Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*)

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Abstract

Hydrilla (*Hydrilla verticillata* L.f. Royle) was introduced to the surface water of Florida in the 1950s and is today one of the most serious aquatic weed problems in the USA. As a result of concerns associated with the applications of pesticides to aquatic systems, fluridone is the only USEPA-approved chemical that provides systemic control of hydrilla. After a decrease in fluridone's efficacy at controlling hydrilla, 200 Florida water bodies were sampled to determine the extent of the problem and the biological basis for the reduced efficacy. Our studies revealed that hydrilla phenotypes with two- to six-fold higher fluridone resistance were present in 20 water bodies. Since fluridone is an inhibitor of the enzyme phytoene desaturase (PDS), the gene for PDS (*pds*) was cloned from herbicide-susceptible and -resistant hydrilla plants. We report for the first time in higher plants three independent herbicide-resistant hydrilla biotypes arising from the selection of somatic mutations at the arginine 304 codon of *pds*. The three PDS variants had specific activities similar to the wild-type enzyme but were two to five times less sensitive to fluridone. *In vitro* activity levels of the enzymes correlated with *in vivo* resistance of the corresponding biotypes. As hydrilla spread rapidly to lakes across the southern United States in the past, the expansion of resistant biotypes is likely to pose significant environmental challenges in the future.

Keywords: aquatic weed, herbicide resistance, invasive species, molecular adaptation, nonindigenous species, somatic mutations

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Introduction

Nonindigenous invasive species have had tremendous ecological and economic impacts worldwide, and they are estimated to be the leading threat posed to nearly half of the endangered species (Wilcove *et al.* 1998). Environmental damage and losses resulting from the spread of non-native organisms amount to $125–140 billion per year in the United States alone (Pimentel *et al.* 2000; Baker 2001;

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National Research Council 2002). Aquatic ecosystems are particularly vulnerable because health and environmental concerns restrict the use of chemicals to eradicate these noxious species. In the early 1950s a female form of the dioecious strain of hydrilla (*Hydrilla verticillata*) was released into the surface water of Florida in Tampa Bay and spread rapidly throughout the state (Schmitz *et al.* 1991). Random amplified polymorphic DNA analysis shows that hydrilla accessions from Florida, Texas and California are dioecious (although only the female form is present in these populations), and clusters them together, close to accessions from Bangalore, India, indicating one common origin for the dioecious plants (Madeira *et al.* 1997, 1999). This monocotyledonous species is now the most abundant, nonindigenous submerged aquatic plant in Florida and is one of the most serious aquatic weed problems in the southern and

Fig. 1 Geographical distribution of the nonindigenous aquatic weed hydrilla in the USA. The dioecious form (red) was released to the environment in Tampa and Miami, FL and spread to the southern and western United States. The monoecious form (green) was introduced in the east coast and California. Areas in purple have both dioecious and monoecious forms present. The lighter coloured States do not have records whereas the darker coloured States do have records of hydrilla. The map is from the United States Geological Services and was last updated September 2002 USGS (2003). Arrows designate points of appearance of resistance.
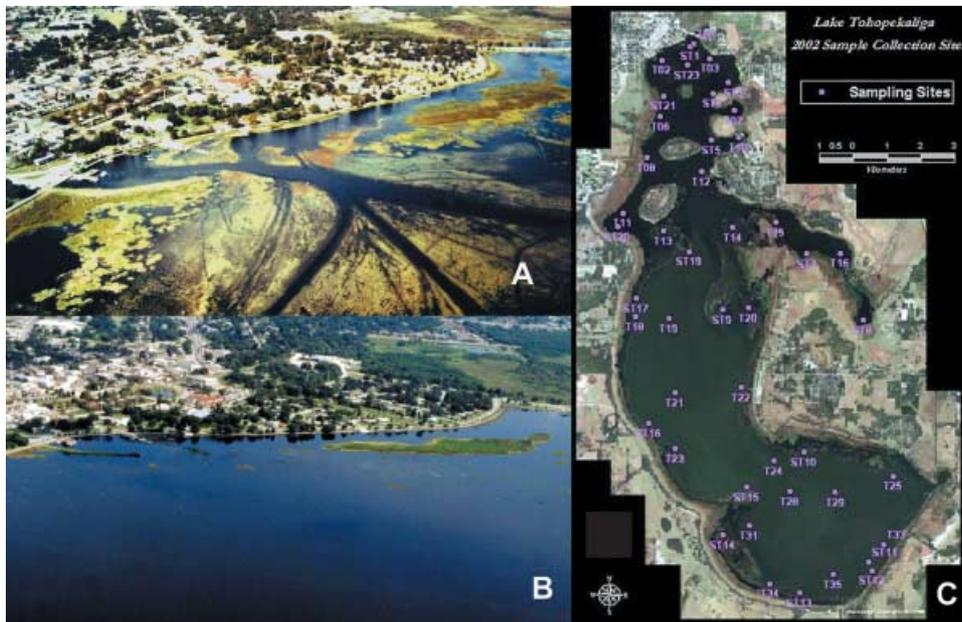

