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Molecular evolution of herbicide resistance to phytoene desaturase inhibitors in *Hydrilla verticillata* and its potential use to generate herbicide-resistant crops†

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Abstract: Hydrilla [*Hydrilla verticillata* (Lf) Royle] is one of the most serious invasive aquatic weed problems in the USA. This plant possesses numerous mechanisms of vegetative reproduction that enable it to spread very rapidly. Management of this weed has been achieved by the systemic treatment of water bodies with the herbicide fluridone. At least three dioecious fluridone-resistant biotypes of hydrilla with two- to fivefold higher resistance to the herbicide than the wild-type have been identified. Resistance is the result of one of three independent somatic mutations at the arginine 304 codon of the gene encoding phytoene desaturase, the molecular target site of fluridone. The specific activities of the three purified phytoene desaturase variants are similar to the wild-type enzyme. The appearance of these herbicide-resistant biotypes may jeopardize the ability to control the spread of this non-indigenous species to other water bodies in the southern USA. The objective of this paper is to provide general information about the biology and physiology of this aquatic weed in relation to its recent development of resistance to the herbicide fluridone, and to discuss how this discovery might lead to a new generation of herbicide-resistant crops.

Keywords: non-indigenous species; invasive species; somatic mutations; herbicide resistance; aquatic weed; molecular adaptation

1 INTRODUCTION

Non-indigenous invasive species have a significant ecological impact and they threaten the survival of nearly half of the endangered species.[1] Furthermore, the economical and environmental damage caused by non-native organisms amount to US$ 125–140 billion per year in the USA alone.[2–4]

Since most eradication programs for invasive weeds rely on the use of selective herbicides, the management of these noxious plants is set back every time an invasive plant develops resistance. Recently, fluridone-resistant biotypes of hydrilla [*Hydrilla verticillata* (Lf) Royle], one of the most serious aquatic weed problems in the southern and western USA, have emerged in the waterways of Florida. *In vitro* assays have shown that mutations at the codon for the amino acid 304 of phytoene desaturase (*pds*) gene of hydrilla rendered this enzyme less sensitive to the herbicide fluridone.[5] Fluridone is the only herbicide approved by the USA Environmental Protection Agency (USA-EPA) for systemic treatment of large water bodies that efficiently controls hydrilla.

This review covers the recent findings on fluridone-resistant hydrilla, and the biological and physiological aspects that could have contributed to the emergence of herbicide-resistant biotypes. Furthermore, we discuss the potential use of these genes in creating herbicide-resistant crops.

2 DISTRIBUTION OF HYDRILLA IN THE USA

In the early 1950s, the female form of the dioecious hydrilla was brought from Ceylon (now Sri Lanka) to Missouri, and from there it was sent to Tampa

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Bay, Florida.[6] The monoecious form of hydrilla was introduced years later and first identified in North Carolina in 1980.[7] Dioecious plants have staminate and pistillate flowers on different individuals, whereas in monoecious plants both types of flowers are separated but borne on the same individual. Extensive studies with random amplified polymorphic DNA (RAPD) analysis of hydrilla samples from the USA and from around the world have indicated that hydrilla accessions from Florida, Texas and California are dioecious and related to accessions from Bangalore, India.[8,9] The monoecious plants from the USA cluster with hydrilla strains from Seoul, Korea.[8] This supported the hypothesis that there is a single common origin for the dioecious hydrilla population found in the USA, and confirmed its geographical point of origin close to Sri Lanka.[6] Apparently, there has been more than one introduction of the monoecious hydrilla in the USA. Information on the distribution of monoecious and dioecious hydrilla in the USA has been compiled by the USA Geological Survey (USGS) (http://nas.er.usgs.gov/plants/maps/hy_vert_bio.jpg). Hydrilla is widely distributed around the world, having been reported in Europe, Asia, Africa, Australia and America.[10,11]

3 ECOLOGICAL AND ECONOMICAL IMPACT

According to the Office of Technology Assessment, at least US$ 100 million is spent annually to control aquatic weeds.[12] Additional losses and damages are estimated at around US$ 10 million, giving a total cost of US$ 110 million per year.[2] In Florida, aquatic exotic plants, including *H verticillata* (hydrilla), *Eichhornia crassipes* (Martius) Solms-Laubach (water hyacinth) and *Pistia straiotes* (L) (water lettuce) are affecting fish and other aquatic animal species, choking waterways, changing nutrient cycles and reducing recreational use of rivers and lakes.[2]

Hydrilla has spread throughout the country's waterways, clogging irrigation and drainage canals, degrading water quality, reducing productivity of recreational fisheries and impeding navigation.[12] Even with an expenditure of US$ 50 million during the 1980s, the percentage of Florida waters invaded by hydrilla increased from 37 to 41%.[13] The cost of using herbicide to control hydrilla is US$ 1235 per hectare (www.ucsusa.org/global_environment/invasive_species) while the mechanical control for hydrilla can cost as much as US$ 2470 per hectare (www.invasive.org/eastern/eppc/HYVE.html).

