an enzyme mixture (1.5% cellulase, 1.5% pectolyase in citric acid buffer) for 1 hour, and in aceto-orcein dye for 10 minutes. The root section was rinsed with water in between the treatments. The slide with root tip cells submersed in dye was then heat-fixed. A cover slip was placed over the slide, and the squashing method was applied to spread out the cells for better visualization. Slides were observed under a light microscope within 24 hours. Chromosome counts were obtained from 29 metaphase cells from different samples from the same clone.

2. DNA extraction of the standard sample

DNA was extracted from fresh hydrilla collected from the Texas population using the IBI Genomic DNA Plant Minikit according to the manufacturer's instructions. This is the sample whose ploidy level had been confirmed as triploid using karyotyping. This sample was the DNA used for genome walking and the known control sample for qPCR.

3. Genome walking-based method for determining PDS copy number

We designed two different primers, GW_F and GW_R, using Primer3 (V 0.4.0, www.bioinfo.ut.ee)(Table 1). Each of these primers anneal to a sequence that is approximately 60-70 bp away from each end of the complete cDNA sequence of the PDS gene (GENBANK accession number AY639658.1). The direction of DNA synthesis was towards the ends of the PDS sequence.

DNA extracted from the standard samples was digested for 4 hours at 37°C with four-base cutters *Alu I* and *Rsa I* to cut the genome into many small fragments. The studied cDNA sequence of PDS was analyzed to ensure there were no recognition sites of *Alu I* and *Rsa I* on the sequence. The success of the digestion was confirmed by performing gel electrophoresis. Cut DNA was cleaned with the Zymoclean Gel DNA Recovery Kit (The Epigenetics Company) to remove all restriction enzymes. We then ligated the cleaned DNA fragments with a 20 bp adapter called SNX linker whose ligation was specific to the blunt ends generated by the restriction enzymes. A PCR (polymerase chain reaction) amplification with the adapter primer was conducted with diluted, ligated DNA to see if a smear would appear, indicating the success of the ligation. All PCR reactions for this method was set up as 10 µl reaction containing 1 µl of DNA, 1 µl of primer mix (concentration of 5 µM for each primer), 5 µl of Qiagen multiplex PCR Master Mix, 0.2 µl BSA, and 2.8 µl dH_2O.PCR reactions were run for 15 min at 95°C; 30 cycles of 30 s at 94°C, 1.5 min at 60°C, 1 min at 72°C; and 5 min at 72°C.

Both the DNA sample amplified with SNX linker primer and the non-amplified one underwent PCR amplification with either the GW_L primer or the GW_R primer. PCR products were run on a gel and produced multiple bands on each lane for some samples. Each individual band was punched out from the gel and place into a 1.5 mL Eppendorf tube containing 100 µL of 10mM Tris. Tubes were vortexed and heated at 60°C for 15 min to release DNA from the gel into the solution, then centrifuged for 5 min to pellet the gel on the bottom of the tube. This gel punch step served to eliminate non-targeted DNA fragments ligated to the adapter. The supernatant containing DNA underwent another PCR amplification with either the primer set of SNX linker primers and GW_L or the set of SNX linker primer and GW_R, depending on what previous PDS primers were used in the previous amplification. PCR products were run on a gel and another gel punch was done to retrieve DNA material from each band on the gel. The extracted DNA underwent nested PCR amplification (Fig. 2) with either the primer set of SNX linker primers and GW_nestL or the set of SNX linker primer and GW_nestR, depending on whether GW_L or GW_R primers were used to generate the amplicons. Gel punch and PCR amplification of the extracted DNA using the same set of primers were repeated until we completely separated different DNA fragments from one another on a gel.

Sequencing of the PCR products was done by ligation into pGEM vectors (Promega), cloning with JM109 competent cells (Promega), and sequencing using vector primers and the Bright Dye Sequencing Kit (MCLAB). Sequences were then electrophoresed on an ABI 3130XL Genetic Analyzer (Life Technologies). Sequences were trimmed of vector sequences and low quality reads and put into contigs using Sequencher v. 5.0 (GeneCodes).