Fig. 2 Views of the Lake Toho (Osceola county, Florida), (A) before treatment with fluridone and (B) after treatment with fluridone. Courtesy of Mike Hulon, Florida Fish and Wildlife Conservation. (C) Aerial view of the sampling site locations on Lake Tohopekaliga. All the lakes surveyed in this study were sampled over several weeks.

western USA (http://nas.er.usgs.gov/plants/maps/hy _vert_bio.jpg) (Fig. 1). Left unmanaged, this invasive plant can rapidly cover thousands of contiguous hectares (Fig. 2A), displacing native plant communities and causing significant damage to the ecosystems (Colle & Shireman 1980; Schmitz & Osborne 1984; van Dijk 1985; Schmitz *et al.* 1993; Bates & Smith 1994). Hydrilla is controlled in large water bodies (> 100–12 000 ha) by sustaining between 12 and 36 nм (4–12 μg/L) concentrations of the chemical fluridone (Sonar®) in lake water for several weeks (Netherland &

Getsinger 1995; Fox *et al.* 1996) (Fig. 2B). Fluridone is an inhibitor of phytoene desaturase (PDS), a rate-limiting enzyme in carotenoid biosynthesis (Chamovitz *et al.* 1993). PDS is a nuclear-encoded protein and has activity in the chloroplasts, the site of carotenoid synthesis (Bartley *et al.* 1991). Under high light intensities, carotenoids stabilize the photosynthetic apparatus by quenching the excess excitation energy; therefore, inhibition of PDS decreases coloured carotenoid concentration and causes photo-bleaching of green tissues (Böger & Sandman 1998).

In response to an apparent decrease in the efficacy of fluridone at controlling hydrilla, a major sampling effort was launched in 2001 and 2002 to determine the extent of herbicide-resistant hydrilla populations in public and private water bodies throughout Florida. In higher plants, evolution of herbicide resistance is considered unlikely to occur in the absence of sexual reproduction (Hill 1982; Maxwell & Mortimer 1994). Furthermore, even in terrestrial weed species that reproduce sexually, resistance to PDS-inhibitor herbicides with significant soil persistence (e.g. norflurazon) has not been documented. Nevertheless, we report that three herbicide-resistant biotypes of hydrilla have evolved in populations restricted to vegetative means of propagation. The molecular basis for herbicide resistance of these biotypes, including cloning of the nuclear-encoded *pds* gene and biochemical characterization of PDS enzymes from various hydrilla populations is discussed.

Materials and methods

Collection and analysis of field samples

Hydrilla samples collected from 200 Florida lakes were tested for their sensitivity to fluridone. Samples of hydrilla were collected systematically across each lake selected in this study and the location of each sample site was determined by GPS (Garmin GPSMap 76) (see example Fig. 2C). Hydrilla populations from the majority of the large lakes (> 2000 ha) in Florida were sampled, as well as many lakes between 200 and 2000 ha, and numerous lakes between 40 and 200 ha. The study included a total of 200 lakes with 25–100 samples per lake collected from fixed locations over several weeks. The samples were brought to the laboratory, cleaned, excised as 4 cm sections and grown at 25 °C with a 14 h light/10 h dark cycle in flasks containing 10% Hoagland's solution (pH 7.5). Light intensity was 200 µmol/m²/s photosynthetically active radiation.

The herbicide sensitivity of hydrilla samples from each site was tested in triplicate in the presence of fluridone concentrations ranging from 2.3 to 145 nM (0.75–48 µg/L). After 14 days, all new tissues were excised from the original 4 cm and analysed for biomass accumulation (dry weight), chlorophyll, phytoene and β-carotene content as described by Sprecher *et al.* (1998). Data collected for each sample site were subjected to an analysis of variance (ANOVA) to determine possible differences within water bodies or inter-site in response to various concentrations of fluridone. Mean values were compared by Fisher's least significant difference.