4 BIOLOGY OF HYDRILLA

Hydrilla verticillata is a member of the family Hydrocharitaceae. This submersed macrophyte is well adapted to freshwater environments,[14] although it can also grow at 0.7% salinity, approximately a fifth of seawater concentration.[15] There are dioecious and monoecious forms of this species, and both are present in the USA.[8,9,11] In the overall distribution of hydrilla throughout the world, the monoecious form is more prevalent in warmer tropical areas, while the dioecious form occupies lower temperature areas. Monoecious hydrilla might be well adapted to high temperatures, making it highly feasible for both biotypes to overlap in the southern states of the USA.[16] Hydrilla has the general appearance to the untrained observer of being a dicotyledonous plant, but is in fact classified as a monocotyledonous plant. The lamina of a hydrilla leaf is only two cell-layers thick.[17] Since the harmful effects of UV-B on sensitive photosynthetic tissues is reduced by internal light scattering in thick leaves,[18] it is very likely that the reduced leaf thickness of hydrilla makes it more susceptible than other plants to a herbicide that induces photodamage, such as fluridone. Hydrilla has low light compensation and saturation points, and a low carbon dioxide compensation point, enabling it to grow in only 1% of full sunlight.[19] An interesting physiological feature of hydrilla is that it is the only known plant to operate a C_4 photosynthetic carbon dioxide concentration mechanism without possessing Kranz anatomy.[17,20]

Hydrilla has developed an inducible C_4-acid cycle to combat adverse conditions, such as limiting carbon dioxide, high oxygen concentration, high temperature and irradiance. Therefore, hydrilla can shift between C_3- and C_4-type photosynthesis, depending on the environment.[20] It is believed that the hydrilla system represents an archetypal form of C_4 photosynthesis among angiosperms, and that this process may have occurred in water before its appearance on land.[20]

4.1 Reproduction

Hydrilla reproduces in nature through a variety of means, including fragmentation, seeds, tubers, stolons and rhizomes. Stem fragments containing a single node can form a mature plant and as much biomass as 16 shoot tips.[21,22] Such fragmentation can occur, for example, by a boat propeller passing through a patch of hydrilla. However, the main reason for its persistence and longevity in natural environments is through production of specialized dormant buds called 'turions'. These structures pose the greatest challenge for controlling this aquatic weed species. Turions that form in the leaf axils and grow above ground are named axillary turions; those that form at the end of positively geotropic rhizomes extending into the hydrosoil are named subterranean turions.[23–25] Subterranean turions can remain dormant for as long as 5 years and are thought to maintain hydrilla growth within a given area, even through periods of drought.[26] Axillary turions are much smaller than subterranean turions, and are generally formed on detached floating mats of hydrilla. They last for about 1 year in the hydrosoil,[26] and provide a mechanism for intermediate distance dispersal within a waterbody and between waterbodies.[27–29] The two biotypes, monoecious and dioecious appear very similar, but differ in their reproductive cycle. In general, the

monoecious hydrilla biotype is more prolific in the formation of both subterranean and axillary turions (two- to sevenfold greater) than the dioecious biotype.[30] Turion formation is a photoperiodic effect and most production occurs under short days for the dioecious biotype.[11,31–33] In contrast, the monoecious biotype produces turions throughout the year.[31,34]

Seed production has been reported for the monoecious biotype in different parts of the world including India,[35] Australia[36] and the USA.[37] In addition, sexual compatibility exists between the monoecious and dioecious biotypes. In particular, the female form of Florida's dioecious hydrilla produced viable seed in crosses with monoecious strains from Malaysia, Bangalore, Kashmir, New Zealand and Panama under culture conditions.[11] Out of 56 crosses, 71% produced seeds, of which 90% were viable. Also, the female hydrilla biotype from Florida was the second largest seed producer out of 24 crosses with the monoecious hydrilla from Penang Island (Malaysia).[11] Monoecious and dioecious biotypes have already been reported in different lakes in California,[25,38] and also growing together at Lake Gaston on the Roanoke River located on the Virginia–North Carolina border, USA.[39] The potential cross-pollination between monoecious and dioecious hydrilla in areas where both biotypes grow together could result in increased genetic variability and possible enhanced adaptability of hydrilla to the environment.

4.2 Growth

A hydrilla colony originating from a single stolon can expand radially at a rate of 4 cm day^{-1}, with an average production of one new ramet m^{-2} day^{-1}. Root crowns develop stolons (horizontal above-ground shoots) that extend into the area surrounding the parent plant and establish new plants.[27] For the dioecious hydrilla, most of the colony expansion (99.9%) is by stoloniferous growth, while the spread from fragmentation is only 0.02 ramets m^{-2} day^{-1}.[27] There have been reports of 1250 to 1976 tubers m^{-2} being produced by the dioecious hydrilla within a period of four months in various Florida lake sediments.[40] However, in other areas, up to 2812 tubers m^{-2} were produced by the dioecious hydrilla during winter, while for the monoecious hydrilla the production of tubers during the summer was 5366 tubers m^{-2} and 2740 tubers m^{-2} during winter.[22] Hydrilla can grow from the substrate to the water surface and reach up to 15 m in length,[41] the stems can branch and they harbor leaf whorls (nodes) every 11–12 mm in the dioecious hydrilla and every 16 mm in the monoecious,[39] and each node can regenerate a new plant.[23]