4. Quantitative real-time PCR for determining PDS gene copy number

a. Preparing samples

DNA of all samples except for the known control were extracted from fresh hydrilla 10 – 15 years ago and stored at 4ºC. Samples were classified as diploids, triploids and tetraploids using microsatellite loci in a previous study (Williams unpublished data). We classified individuals with two, three, and four alleles at a locus as possible diploids, triploids, and tetraploids respectively. Diploid samples were from East Africa, where hydrilla populations were known to be diploid both from karyotyping and microsatellite analyses (Pieterse et al. 1985, Williams unpublished data). Triploid samples were comprised of two groups: fluridone-susceptible Texas hydrilla, and fluridone-resistant Florida hydrilla. All tetraploid samples were from the resistant Florida population.

We used 18S ribosomal RNA as the housekeeping gene. DNA concentration of all samples was measured via the absorbance at 260 nm on a Nanodrop spectrophotometer. The purity of the samples was analyzed via the 260/280 nm ratio. Samples with 260/280 ratio between 1.7 and 2.1 were chosen for qPCR. All samples were diluted to a standardized concentration of 3 ng/µL.

b. Designing primers

Primers were designed with Primer3 (V 0.4.0, www.bioinfo.ut.ee). We designed two pairs of primers for each of the following regions: 18S ribosomal RNA (GENBANK accession number KM982361.1), exon 1, and exon 2 (which correspond to the second and third exons in the PDS sequence in Tallent 2012) of the PDS gene. Sequences and sizes of primers are detailed in Table 1.

We ran regular PCR amplification using the same cycle number (40x) for qPCR on all primers pairs to confirm that each pair only gave rise to one PCR product. Under this condition, both the primers for exon 1 and the Ex2_a primer were removed from the experiment because there was more than one product on the gel (Fig. 3). 18S_b and Ex2_b primer pairs were chosen for qPCR analysis.

c. Setting up quantitative real-time PCR reaction

We ran the qPCR amplification reactions of each sample in triplicate. Each reaction contained 5 µL of Power SYBR® Green Master Mix (Applied Biosystems), 1 µL of primers (primer concentration of 3 µM each), 0.2 µL BSA and 2 µL of DNA (6 ng of DNA in total), and were brought to a final volume of 10 µL with dH_2O. 1 µL of primers were deposited into each well of the 96-well reaction plate. A master mix containing SYBR, DNA, BSA and dH_2O with volume enough for 6.5 reactions was prepared for each sample. 9 µL of the master mix was then pipetted into each of the 6 wells containing 18S_b or Ex2_b primers for that single sample. We conducted amplification in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with

the following setup: a holding stage of 10 min at 95°C; 40 cycles of 15 s at 95°C and 1 min at 59°C; and a melt curve stage of 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C.

d. Data analysis

The StepOnePlus™ Real-Time PCR System instrument provides the cycle number at which the accumulation of fluorescence from PCR products of a particular reaction crosses the threshold (C_t) into an exponential phase of amplification. Relative gene copy numbers were obtained using the comparative C_t ($2^{-\Delta\Delta Ct}$) method. The PDS C_t value was compared to the 18S value of each sample to the level of total nucleic acids present, to yield $\Delta C_t = C_{t\,(PDS)} - C_{t\,(18S)}$. Because the copy number of the endogenous control gene per genome remains constant, a change in ΔC_t corresponds to a change in quantity of the gene of interest. By comparing the ΔC_t value of each unknown sample to the ΔC_t of the known control, $\Delta\Delta C_t$ was obtained via $\Delta\Delta C_t = \Delta C_{t\,(sample)} - \Delta C_{t\,(known\ control)}$. The gene copy number relative to the number of the known control was equal to $2^{-\Delta\Delta Ct}$.

One-way ANOVA with a Tukey post-hoc test was used on $2^{-\Delta\Delta Ct}$ to determine whether the gene copy number was significantly different between the populations. Mean and standard deviation (SD) of $2^{-\Delta\Delta Ct}$ were calculated for all samples of each population.

RESULTS

1. Ploidy level of standard sample

The average chromosome count for each cell was 19.9 ± 1.7 (range: X-X) chromosomes. This result exceeds the chromosome number found in diploids (2n = 2x = 16) and is closer to the triploid count (2n = 3x = 24) rather than the tetraploid count (2n = 4x = 32), indicating that the ploidy level of the standard is triploid.