For molecular and biochemical analyses, hydrilla propagules representative of low, moderate and high levels of fluridone resistance were collected in lakes Lulu (Polk county, 27°59′43″-N, 81°43′13″-W), Pierce (Polk county, 27°58′29″-N, 81°31′17″-W) and Okahumpka (Sumter county, 28°49′29″-N, 82°00′24″-W), respectively (Table 1). Hydrilla vegetative propagules from Rainbow River (Marion county, 29°06′08″-N, 82°26′14″-W) were considered wild-types, because this site was never exposed to fluridone and the plants were susceptible to this herbicide.

Sequence analysis and cloning of the pds gene

Phytoene desaturase genes (*pds*) were cloned from hydrilla specimens obtained at different locations. The *pds* gene is a nuclear-encoded gene expressed in chloroplasts. Partial *pds* sequences were obtained by polymerase chain reaction (PCR) using degenerate primers (PDS-819: 5′-TAA AYC CTG ATG ARY TWT CAN TGC-3′ and RPDS-1219: 5′-GTG TTB TTC AGT TTT CTR TCA A-3′) to regions in *pds* genes conserved among many plant species. The resulting 400 base pair fragment was used to amplify the unknown 3′ and 5′ ends of the hydrilla *pds* gene using 3′ and 5′ rapid amplification of cDNA ends RACE-protocols (Clontech). Alleles for the *pds* gene were amplified via PCR with a

Table 1 Differential response of the susceptible (Rainbow River) and resistant hydrilla biotypes (Lulu, Pierce, Okahumpka) from representative Florida water bodies

Location	Amino acid[304]	LCIC nM	R/S	Biomass DW (g)	%	β-carotene µg/g FW	(%)	Chlorophyll mg/g FW	(%)	Phytoene µg/g FW	(%)
Rainbow River (5)	arginine	12	1.00	0.5 ± 0.3	10	2.9 ± 0.7	10	0.13 ± 0.05	12	127.4 ± 14.9	728
Lulu (10)	serine	27	2.25	2.4 ± 0.3	38	11.9 ± 1.1	42	0.47 ± 0.12	43	109.1 ± 15.2	623
Pierce (12)	cysteine	45	3.75	3.4 ± 0.5	58	15.3 ± 2.1	57	0.72 ± 0.10	65	94.2 ± 12.8	538
Okahumpka (15)	histidine	73	6.00	4.2 ± 0.6	72	21.6 ± 1.3	77	1.07 ± 0.10	97	86.1 ± 13.1	492

Mean and standard deviation values of biomass, β-carotene, chlorophyll and phytoene in samples exposed to 12 nM (4 µg/L) fluridone. Numbers in parentheses indicate the total of independent sites sampled at each location. Percentages are relative to values in the untreated samples.
LCIC, lowest complete-inhibition concentration of fluridone (complete bleaching); DW, dry weight; FW, fresh weight; *R/S*, ratio of resistance to sensitivity.

proof-reading polymerase mixture (Advantage2, Clontech) and the primers ORF-Primer (5′-ATG ACT GTT GCT AGG TCG GTC GTT-3′) and RPDS-1849 (5′-TAC CCC CTT TGC TTG CTG ATG-3′) on cDNA from fluridone-sensitive and -resistant hydrilla biotypes. The sequence of the *Hydrilla verticillata pds* gene is available from GenBank (Accession number AY639658). PCR fragments were cloned into vector Topo 4 (Invitrogen), used for double-strand sequencing, and for detection of possible mutations in the resistant biotypes. When cloning the hydrilla *pds*, we found three putative start codons (codon 1, 39 and 76) in frame within the sequence of a putative transit peptide. Using a forward primer designed to start at codon 77 (3ORF-ATG: 5′-GATTTCCCAAGACCTGATATAGATAAC-3′) a deletion sequence of hydrilla *pds* was amplified by PCR and sub-cloned into the vector TOPO 4 (Invitrogen), the resulting plasmid was named p3ORF-ATG. Using a QuickChange Site-directed Mutagenesis Kit (Stratagene, CA, no. 200518) three clones were generated to replace the arginine codon 304 (Arg304) of *pds* by histidine, serine and cysteine. The resulting plasmids were named p3ORFHis, p3ORFSer and p3ORFCys, respectively. The primers used for the mutagenesis were as follows. For histidine mutation: Hyd-His forward primer, 5′-GCATCCTGATTGCCTTA-AACCATTTCCTTCAGGAAAAGC-3′, Hyd-His reverse primer, 5′-GCTTTTCCTGAAGGAAATGGTTTAAGG-CAATCAGGATGC-3′; for serine mutation: Hyd-Ser forward primer, 5′-GCATCCTGATTGCCTTAAACA-GTTTCCTTCAGGAAAAGC-3′, Hyd-Ser reverse primer, 5′-GCTTTTCCTGAAGGAAACTGTTTAAGGCAATCAG-GATGC-3′; and for cysteine mutation: Hyd-Cys forward primer, 5′-GCATCCTGATTGCCTTAAACTGTTTCCTT-CAGGAAAAGC-3′, Hyd-Cys reverse primer, 5′-GCTTTT-CCTGAAGGAAACAGTTTAAGGCAATCAGGATGC-3′.