Biomass allocation studies of the dioecious hydrilla show that this plant can accumulate up to 1200 g m^{-2} dry weight of above-ground shoot tissue.[42] Hydrilla has several organs for storage of carbohydrates, eg tubers, turions, stolons, stems and root crowns.[42] Of these, the upper and lower stems contain the largest amounts of total non-structural carbohydrates, ranging from 100 to 700 g m^{-2} in the upper and lower stems, respectively. Stolons and root crowns are the main source for re-growth in spring, rather than from tubers. Tubers can remain viable in the sediment for up to 5 years. In Texas, the dioecious hydrilla could accumulate 200 g m^{-2} dry shoot weight during the winter months, and about 600 g m^{-2} day^{-1} during the summer months.[42]

4.3 Genetic diversity

The genetic diversity level in aquatic macrophytes is known to be lower than that in terrestrial plants.[43] Hydrilla, however, is regarded as a species of high genetic variation.[44] Genetic variation between hydrilla strains from the USA,[10] Africa[45] and other regions has been examined by comparative isozyme studies[46,47] and random amplified polymorphic DNA (RAPD).[8,48,49] These studies were used to identify hydrilla strains in infected areas, and to determine the genetic relationship between geographically diverse hydrilla populations. Generally, hydrilla populations from Europe showed isozyme patterns distinct from all other hydrilla strains, which may be due to ecological adaptation and genetic drift.[45] Isozyme patterns of African biotypes are not very distinctive, probably due to local infestations and the non-weed nature of hydrilla in that continent.[45] The non-invasive nature of hydrilla in Africa could be due in part to the presence of numerous species of insects (Diptera and Lepidoptera) that feed on this species.[50] Genetic diversity in hydrilla was especially large in hydrilla collections from Southeast Asia and even between plants collected from a single lake in that region (Curug, Indonesia).[47] Southeast Asia is considered the origin of distribution of this noxious weed species.[51]

The dioecious and monoecious biotypes of hydrilla from the USA can be differentiated by isozyme patterns[52] and by RAPD analysis.[39] Using RAPD analysis, the monoecious biotype produces a single amplification product (850 base pairs), while the dioecious hydrilla produces two amplification products (850 and 450 base pairs).[39]

4.4 Monoecious and dioecious biotypes

Dioecious hydrilla was reported to have been introduced into the USA in the 1950s and was first observed in a Florida lake in 1959.[53] This dioecious female plant has spread throughout Florida and much of the southeastern USA.[54] It has been reported in different parts of Georgia, Tennessee, and South and North Carolina,[25] as well as Texas, California and Connecticut.[49,55] A second, separate introduction of the monoecious biotype was reported in Delaware in 1976 and in the Potomac River in 1980, and is also distributed throughout Virginia, Maryland and North Carolina.[56] Besides flowering and turion production, these two biotypes are also different in terms of growth habits. Growth of monoecious hydrilla is generally prostrate, near the hydrosoil, with many horizontal stems and higher densities than dioecious hydrilla.

The monoecious biotype can tolerate, and is even able to sprout at, cooler temperatures than the dioecious biotype.[33,48] In contrast to its worldwide distribution, the monoecious hydrilla possesses an annual growth habit, along with rapid turion production, adapting this biotype to the northern USA, which has cooler temperatures and shorter growing seasons.

4.5 Ploidy

Hydrilla does not sexually reproduce in Florida and southeast USA, ie seeds are not produced, since only the female form of the dioecious biotype is present.[23] In general, sexually reproducing species are characterized by high genetic diversity compared with asexually propagated species.[57] However, it has been documented that hydrilla is a polyploid plant. Its chromosome counts vary widely within a vegetative population.[10,51] Plants in Asia, India and Europe are either diploid ($2n = 2x = 16$) or triploid ($3n = 3x = 24$),[51] while the presence of tetraploid plants ($2n = 2x = 32$) has been reported in Alabama.[38] Both diploid and triploid plants have been collected from different parts of Washington DC, Maryland and Texas,[10,58] and those collected from California, Florida, Texas and Connecticut have been recorded as triploid.[58,59]

Hydrilla plants in various dioecious populations in Japan are either diploid or triploid, whereas monoecious strains in Japan are always triploid.[60] However, in different parts of the USA, the presence of diploid and triploid plants has been reported within the monoecious strain.[59] It has been suggested that hydrilla may be an endopolyploid plant resulting from chromosomal mutations in the original triploid female stock,[49] as an explanation for the occurrence of different ploidy levels in plants within the same population.[58,61,62] Various combinations of diploid, triploid and tetraploid cells have also been observed in root tips of hydrilla from the same plant.[54] Some plant species apparently have no consistent ploidy level in the developing root tissue.[63] The higher frequency of triploid plants than diploid ones in a hydrilla population may suggest an ecological advantage for triploid plants.[46] The molecular process of polyploidy evolution is the primary mechanism to generate genomic redundancy.[64] In young polyploids, both copies of a duplicated gene usually retain expression, and some authors have observed a direct relationship between the polyploidy and proliferation of transposable elements.[64] Genome duplication could allow for gene function of duplicated genes to diversify and bring about evolutionary innovation in general.[65] In plants, it has been shown that there is also a positive correlation between the expression level of a gene and the increase of the ploidy.[66] Although the relative importance of genome duplication in evolution is still not clear, it is possible that the variable ploidy of hydrilla could contribute to its adaptation.[65] These large genetic variations within a population, along with endopolyploidy, may offer the species an opportunity to have clones[10,54] that may react differently to varying environmental conditions. In the case of hydrilla, these factors may have contributed to the rapid development of herbicide resistance.