2. Genome walking

PCR amplification using the first set of primers, GW_L or GW_R along with SNX linkers primer, produced several PCR products of different sizes (Fig. 4A). These bands were extracted and amplified with the nested primers and the SNX linker primers. Nested PCR products were observed (Fig. 4B). We gel punched those PCR bands and the nested PCR twice to separate the different products. No PCR products were observed for the last nested PCR. Using cloning and Sanger sequencing, we sequenced any PCR product that was >400 kb from the gel of the initial PCR reaction and compared it with the PDS gene sequence using BLAST from NCBI. None of the sequences aligned with PDS or any plant genes. Some of the sequences partially aligned to genes from bacteria or insects. The sequences were also aligned with the PDS gene using Sequencher but no contig could be assembled.

We ran PCR on the non-ligated DNA with only GW_R or GW_L for 40 cycles (Fig. 4C), then extracted the products for nested PCR. However, we did not observe any amplified products for the nested PCR.

3. Quantitative real-time PCR

In general, there was little variation in C_t between the triplicate reactions ($SD < 0.2$) for most samples. Any measurement with SD higher than 0.3 was eliminated from subsequent analyses. Since we did not know the PDS copy number in the standard sample, we could only infer information on whether other samples have higher or lower PDS copy numbers than our standard sample. Information on exact gene copy number could not be deduced. Table 2 summarizes the average $2^{-\Delta\Delta Ct}$ and the SD $2^{-\Delta\Delta Ct}$ of the samples of each population.

Although the SD $2^{-\Delta\Delta Ct}$ for tetraploids was not high ($SD = 0.29$), the range of the $2^{-\Delta\Delta Ct}$ values was large (0.25 to 1.04) and the $2^{-\Delta\Delta Ct}$ values varied tremendously. Therefore, tetraploid samples were not included in further statistical tests and comparisons. Other reasons for the exclusion of tetraploid samples are stated in the discussion.

One-way ANOVA with a Tukey post-hoc test suggested that there was a significant difference in the $2^{-\Delta\Delta Ct}$ value between the Africa diploid hydrilla and the Texas and Florida triploid hydrilla ($F_{2,24} = 7.67$, $p = 0.003$). However, the $2^{-\Delta\Delta Ct}$ values of the two triploid populations did not differ significantly (Fig. 5).

DISCUSSION

Several studies have suggested multiple mechanisms, such as PDS mutations, behind the fluridone resistance in hydrilla (Puri et al. 2007a). However, no study has ever examined PDS gene amplification as one of the possible mechanisms in hydrilla. A similar mechanism of herbicide resistance was reported for *Amaranthis palmeri*. Amplification of EPSPS, the gene targeted by glyphosate herbicide and the correlated high expression of EPSPS were likely the cause of *A. palmeri* resistance to glyphosate (Gaines et al. 2010). Similarly, this study aimed to confirm whether the increase in PDS gene copy number was associated with the fluridone resistance in hydrilla.

A genome walking-based method was conducted to measure the number of PDS gene copy. The idea is that if the PDS gene gets duplicated and inserted back into the genome elsewhere, then the DNA regions lying before the promoter area will differ from one duplicate copy to another and between the duplicate copy and the parental copy. If that region contains any recognition site of *Alu I* and *Rsa I*, then we would expect to retrieve products of different sizes and sequences representing different PDS copies via amplification with an adapter primer and a PDS primer. If the PDS gene along with its promoter region was duplicated and inserted somewhere else in the genome, we would expect to see multiple bands representing PCR products of different sizes and sequences. If the PDS gene was not duplicated, then we would

expect to observe only one PCR product. In this experiment, we used two different primers specific to the sequence at both ends of the PDS gene because we had no information on which end the promoter was located.

This genome walking-based attempt was fully based on luck, since it depended on many unknown factors, such as whether there was any enzyme recognition site flanking each PDS copy. One major limitation was using the cDNA sequence instead of the genomic DNA sequence of PDS for designing primers and checking for enzyme recognition sites. Since we did not have any information about the introns and exons at the end of the gene, if the primers that we designed based on the all-exon cDNA sequence spanned two or more exons, then the primers would not anneal specifically to the PDS sequence. Since there were multiple PCR products retrieved from the PCR reactions using non-ligated DNA and only GW_L or GW_R primers (Fig. 4B), the primers might have annealed to other untargeted regions within the genome. This also explains why the nested PCR yielded no products. Moreover, if any *Alu I* or *Rsa I* restriction sites were located where an intron meets an exon, the PDS could have been truncated into smaller pieces and failed to bind to its primers. Ideally, had a genomic DNA sequence of PDS gene been available, using it for primer design and restriction enzyme selection could have helped us avoid these issues.