Bacterial expression vectors were constructed by sub-cloning the 1680 base pair *Eco*RI fragment from p3ORF-ATG, p3ORFHis, p3ORFSer and p3ORFCys into the *Eco*RI site of pRSETB vector (Invitrogen) to add a 6XHistidine tag at the N terminus. The resulting plasmids named p3ORF-ATGSet, p3ORFHisSet, p3ORFSerSet and p3ORFCysSet, were transformed into BL21(DE3)pLysS cells for protein expression.

Biochemical analysis of PDS

Separate BL21(DE3)pLysS cultures harbouring the expression vectors p3ORF-ATGSet p3ORFHisSet, p3ORFSerSet and p3ORFCysSet were grown overnight in 500 mL Luria Broth supplemented with carbenicillin (100 mg/L) and chloramphenicol (60 mg/L) at 37 °C, and induced with 0.3 mM isopropylthio-β-D-galactoside for 3 h. Cells were lysed using a French press (Spectronics Instrument) at 140 MPa and the His-tagged PDS protein expressed was purified on a nickel-activated Hitrap Chelating HP column (Amersham Bioscience). The pure protein eluted between 100 and

250 mM imidazole. Western blots using Anti-HisG (cat. no. R940-25, Invitrogen) were run after every extraction. The protein was transferred to the assay buffer on a PD10 column (Amersham Bioscience), and the concentration was determined using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as the standard and adjusted to 100 μg/mL. Crude extracts containing phytoene were produced in *Escherichia coli* JM101/pACCRT-EB (EB extract) containing geranylgeranyl pyrophosphate synthase and phytoene synthase enzymes from *Erwinia uredovora* (Misawa *et al*. 1995). The reaction assays consisted of 50 μg PDS in 500 μL of assay buffer (200 mM sodium phosphate, pH 7.2) and 500 μL of EB extract. Fluridone (10 μL in methanol) was added to the 500 μL of PDS extract and incubated on ice for 15 min prior to mixing it with the EB extract. Seven final concentrations of fluridone were tested in duplicate ranging from 3 nM to 1 mM with two biological and two technical replicates. The assay was carried out for 30 min at 30 °C and 350 r.p.m. on an Eppendorf ThermoMixer-R (Brinkmann Instruments). All experiments were repeated. The rate of reaction was linear during the length of the enzyme assay. ζ-Carotene produced was extracted according to Sandmann *et al*. (1996) and quantified spectrophotometrically at A_{425} using an extinction coefficient (mM) εmax 138. Dose–response curves were fitted to a four-parameter logistic function. Since minimum and maximum values in the model were 0 and 100, respectively, the equation was simplified to the following:

$$f = \frac{100}{1 + e^{b*[\ln(x) - \ln(I_{50})]}}$$

where *b* is the slope, *x* is concentration of inhibitor, I_{50} is the concentration for 50% inhibition of enzyme activity, and *f* (μg/mg/h) is the specific enzyme activity expressed as μg of ζ-carotene produced per mg enzyme per hour. I_{50} values were calculated from the regressions. Resistance factors (*R/S*) were determined as the ratio of the I_{50} of the resistant enzyme over the I_{50} of the wild-type enzyme.