5 MECHANICAL, BIOLOGICAL AND CHEMICAL CONTROL

Several methods have been investigated for the control of hydrilla populations, including mechanical, biological and chemical control. Drawdowns and desiccation of hydrilla tubers have been shown to reduce the viability of the tubers. However, the efficacy of the desiccation treatment depends on several factors. Although a reduction of 90% in the number of tubers was achieved by this method, it did not completely eliminate the tubers from the sediments.[67] This study showed that, in some cases, drawdowns of 12 months were not enough to reduce the number of tubers in the banks or to reduce their viability.[67] In fact, it has been reported that if the drawdown is not long enough to kill the shoot biomass of hydrilla, it could stimulate hydrilla tuber production, which translates into more plants sprouting and colonizing a given area.[68] In the monoecious hydrilla, vegetative biomass and tuber number production were usually suppressed by one-week drawdown periods.[68]

Several biological agents have been studied for the control of hydrilla. For example, the fungal pathogen *Mycoleptodiscus terrestris* Ostazeski, an endemic pathogen that causes a short duration disease on hydrilla without persistence in plant debris or plant tissue, has been used as a mycoherbicide.[69] It can reduce hydrilla biomass in 80 days by up to 40% when applied alone, or by 93% when used in combination with fluridone treatments.[70] The use of this mycoherbicide in combination with the systemic treatment of lakes with the herbicide fluridone seemed to increase the susceptibility of hydrilla to the herbicide.[71] A complete list of insects evaluated as candidates for biological control has been surveyed and published.[72]

Mechanical control of hydrilla involves the removal of the vegetative tissues and/or the tubers/turions by dredging. This process is not practical for large lakes and is considered too expensive, costing about twice as much as chemical treatment (www.invasive.org/eastern/eppc/HYVE.html). At present, although much is known about hydrilla population biology, this knowledge has not been translated into effective control of this invasive species by mechanical or biological control methods.[73]

Registered herbicides for hydrilla management are limited to the contact herbicides endothall, diquat and chelated copper, and the systemic compound fluridone. Contact herbicides have been used to control hydrilla since the mid-1960s,[74] but they are only

effective to control newly emerging and smaller infestations. These compounds have short residual effect and the cost of product and application is in the range 700–1600 US$ ha^{-1}. The herbicide fluridone is the only cost-effective option to control large-scale hydrilla infestations. The use rates of fluridone are between 5 and 30 $\mu g\,liter^{-1}$, which result in up to a 30-fold reduction in product volumes compared with contact herbicides, and the cost for fluridone applications is between 125 and 600 US$ ha^{-1}.[75] Hydrilla is susceptible to very low concentrations of this herbicide that are sub-lethal to other aquatic vegetation, and this herbicide is the only EPA-approved systemic compound that can be used for treatment of large water bodies (www.sepro.com/pdf_lit/aquatics/sonar/Sonar_Q_Label.pdf).

6 EVOLUTION OF RESISTANCE TO PHYTOENE DESATURASE INHIBITORS

6.1 Biosynthesis and physiological functions of carotenoids in plants

Carotenoids play important roles in plants. These polyunsaturated molecules are antioxidants that have essential functions in photosynthetic organisms (plants, algae and cyanobacteria). In chloroplasts, carotenoids accumulate in the thylakoids, in association with the photosynthetic apparatus. They dissipate excess light energy trapped by the antenna pigments, while concomitantly participating in trapping light for photosynthesis and protecting chlorophylls from photodegradation under high light intensities. The protective role of carotenoids is achieved by quenching the excess excitation energy (in the form of electrons) released when photoenergized chlorophyll returns to ground state.

Carotenoids are also important precursors in the synthesis of the plant hormones abscisic acid[76,77] and gibberellic acid.[76] Mutant plants with deficient carotenoid pathways can exhibit dwarfism and altered foliar development because of the resulting hormone imbalance.[78] Finally, carotenoids are responsible for the yellow, orange and red coloring in many flowers and fruits.

The biosynthesis of carotenoids is compartmentalized in plastids and the steps are well characterized.[79] In brief, the first committed step in the biosynthetic pathway of carotenoids is the head-to-head condensation of two geranylgeranyl pyrophosphate molecules catalyzed by phytoene synthase that yields phytoene, a 40-carbon colorless carotenoid. Under normal conditions, phytoene does not accumulate in plant cells but is rapidly converted to the colored carotenoids phytofluene and ζ-carotene by phytoene desaturase (PDS). PDS is encoded by the *pds* gene, a member of a low-copy-number nuclear gene family,[80] and the protein is imported into the chloroplasts,[80,81] where, as the mature PDS protein, it is detected in soluble and membrane fractions of chloroplasts.[80] ζ-Carotene is subsequently converted to neurosporene and lycopene by the action of ζ-carotene desaturase. Most carotenoids downstream from lycopene (ie β-carotene, lutein, zeaxanthin and violaxanthin) undergo various levels of cyclization and oxidation (Fig 1).