Usually an adapter with a special non-linear structure is used, so that DNA fragments flanked by adapters on both ends can be removed by certain techniques that target such fragments, leaving only DNA sequences flanked by the adapter on one side and the target gene primer on the other side. Since we did not utilize this technique, the PCR products that we obtained from the PCR reaction using the SNX linker primer and GW_L or GW_R could be mostly DNA fragments flanked by the adapter on both ends. Although we tried to prevent this by performing a PCR reaction with only GW_L and GW_R primers in the first place and gel punching the products to select for only fragments flanked by these primers on at least one end, some fragments, flanked by SNX linkers on both ends might have escaped this filtering step and got amplified in the following PCR reactions. This explains why when we sequenced these products, they aligned to random genes. The lack of a restriction site upstream of the promoter could exacerbate the situation, because in this case, all the PCR products that we obtained from the reaction using the SNX linker and GW primers might have all been random DNA fragments flanked by SNX linkers on both ends. Despite the many shortcomings of this genome walking-based method due to the lack of information required for experimental design, the fact that the procedure was so simple yet potential makes it worth an attempt.

With respect to qPCR experiment design, ideally, we would use two different reference genes, and two exons of the target gene, PDS, for stronger validation of our analysis. However, we encountered difficulty finding the reference genes for quantitative RT-PCR for hydrilla, because most of the common housekeeping genes for qPCR of plants, such as ACT, GADPH, 25S rRNA, TEF2, etc. have not been sequenced for hydrilla, a non-model organism (Tong et al. 2009). 18S rRNA sequence was the only housekeeping gene found on GENBANK for hydrilla.

Regarding the PDS exons, two sets of primers were designed for each exon. However, all but only one pair of primers for exon 2 (Ex2_b) showed multiple PCR products (Fig. 3). It is critical to ensure that primers will generate one single product for qPCR amplification, since the extra PCR products could potentially allow to these samples to pass the threshold in fewer cycles and interfere with the Ct values.

Microsatellite genotyping was used to tentatively predict ploidy levels of all samples. Based on our microsatellite results and the findings of a large body of literature on hydrilla, tetraploidy in hydrilla was found to be rare (Puri et al 2007b). Using flow cytometry, Puri et al. (2007b) reported the presence of tetraploid individuals in limited abundance among resistant hydrilla populations from Florida. Our microsatellite data confirmed the existence of hydrilla with tetraploidy, but they were also rarely observed. The high variation and the wide range of the $2^{-\Delta\Delta Ct}$ results for potential tetraploids, may have occurred because diploid and triploid samples were inaccurately identified as tetraploids due to PCR artifacts in the original microsatellite analysis. Since tetraploidy was rare and not confidently confirmed, we decided to exclude tetraploid samples from subsequent comparisons and focused on the more abundant diploid and triploid individuals.

For the comparison of PDS copy number between diploid and triploid samples, we expected that the diploid African population would have a lower mean $2^{-\Delta\Delta Ct}$ value compared to the triploid populations. This prediction was based on the fact that since African hydrilla was not exposed to fluridone, so PDS duplication was unlikely to be selected for and the diploid genome should contain only two PDS copies. Surprisingly, the diploid samples from Africa had the highest mean $2^{-\Delta\Delta Ct}$ value that was significantly different from either of the triploid populations. The result indicates that African diploid hydrilla has higher PDS gene copy numbers compared to the U.S. triploid hydrilla. The high SD $2^{-\Delta\Delta Ct}$ of this diploid population suggested high variation in the quantification of PDS copy number. One possible explanation for this high variation in diploid samples is endopolyploidy. Endopolyploidy, a phenomenon that is widespread in plants and particularly common among angiosperms, produces elevated nuclear DNA quantities resulting from consecutive doubling of the original unreplicated 2n ploidy level (Melaragno et al. 1993). Langeland et al. 1992 documented that hydrilla may be an endopolyploid plant. The study by Puri et al. 2007b on the variation of ploidy level in Floridian hydrilla observed endopolyploidy in only diploid samples, but not in higher ploidy level (triploid and tetraploid) samples. Therefore, there is a possibility that diploid hydrilla from Africa may be inclined to endoduplication events like Floridian hydrilla, inducing the existence of cells with different ploidy levels within an individual and creating substantially high variation of the $2^{-\Delta\Delta Ct}$ results for this population. However, the findings in Puri et al. 2007 were made on rapidly growing root tissue of hydrilla, while our samples were collected from leaf tissue. Whether diploid hydrilla from Florida and Africa share the same propensity for endoreduplication, and whether the endoreduplication pattern is the same between different hydrilla tissues remain to be investigated.