Results

The majority of the collection sites contained hydrilla plants that were highly susceptible to fluridone and no intra-site variation in response to this herbicide was detected ($P = 0.479$). However, there was a significant ($P < 0.05$) inter-site variation, with fluridone-resistant hydrilla populations detected in 20 water bodies of central Florida ranging in size from 120 to 8500 ha. Based on ANOVA and mean comparisons ($P < 0.05$) for each fluridone concentration in the range of 12–48 nM four phenotypes were separated (Fig. 3). The susceptible hydrilla phenotype (wild-type) was completely controlled at 12 nM fluridone, whereas the low-, intermediate- and high-resistance phenotypes required 36,
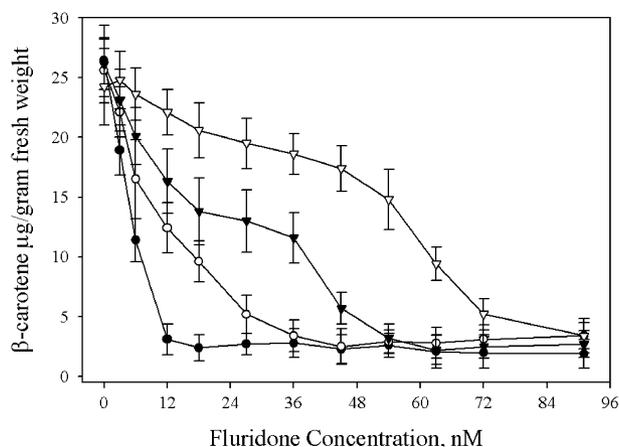
Fig. 3 Mean and standard deviations obtained from laboratory assays. β-carotene content of hydrilla shoot apices following a 14-day exposure to fluridone concentrations ranging from 0 to 91 nM. Phenotypes: ●, susceptible (179 lakes); ○, low resistance (eight lakes); ▼, intermediate resistance (seven lakes); and ▽, high resistance (five lakes).

Fig. 4 Phytoene desaturase sequences and mutations. (A) Fragments of sequences of phytoene desaturases from various organisms harbouring arginine codons at the amino acid position 304 of hydrilla's PDS. (B) Three independent single base mutations found at amino acid 304 of the hydrilla *pds* gene that convert the susceptible (Rainbow River) into resistant biotypes (Lulu, Pierce and Okahumpka). Segments of nucleic acid sequence and their corresponding deduced amino acids.

54 and 91 nM fluridone for complete control, respectively (Fig. 3). At 12 nM fluridone, the susceptible plants had a β-carotene concentration of 3 μg/g, whereas the β-carotene concentration in some resistant plants exposed to 50 nM fluridone was 15 μg/g, half the content of the untreated controls (Fig. 3). In our experiments, the shoots of hydrilla that had not been treated with fluridone contained 1.0–1.2 mg chlorophyll/g fresh weight, and 10–25 μg phytoene/g fresh weight. Percentages for treatments were calculated using the average values (1.1 mg/g and 17.5 μg/g) for chlorophyll and phytoene, respectively. Samples of hydrilla from water bodies representative of each of those four biotypes were selected for further molecular and biochemical analysis. The selected sites were: Rainbow River, Lulu, Pierce and Okahumpka, for the susceptible, low, intermediate and high levels of resistance. Comparison of β-carotene, among samples of the four selected sites after 14 days exposure to various fluridone concentrations confirmed the distinct patterns of response (Table 1) ($P < 0.05$). At 12 nM fluridone, the phytoene concentration was seven times higher in the susceptible biotype and five times higher in the highly resistant biotype compared to the untreated control (Table 1). All of the hydrilla samples could be categorized as exhibiting one of the four phenotypes shown in Fig. 3. Most lakes (179) were populated with the fluridone-susceptible phenotype. The hydrilla phenotype with low-level resistance was found in eight lakes, the phenotype with intermediate resistance was found in seven lakes, and the most resistant phenotype was found in five lakes.