6.2 Appearance of herbicide resistance

PDS inhibitors have been commercialized as herbicides for about 30 years.[82] All PDS inhibitors except fluridone have been registered for use in agricultural crops. The herbicide fluridone was discovered in the mid-1970s and approved by the USA-EPA for use in aquatic systems in 1986 (http://aquat1.ifas.ufl.edu/guide/sup3herb.html). Since then, it has been highly effective in the control of hydrilla.[83] However, there has been a recent report of the decrease in the efficacy of fluridone to control this aquatic weed.[5] Three dioecious hydrilla phenotypes have been described with resistance to 36, 54 and 91 nM fluridone in Florida lakes; these three were subsequently associated with mutations on the *pds* gene (Fig 2).[5]

6.3 Mechanism of action of PDS inhibitors

The carotenoid biosynthetic pathway is an excellent target for herbicides because it is essential for plant development while being absent in animals. Several chemical classes of PDS inhibitor have been developed, including pyridazinones, pyridinecarboxamides and phenoxybutanamides.[84] However, only a few inhibitors of this pathway have been commercialized because most of these compounds lack sufficient crop selectivity. Of the many enzymes involved in the formation of carotenoids, PDS is the primary herbicide target site in this pathway. Inhibition of this enzyme stops the synthesis of carotenoids in developing tissues, causing the destruction of chlorophylls and resulting in white foliage. Consequently, these herbicides are often referred to as bleacher or bleaching herbicides.

Mechanistically, PDS catalyzes the removal of two pairs of electrons (four electrons total) required to convert phytoene to ζ-carotene (Fig 1). Upon inhibition of this enzyme, phytoene (a colorless carotenoid) accumulates. Many kinetic studies have shown that the inhibitors do not compete for the binding of phytoene on PDS (Fig 3a).[85] While the exact mechanism of action for these inhibitors is not completely understood, it was recently demonstrated that these compounds compete for the binding site of plastoquinone on PDS (Fig 3b).[86] Plastoquinone is an essential cofactor of PDS.[87] It is interesting that the quinone binding site is also the herbicide target site of inhibitors of photosystem II,[88] and a vestige of a quinone binding site appears to be the herbicide binding site of acetolactate synthase.[89] In the resistant hydrilla biotypes, this binding site might be modified to prevent interaction with the herbicide, thus causing resistance, but this change may not hinder the interaction with plastoquinone since enzyme activity is not altered.[5]