Initially, we hypothesized that duplication events of the PDS gene within the genome could potentially be a mechanism behind the resistance to fluridone. The increased expression of the PDS gene due to higher copy number could enhance PDS polypeptide production and abundance, allowing the PDS protein to overcome the effect of fluridone. By offering hydrilla an adaptation to survive fluridone, PDS duplication could undergo positive selection and become prevalent, leading to increased resistance. If the duplicate PDS copy number varies in number among populations, it could induce the varied levels of fluridone resistance observed in hydrilla. Based on this mechanism, we would expect the resistant population from Florida to have significantly higher PDS copy number relative to the susceptible population from Texas, if gene duplication was partially responsible for the resistance trait. However, there was no significant difference in PDS copy number between Florida and Texas. The average $2^{-\Delta\Delta Ct}$ values of both populations were consistent with that of the triploid standard sample from the Texas population, indicating the same number of PDS copies in both the resistant and susceptible biotypes. This result suggested that PDS gene amplification was unlikely to be the mechanism behind the resistance to fluridone in hydrilla. Unknown molecular mechanisms other than PDS duplication might be involved in the development of fluridone resistance. Possibilities could include undescribed PDS mutations, modifications of the promoter region that affect gene expression, or non-target site resistance factors (Puri et al. 2007a, Michel et al. 2004, Vaillant and Paszkowski 2007).

It is important to understand that the approach using qPCR and microsatellite loci for ploidy level gene copy number determination has certain limitations. Microsatellite genotyping was used to initially predict the ploidy level of samples and generate reference information for qPCR. However, the data inferred from microsatellites might not accurately reflect the level of ploidy in plants, because PCR artifacts can produce false alleles increasing the observed ploidy or homozygous alleles can generate fewer peaks and be mistaken for lower ploidy levels. Regarding qPCR, he accuracy of the obtained data largely depends on multiple factors, such as sample preparation, DNA quality, choice of housekeeping gene, and primer specificity. Although standardization of the DNA concentration was done for all samples to minimize differences in the initial DNA amount and reduce the variation in ΔC_t values between samples, the procedure using 260 absorbance is not considered highly accurate. To test for variance between qPCR runs, we applied one-way ANOVA analysis with a Tukey post-hoc test on the ΔC_t values of two samples that were consistently run on all plates. The test resulted in significant variance between the runs ($F_{3,4} = 6.59$, $p = 0.002$), which could reduce the confidence of our interpretation of the combined data. In most studies of gene copy number that adopt the qPCR approach, Southern blot analysis is often also conducted as a method to validate the results of qPCR (Bubner & Baldwin 2004).

Overall, the PDS gene copy number varied tremendously between different populations and ploidy levels, suggesting that PDS copy number did not correspond simply to ploidy levels. Technical errors (limitations of microsatellite genotyping and qPCR) or biological factors

(endoreduplication) could explain this discrepancy. The qPCR results suggested that amplification of the PDS gene was unlikely to be one of the mechanisms behind fluridone resistance in hydrilla, yet future studies should repeat qPCR with other reference genes and exon targets and perform Southern blot analysis to further validate the results of this study.

TABLES & FIGURES

Table 1. Primers used in the qPCR and genome walking experiments.

Primers	Sequences (5’ → 3’)
GW_L	GGACTTGAACATGGCCTCTG
GW_R	GACTGATGAACTGGCTGGGTA
GW_nestL	AGCAAGCAAAGGGGGTAAGT
GW_nestR	TTTCCCTACCGCAGAATCC
18S_b F	TGAGAAACGGCTACCACATC
18S_b R	ACTCGTAGAGCCCGGTATTG
Ex2_b F	GGCCAAGTCATCCTTAATTCC
Ex2_b R	CATTGGTGAGCACAAAATGC

Table 2. qPCR results for hydrilla samples.