The lowest complete-inhibition concentrations (LCIC) of fluridone for the resistant biotypes were between 2.25–
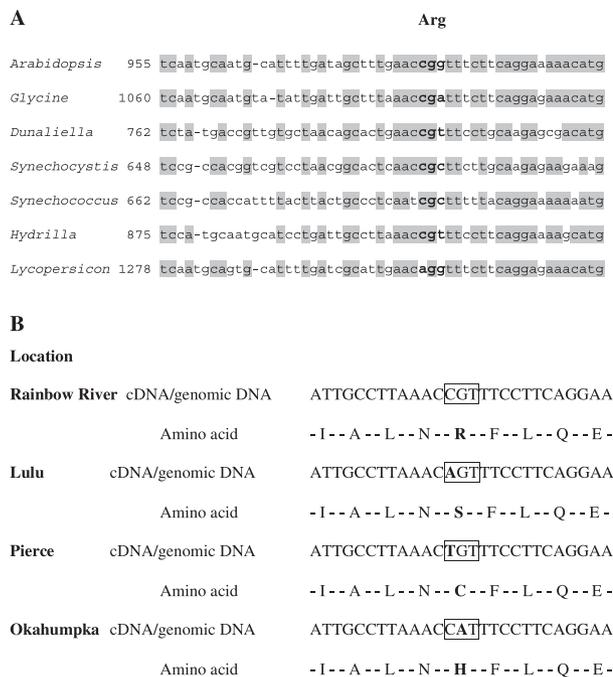
and 6-fold greater than for the representative susceptible hydrilla population located at Rainbow River (Table 1). This last population was collected near a natural spring and had never been exposed to fluridone or other PDS inhibitors. In the susceptible population, 12 nM (4 μg/L) fluridone strongly inhibited biomass accumulation and β-carotene accumulation, showing only 10% of the levels found in untreated plants. The resistant biotypes were found in lakes that had been treated with fluridone for several years. Following a 14-day exposure to 12 nM fluridone, these plants had biomass and β-carotene accumulations of up to 72% and 77% of the content in untreated plants, respectively (Table 1).

The *pds* gene was cloned from the herbicide-susceptible as well as from the herbicide-resistant biotypes of hydrilla from locations in Table 1. Three separate and independent single-point mutations of the codon 304 encoding for Arg (Arg304) in *pds* were identified. The alignment of *pds* genes from cyanobacteria, microalgae and higher plants showed conserved Arg codons corresponding to the position 304 of *pds* in hydrilla (Fig. 4A). The codon usage for Arg304 in the wild-type hydrilla is CGT and single-point mutations yielding either Ser (AGT), Cys (TGT), or His

Table 2 Hydrilla phytoene desaturases with mutations at the amino acid 304, and the corresponding fluridone concentrations that resulted in 50% inhibition of enzyme activity (I_{50})

Location	Amino acid[304]	Specific activity (f)	I_{50} (nM)	R/S
Rainbow River	arginine	11.0	300	1.0
Lulu	serine	8.1	600	2.0
Pierce	cysteine	7.3	800	2.7
Okahumpka	histidine	10.5	1500	5.0

Locations are the names of the water bodies where those mutations were detected; Rainbow River was the origin of the herbicide-susceptible strain. R/S, ratio of resistance to sensitivity. Specific enzyme activity (f): μg of ζ-carotene produced per mg enzyme per hour).

(CAT) substitutions were identified in hydrilla populations from Lulu, Pierce and Okahumpka lakes, respectively (Fig. 4B).

The wild-type PDS as well as the Cys, Ser and His mutations were expressed in bacterial cells and detected using the 6XHis epitope tag. PDS proteins as expressed in pRSET vectors consisted of 550 amino acids and had a molecular weight of 61 329. Examples of Western blot and Coomassie staining of the purified PDS are shown in Fig. 5. Although there was only evidence for amino acid 304 being related to fluridone resistance, we also found other mutations in the *pds* sequences. Amino acid substitutions were found in the sequences of the resistant alleles [i.e. a *pds* gene with Ser304 also had mutations of Ile341 (ATC) to Val341 (GTC); and Ile329 (ATT) to Phe329 (TTT)]. However, reversion of the amino acid at 304 to the wild-type Arg by site-directed mutagenesis restored herbicide sensitivity to this enzyme (data not shown), indicating that those other mutations did not provide herbicide resistance. Several silent mutations were also found in resistant and susceptible *pds* alleles [i.e. Ala149 (GCG to GCT), Pro321 (CCA to CCG), Ile298 (ATC to ATA), Lys199 (AAG to AAA), Ile219 (ATA to ATT), Pro328 (CCA to CCT)]. To facilitate the construction of all the clones for the reported *in vitro* experiments, the wild-type sequence was used and the indicated mutations at amino acid 304 were introduced. All of the resistant plants tested were heterozygous, each possessing resistant and susceptible alleles of the *pds* gene. After being expressed in *Escherichia coli*, purified and tested *in vitro* with fluridone, all three PDS enzymes containing mutations had two to five times higher I_{50} values than the wild-type PDS (Table 2). However, the wild-type PDS and all three mutated enzymes had similar *in vitro* specific activities (f), ranging between 7.3 and 11.0 (μg/mg/h) (Table 2).
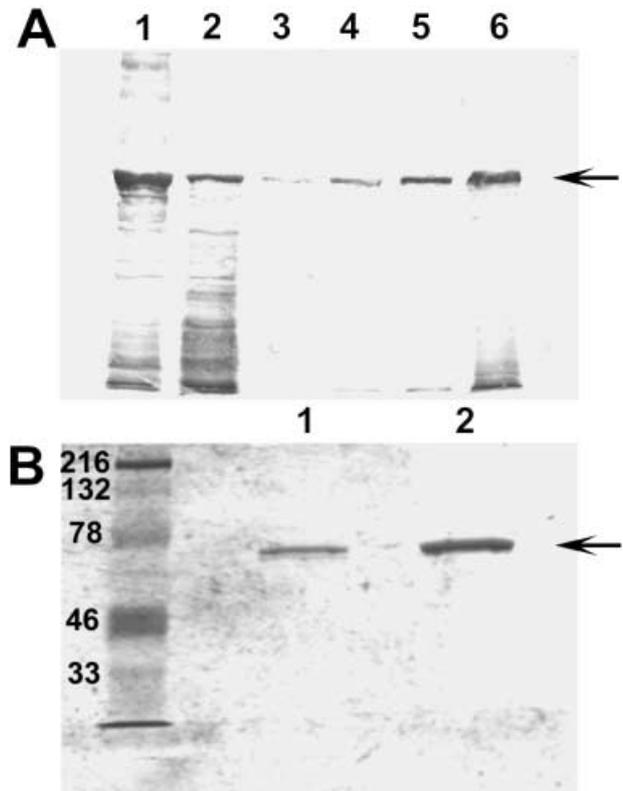