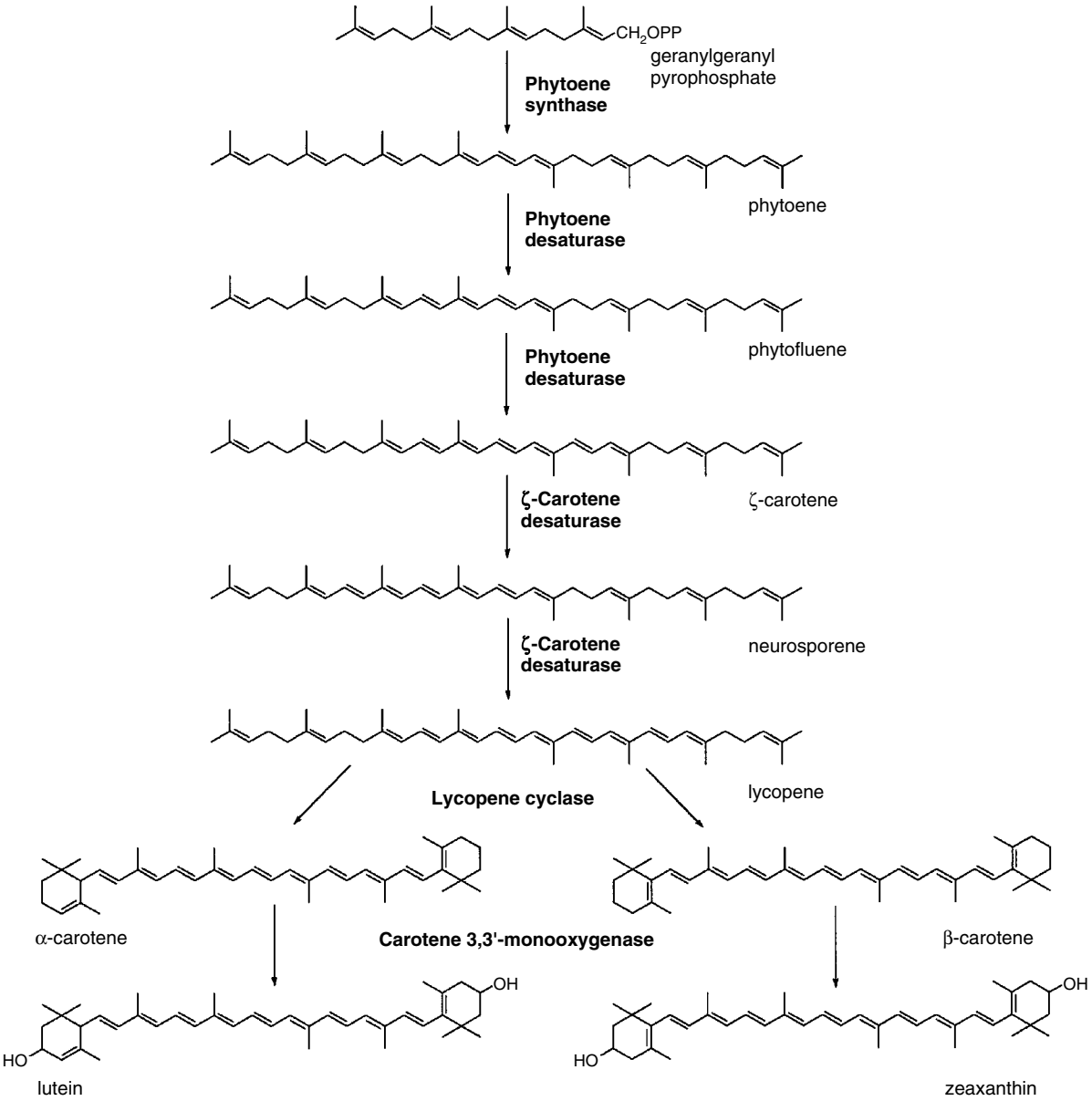

Figure 1. Simplified biosynthetic pathway of carotenoids. Enzyme names are indicated in bold. Compound names are indicated in normal font.

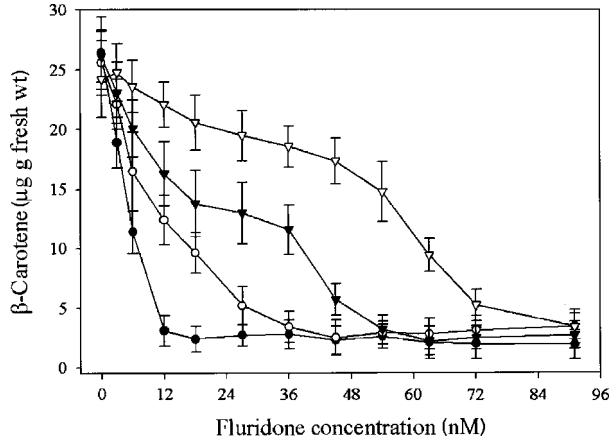

Figure 2. Mean and standard deviations obtained from laboratory assays of the β-carotene content of hydrilla shoot apices following a 14-day exposure to fluridone concentrations ranging from 0 to 91 nM. Phenotypes: ●: susceptible (179 lakes); ○: low resistance (8 lakes); ▼: intermediate resistance (7 lakes); ▽: high resistance (5 lakes) (reprinted with permission of Blackwell Publishing from Michel *et al*[5]).

6.4 Molecular basis for resistance to PDS inhibitors

Since the early 1990s there have been reports of several mutations on the *pds* gene of cyanobacteria that conferred resistance to PDS-inhibiting herbicides, ie Val403 to Gly, Leu320 to Pro, Arg195 to Pro and Leu436 to Arg, and a 20-nucleotide deletion in the transit peptide.[90] Cyanobacteria as a model system has the advantage of combining the presence of a photosynthetic apparatus with the fast growth rate of prokaryotes. Using this model system, screening methods to discover resistance to bleaching herbicides were designed. Briefly, cyanobacteria were first exposed to chemical mutagenesis and then selected for herbicide resistance on growth media containing various amounts of inhibitors. In particular, the point mutation resulting in an amino acid substitution (Val403 to Gly) in the phytoene desaturase gene of *Synechococcus* PCC 7942 was responsible for herbicide

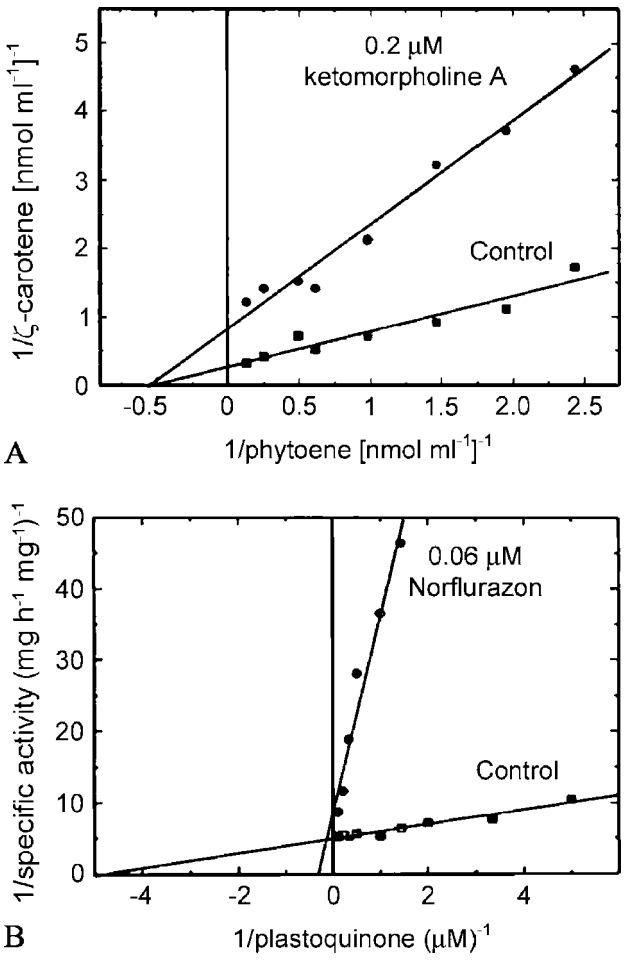

Figure 3. Types of inhibition kinetics of phytoene desaturase. (A) Non-competitive inhibition kinetics pattern obtained when a herbicide (ketomorpholine A) is tested against the enzyme substrate (phytoene) (Sandmann and Mitchell[85]) and (B) competitive inhibition kinetics pattern obtained when a herbicide (norflurazon) is tested against the enzyme cofactor (plastoquinone) (Breitenbach *et al*[86]). Figures are with permissions from publishers.