Population	Sample number	Mean $2^{-\Delta\Delta Ct}$	SD $2^{-\Delta\Delta Ct}$
African diploid	10	1.51	0.72
Texas triploid	6	0.87	0.20
Florida triploid	11	0.74	0.19
Florida tetraploid	6	0.73	0.29

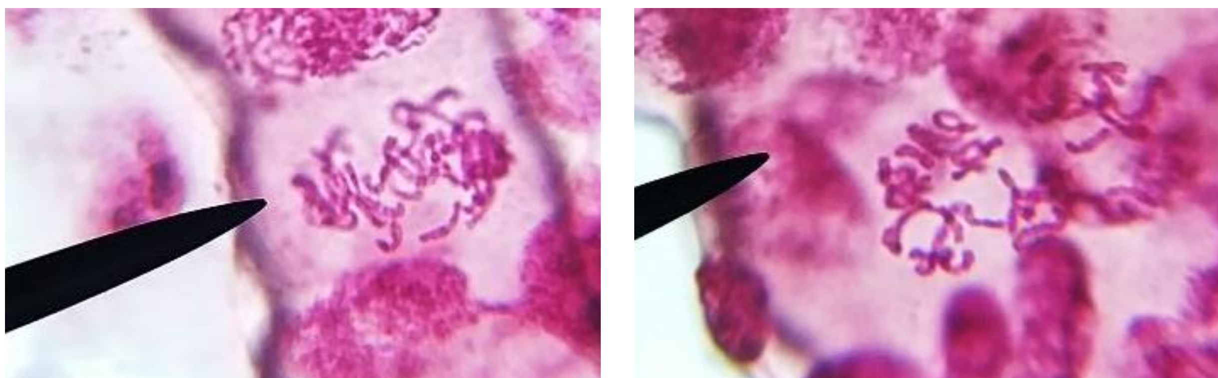

Figure 1. Chromosome spreading for karyotyping under microscope.

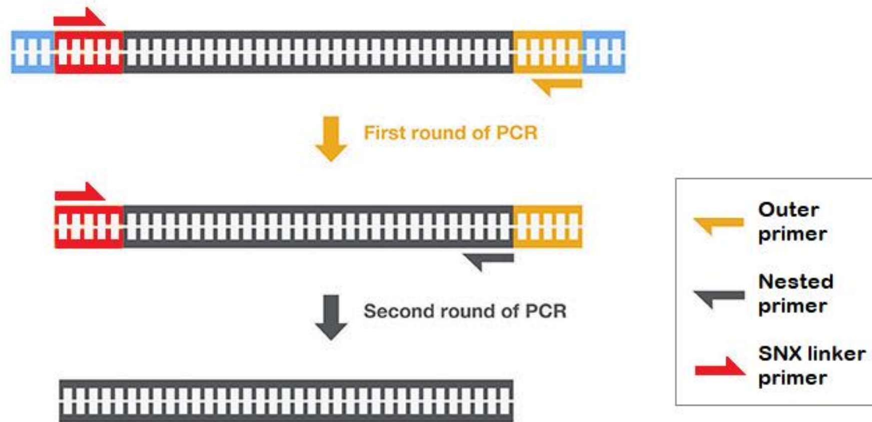

Figure 2. Nested PCR.

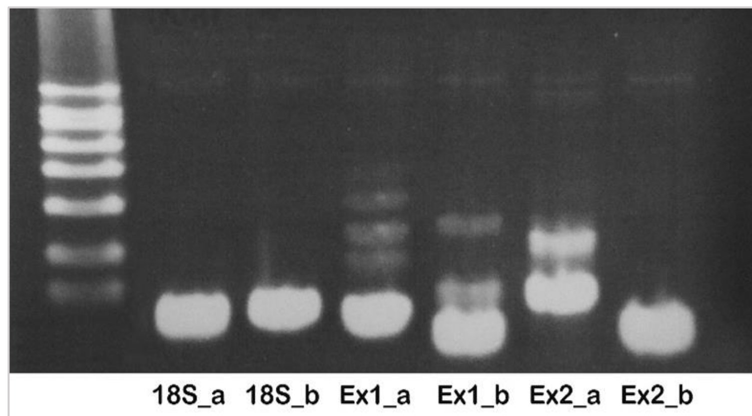

Figure 3. Primer test for qPCR in hydrilla. Only 18S_a, 18S_b and Ex2_b produced a single band representing a single product. Among these three primers, 18S_b and Ex2_b were chosen for qPCR amplification of the housekeeping gene and PDS gene, respectively.