Fig. 5 Examples of phytoene desaturase (PDS) purification. (A) Western blot for His-tagged PDS. Raw cellular extract (lane 1), flow through the nickel column (lane 2), elution of PDS with 50–1000 mM imidazole (lanes 3–6). (B) Coomassie-stained gel. Purified PDS containing cysteine and serine mutation (lanes 1 and 2, respectively). The mass of molecular weight markers in kDa is indicated on the left. The 61.3-kDa PDS is indicated by arrows.

Discussion

Analysis of biomass accumulation and β-carotene content of plants from multiple sampling sites after treatment with fluridone indicated that hydrilla populations within each lake were homogeneous. However, hydrilla populations differed between lakes, each responding to fluridone according to one of four levels of resistance (Fig. 3). This suggests that when a mutation occurred, a single herbicide-resistant biotype rapidly became the dominant population within the lake. Since only female strains of hydrilla from a single geographical origin have been reported to colonize the lakes in Florida (Madeira *et al.* 1997, 1999), the establishment of herbicide-resistant biotypes as the dominant forms in these lakes was not anticipated. Usually, plants restricted to asexual means of reproduction are under strong uniparental constraints that limit their ability to respond to environmental changes (Holsinger 2000). The reduced genetic variability is also thought to lead to accumulation of deleterious mutations called mutational

melt-down that reduce the survival chances of these plants, but in the short term, a single somatic mutation may provide a genotype that is adaptively superior (Klekowski 2003). The uniform genotypes we observed within each lake may have resulted from the background selection of mutants in the presence of fluridone. According to Charlesworth *et al.* (1993) background selection is the selection against deleterious alleles that is facilitated or sustained by mutations and results in the reduced genetic diversity of highly inbreeding and asexual populations. In our case *pds* genes with the Arg304 (wild-type) would have been the deleterious alleles in lakes under the selection pressure of fluridone treatments.

Of the three independent somatic mutations identified in hydrilla, two were transitions from G to A (for histidine) and C to T (for cysteine), and the other was a transversion from C to A (serine) (Fig. 4B). In general, transitions (changes from purine to purine or pyrimidine to pyrimidine) are more common than transversions (changes from purine to pyrimidine and vice versa) (Muse 2000). Similar Arg to Ser, Cys, and His mutations providing herbicide resistance have been reported in the cyanobacteria *Synechococcus* and *Synechocystis* grown on selection media with various PDS inhibitors (Linden *et al.* 1989; Martinez-Ferez & Vioque 1992). However, the mutations observed in hydrilla are the first known naturally occurring cases of herbicide resistance to PDS inhibitors based on an altered target site in higher plants, even though other PDS inhibitors with significant soil persistence (e.g. norflurazon) have been used for terrestrial weed control in crops for many years.