resistance in the mutant NFZ 4.[91] Using this mutated gene to transform tobacco plants, it was found that the gene conferred resistance to norflurazon and fluridone 58- and 3-fold higher than the wild-type controls, respectively.[92]

6.5 Molecular basis for resistance to PDS-inhibitors in hydrilla

The first case of a higher plant having evolved resistance to the PDS-inhibitor herbicide fluridone was discovered in hydrilla and published in 2004.[5] This herbicide has been on the market for 20 years, and when compared with other herbicide classes, it is surprising that resistance to PDS inhibitors has not been found previously. The *pds* alleles from herbicide susceptible and herbicide-resistant hydrilla biotypes have been cloned and sequenced. Alignment of *pds* sequences of various organisms shows conserved Arg codons in the same position (Fig 4). Three independent base pair substitutions at the amino acid 304 codon (Arg to Ser, Cys and His) of the PDS protein have been described in relation to fluridone

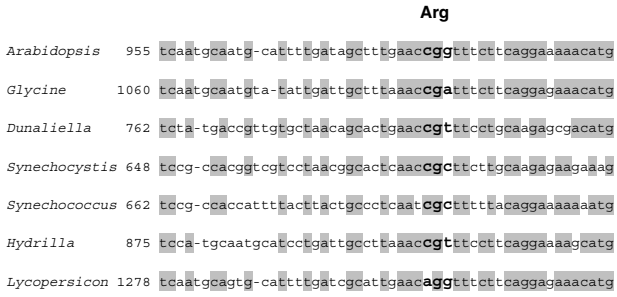

Figure 4. Alignment of partial phytoene desaturase gene sequences from various organisms showing arginine codons at the amino acid position 304 of hydrilla's PDS.

resistance.[5] These mutations were the first known cases of naturally occurring herbicide resistance to PDS inhibitors based on an altered target site in higher plants. Similar substitutions to the ones described for hydrilla (Arg to Ser, Cys and His) were observed at different positions within PDS of the cyanobacteria *Synechococcus* and *Synechocystis* when the cultures were grown on selection media with various PDS inhibitors.[93,94]

Reported mutations in cyanobacterial *pds* genes leading to herbicide resistance have usually been associated with a reduced specific activity of the enzyme.[90] However, in the mutations found in herbicide-resistant hydrilla populations, the specific activity of the PDS enzymes expressed in *E coli* was not significantly affected compared to the wild-type enzyme.[5] Since there were no differences in specific activity of PDS enzymes of hydrilla, when comparing the mutated proteins to the wild-type PDS, plants harbouring the mutated forms of PDS may still be able to compete in the environment without the presence of fluridone. The sustained ecological fitness of hydrilla plants carrying mutations on the *pds* gene may have enabled these biotypes to become the dominant populations within each lake.[5]

PDS is a nuclear-encoded protein with activity in the chloroplasts, the site of carotenoid synthesis.[80] Despite carotenoids being synthesized in all types of photosynthetic tissues, plants usually have very low levels of *pds* transcript. For example, no *pds* transcripts could be detected in tomato leaves,[95] and in soybean, no *pds* transcripts could be detected in any type of tissues by standard methods.[80] Low levels of transcription of a single copy gene have been negatively correlated with its mutation frequency. By using transformed *Arabidopsis thaliana* Heynhoe plants with the β-glucuronidase (*uidA*) gene as a model system, it was found that the frequency of somatic mutations for a single copy gene in plants is two to three orders of magnitude higher than in animals, yeast or even bacteria.[96] The predicted frequency of forward mutations for a single copy gene with a low level of transcription was 10^{-6}–10^{-7} events per base pair in *A thaliana*.[96] According to these predicted values, the 1.7-kb *pds* gene of hydrilla could have mutations in the order of 1.7×10^{-3} to 1.7×10^{-4}.

If genes with low levels of transcription have higher mutation rates, then the *pds* gene that has a low level of transcription could be prone to mutations and this could be an additional factor in the development of hydrilla biotypes with herbicide resistance. In hydrilla samples from only four Florida lakes, a total of six transversions and five transition mutations were found in *pds* alleles; the frequency of transversions was A → T (3×), C → A (2×), G → T (1×), while the rate for transitions was A → G (2×), G → A (2×), C → T (1×).[5] This frequency is much higher than expected for a dioecious plant that undergoes only asexual propagation.