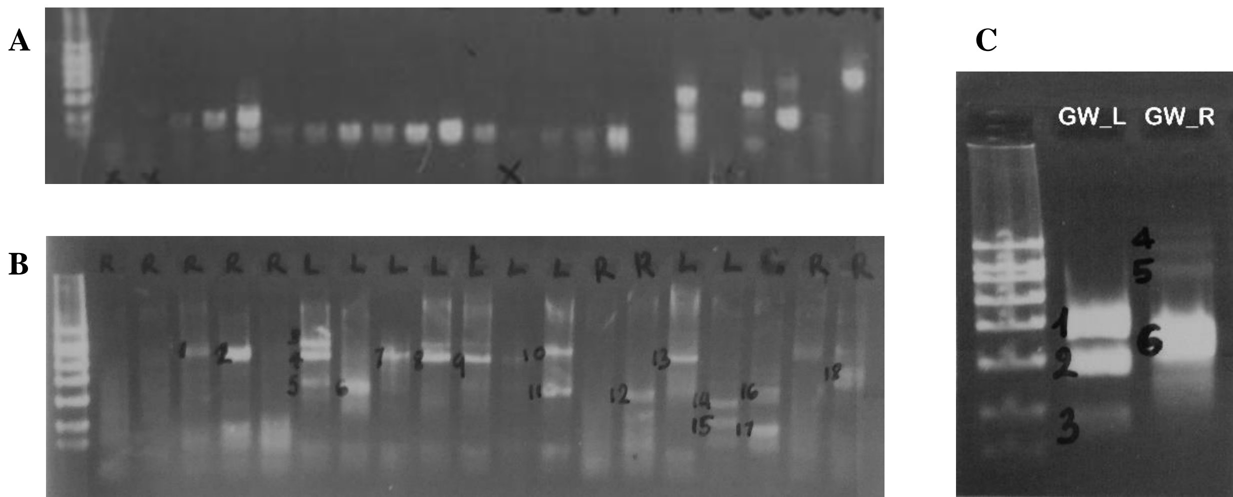

Figure 4. Gel electrophoresis of PCR products retrieved from genome walking-based method in determining PDS gene copy number. The results for the A) the first PCR reaction using SNX linker primer and GW primers and B) the nested PCR reaction using SNK linker primer and the nested primers are shown. Picture C) shows the bands for the 40-cycle PCR amplification using non-ligated DNA and only GW_L or GW_R primers.

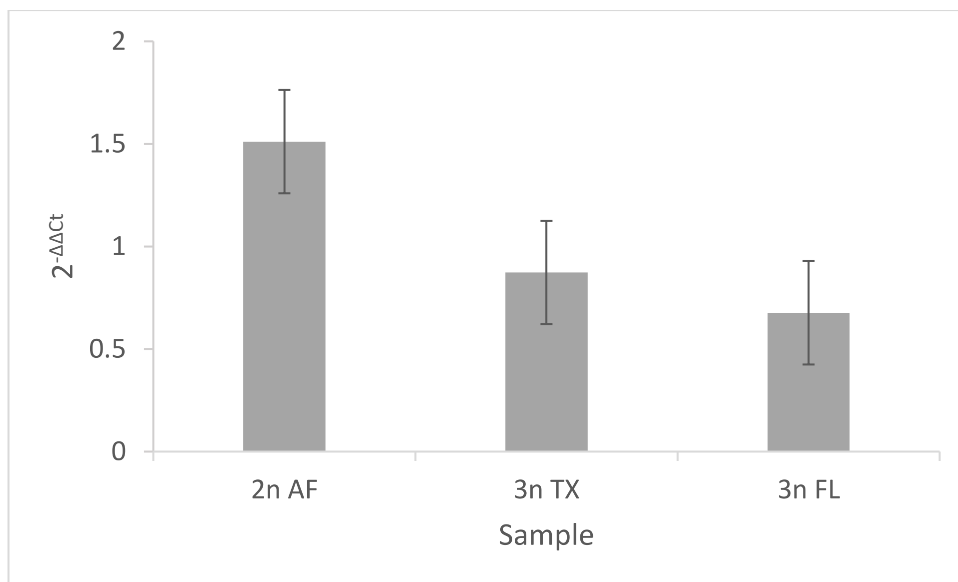

Figure 5. Mean $2^{-\Delta\Delta Ct}$ values with standard error of three hydrilla populations: Africa diploid (2n AF), Texas triploid (3n TX) and Florida triploid (3n FL).

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