The resistant hydrilla plants may develop into seemingly insignificant small patches in treated lakes. Fragments of these biotypes could then spread within a lake and from lake to lake via vectors such as boats and connecting canals, and could become the dominant population within 2–3 years. Given the rapid growth of hydrilla, with hundreds of millions of individuals per hectare, and the number of potential meristematic regions exposed to a sustained selection pressure with fluridone, it may not be surprising that resistance to a PDS inhibitor occurred with this plant. Once a resistant biotype has established itself as the dominant population, it produces millions of axillary and subterranean turions that can remain quiescent for many years, further diminishing the prospect of eradicating herbicide-resistant plants.

The combination of chemical weed control in aquatic environments and the multiple vegetative means of propagation of hydrilla may have favoured the development of resistance in this plant. Indeed, weed management with fluridone is accomplished by maintaining a constant herbicide concentration in lakes over several weeks (http://www.sepro.com/pdf_lit/aquatics/sonar/Sonar_Q_Label.pdf), imposing a sustained selection pressure on an organism that grows as much as 100 mm per day and can regenerate entire plants from a single node (Langeland 1996).

The fact that the molecular target site of fluridone is PDS, one of the key enzymes in carotenoid biosynthesis, may have also played an unforeseen role in the development of resistance in an aquatic environment. The primary physiological role of carotenoids is to protect the photosynthetic apparatus by quenching the excess electrons generated by photoenergized chlorophyll molecules under high light intensities. Plants treated with PDS inhibitors undergo photobleaching because the unquenched electrons cause chlorophyll photodegradation (Böger & Sandman 1998). However, hydrilla has low light compensation and saturation points, and a low CO_2 compensation point, enabling it to grow in only 1% of full sunlight (Van *et al.* 1976). Thus, the aquatic environment provides a gradient of light intensity where, in the presence of herbicide, plant segments harbouring resistant *pds* alleles could survive at high light intensity while being supported by susceptible stems that do not undergo photobleaching at intensities close to the compensation point. At low light incidence, even in the presence of herbicide, photodegradation of chlorophyll would be less likely to occur. Somatic mutations occurring in secondary axillary meristems are normally not expressed because of apical dominance. However, such mutations can be expressed when biotic or abiotic stresses remove the apical meristems (Marcotrigiano 2000). Treatments with fluridone are more effective toward the surface (high light intensities) than in deeper water (low light intensities). Therefore, most apical meristems are destroyed, probably releasing axillary meristems from dormancy. In addition, somatic mutations in meristematic cells can displace ancestral cells through intraorganismal selection. The modelling of this type of selection predicts that if a mutation provides an adaptive advantage to the plants regenerated from the mutated cell, the trait can rapidly spread through the population (Pineda-Krch & Fagerström 1999). This could be the case of the resistant biotypes observed in Florida lakes. In hydrilla, somatic mutations transmitted in either the apical or any of the numerous axillary meristems do not necessarily die with the rest of the plant, as would be typical in terrestrial systems, but fragments of hydrilla possessing a meristem can regenerate into entire plants (Langeland 1996).

The resistance factors observed at the enzymatic level in hydrilla (R/S-values, Table 2) translated to commensurate increases of up to six-fold in resistance at the organism level (R/S-values, Table 1). Stoichiometric equivalence between the *in vitro* and *in vivo* levels of herbicide resistance is unusual in terrestrial plants. In hydrilla, this may be related to the fact that in the aquatic environment where fluridone resistance evolved, the potential role of other physiological factors, such as uptake, translocation and metabolism of the herbicide, are minimized. The specific

activity (*f*) of these mutated PDS enzymes was not greatly affected, suggesting no fitness cost linked to resistance (Table 2). The fluridone-resistant biotypes may be equally competitive against the wild-type hydrilla and may persist as the dominant forms in lakes, even when the selection pressure subsides after the dissipation of fluridone.

Weed management in large water bodies relies and depends heavily on fluridone, the only USEPA-approved synthetic herbicide available for systemic treatments of lakes. The lack of sustainable alternatives has led to a concerted effort between industry, state and federal agencies to devise contingent management plans that take into account evolved fluridone resistance by hydrilla. Lakes are monitored regularly to detect any further changes, and prevent the spread of these herbicide-resistant biotypes. In light of the aggressive spread of hydrilla in aquatic ecosystems, the evolution of resistance to fluridone in Florida may forecast significant and long-lasting ecological and economic problems throughout the southern and pacific coastal states of the USA.

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