Variations in a single gene can be high in cross-pollinated plants, but are expected to be low in self-pollinated plants. An example of this can be found in the gene for acetolactate synthase (ALS), which is related to herbicide resistance. In ragweed (*Ambrosia artemisiifolia* L), a mainly cross-pollinated species, when a 385 nucleotide fragment of the acetolactate synthase (ALS) gene was amplified, 48 nucleotides were polymorphic.[97] In the same study, a single ragweed plant had up to 34 nucleotide polymorphisms per 385-nucleotide sequence. However, in common cocklebur (*Xanthium strumarium* L), primarily self-pollinated, no polymorphisms were detected within the ALS sequences of 24 plants from seven different states (IL, MN, OH, NC, NM, MS and WA).[97] Although, in these particular studies, the authors did not find genetic variability related to resistance to ALS-inhibitor herbicides, they did find significant variability in the ALS genes and that the variability differed among species.[97] Given the high frequency of mutations already reported for the PDS gene of the dioecious hydrilla that reproduces asexually, even higher variations could be expected if cross-pollination occurs.

Factors that are likely to accelerate the selection of resistant biotypes are the repeated use of the herbicide in large areas, no use of alternative mode of action herbicides, high efficacy of the herbicide on the sensitive biotype at the rate used, and residual herbicide activity. Examples of plants that have developed resistance to herbicides via mutations can be found for ALS-inhibiting herbicides and for acetyl-CoA carboxylase-inhibiting herbicides. By 2002, eight different amino acid substitutions for Pro197 had been reported to confer herbicide (ALS-inhibitors) resistance in weeds, and 17 amino acid substitutions that conferred resistance in various organisms such as plants, yeast, bacteria and green algae.[98] The two mechanisms of resistance to ALS-inhibiting herbicides are increased herbicide metabolism resulting in rapid detoxification of the herbicide, and the reduced sensitivity of the target enzyme to inhibition by the herbicide.[98]

Recently, a terrestrial plant showing resistance to the PDS inhibitor diflufenican has been published. Populations of the weed *Raphanus raphanistrum* L developed resistance to the PDS inhibitor diflufenican after only four applications of this herbicide. Up to 16% of these populations of *R raphanistrum* survived four times the commercial application rate of diflufenican.[99] The molecular mechanisms related to the development of resistance in *R raphanistrum* have not been elucidated.

7 POTENTIAL USE OF MUTATED PDS FOR HERBICIDE-RESISTANT CROPS

The widespread adoption of glyphosate-resistant crops has revolutionized traditional agriculture. Some of the advantages of this technology are its broad-spectrum weed control and simpler weed-management practices. Several other transgenic crops possessing herbicide-resistant traits are also commercially available. Engineering plants for herbicide resistance has been the most effective way to obtain extremely high selectivity between crops and weeds.[100]

The phytoene desaturase (PDS) gene from *Erwinia uredovora*, crtI, which is not sensitive to PDS inhibitors, has been expressed in plants to confer resistance to norflurazon.[101] However, bacterial PDS can carry out four desaturation steps so that we can expect with crtI a broad spectrum of resistance to PDS and ζ-carotene desaturase inhibitors. Furthermore, consumers have expressed concerns over plants that have been transformed with prokaryotic genes. A mutated PDS, NFZ 4, from *Synechococcus*, has been expressed in tobacco where it conferred resistance to norflurazon although not significant resistance to fluridone.[92]

The mutations that we reported for hydrilla PDS were directly related to fluridone resistance.[5] On-going work in our laboratory has shown that expression of this gene in other plants confers a significant level of resistance to fluridone and several other PDS inhibitors.[102] The use of the mutations on hydrilla PDS for generating herbicide-resistant crops seems promising and could open new markets for this class of chemistry. This technology may also provide a new tool to help fight the evolution of herbicide resistance in weeds.

8 CONCLUSIONS

It is not possible to ascribe the emergence of herbicide resistance in dioecious hydrilla to a specific factor. Nevertheless, when analyzing hydrilla as a system, possible causes can be hypothesized. Hydrilla is a fast-growing plant with multiple means of propagation; able to generate new individuals from a single node, while each plant can be several meters long. Hydrilla has variable ploidy levels which can result in gene duplication and duplication of gene function, favouring the presence of various alleles able to adapt to variable environmental conditions. In addition, increase in ploidy has been related to increased levels of gene expression, which in the case of PDS could also contribute to herbicide resistance. The ability

of hydrilla to elongate at light intensities below the compensation point and to alternate between C_3 and C_4 metabolism assures nutrient support within the plant in distant tissues that could be exposed to adverse environments. The fact that the leaf lamina in hydrilla is semi-transparent and only two cell-layers thick may explain its susceptibility to fluridone at concentrations that are sub-lethal for other vegetation. However, the same leaf anatomy may contribute to higher rates of mutation due to UV radiation on hydrilla biomass growing on the surface of water bodies. A high rate of somatic mutations has been described in dioecious hydrilla, and these mutations can perpetuate, since this biotype undergoes only asexual propagation in the USA. In addition, the use of a single herbicide at low doses being the most cost-effective method to control this weed may in itself have contributed to the selection of resistant biotypes, given the adaptive potential of hydrilla as a system. Resistance in hydrilla is the result of one of three independent somatic mutations at the arginine 304 codon of the gene encoding phytoene desaturase, the molecular target site of fluridone. The use of these mutated genes offers a new approach to generate herbicide-resistant crops.

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