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Characterization of a higher plant herbicide-resistant phytoene desaturase and its use as a selectable marker

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Summary

Three natural somatic mutations at codon 304 of the phytoene desaturase gene (*pds*) of *Hydrilla verticillata* (L. f. Royle) have been reported to provide resistance to the herbicide fluridone. We substituted the arginine 304 present in the wild-type *H. verticillata* phytoene desaturase (PDS) with all 19 other natural amino acids and tested PDS against fluridone. In *in vitro* assays, the threonine (Thr), cysteine (Cys), alanine (Ala) and glutamine (Gln) mutations imparted the highest resistance to fluridone. Thr, the three natural mutations [Cys, serine (Ser), histidine (His)] and the wild-type PDS protein were tested *in vitro* against seven inhibitors of PDS representing several classes of herbicides. These mutations conferred cross-resistance to norflurazon and overall negative cross-resistance to beflubutamid, picolinafen and diflufenican. The T3 generation of transgenic *Arabidopsis thaliana* plants harbouring the four selected mutations and wild-type *pds* had similar patterns of cross-resistance to the herbicides as observed in the *in vitro* assays. The Thr304 *Hydrilla pds* mutant proved to be an excellent marker for the selection of transgenic plants. Seedlings harbouring Thr304 *pds* had a maximum resistance to sensitivity (R/S) ratio of 57 and 14 times higher than that of the wild-type for treatments with norflurazon and fluridone, respectively. These plants exhibited normal growth and development, even after long-term exposure to herbicide. As Thr304 *pds* is of plant origin, it could become more acceptable than other selectable markers for use in genetically modified food.

Keywords: chloroplast signal peptide, cross-resistance, fluridone, herbicide resistance, selectable marker.

Introduction

Genetic transformation of plants for a particular trait requires the use of a selectable marker gene which allows the identification of individuals that have received the gene of interest. Usually, selectable markers are genes that confer resistance to biocides, such as antibiotics or herbicides. Antibiotic resistance genes have their origin in microorganisms and, in some cases, plants harbouring these markers cannot withstand strong selection pressure, e.g. hygromycin (Nazakawa and Matsui, 2003). Antibiotic markers have come under strong criticism because of the fear that the use of antibiotics will promote the development of resistant bacterial strains, either by horizontal gene transfer or environmental selection pressure, as a result of the application of the antibiotic. Some herbicide resistance genes used as selectable markers also have their origin in microorganisms (Misawa *et al*., 1993; Wagner *et al*., 2002). The possible transfer of a gene for herbicide resistance from a crop to a weed remains a potential problem (Hall *et al*., 2000) and, as weed resistance evolves, new herbicide resistance genes will be needed.

Although no negative health effects have been associated with the use of antibiotic or herbicide markers in genetically engineered food, consumer surveys indicate a higher acceptability of genes of plant origin in food products than genes from other organisms (Lusk and Sullivan, 2002). To satisfy public concern, Rommens *et al*. (2004) proposed the use of all-native plant DNA for transformation. A desirable marker would be of plant origin, with a site of action of the selection agent that is not present in mammalian systems, and has a selection agent that can be easily incorporated in standard transformation systems.

The enzyme phytoene desaturase (PDS) has been the main target for herbicides that inhibit the carotenoid biosynthetic pathway. In plants, PDS converts phytoene to ζ-carotene. Carotenoids are essential components of the photosynthetic apparatus. They participate in light harvesting and protect the chloroplasts from the harmful effect of singlet oxygen formed during photosynthesis (Sandmann and Böger, 1997). PDS-inhibiting herbicides prevent the formation of carotenoids, resulting in the degradation of chlorophyll and the destruction of chloroplast membranes, which is characterized by the photobleaching of green tissues (Böger and Sandmann, 1998). Mutations of the cyanobacterium *Synechococcus* PDS have resulted in herbicide-resistant microbial enzymes (Chamovitz *et al*., 1991), and have conferred herbicide resistance when expressed in transgenic tobacco (*Nicotiana tabaccum*) plants (Wagner *et al*., 2002).

Recently, naturally occurring mutations at amino acid 304 of PDS in the aquatic weed *Hydrilla verticillata* (L. f. Royle) have been reported to impart herbicide resistance (Michel *et al*., 2004). This is the first known case of evolved resistance to PDS inhibitors in higher plants. Factors that may have contributed to this unique case, such as the unusual growth habits and multiple means of vegetative reproduction of *H. verticillata*, as well as the method of control in aquatic environments using fluridone, have been reviewed (Arias *et al*., 2005).

To further investigate the potential use of this herbicide-resistant gene from a higher plant as a selectable marker, amino acid 304 of *H. verticillata* PDS was substituted with the other 19 main amino acids, and the activity of the enzymes was tested *in vitro* against fluridone. Four of these mutations, in addition to the wild-type PDS, were selected for expression in *Escherichia coli* and *Arabidopsis thaliana* for further characterization. Cross-resistance of the enzymes and of the T3 generation of transgenic plants was tested against seven PDS-inhibiting herbicides.

Results

Analysis of the nucleotide sequence of a higher plant phytoene desaturase

The sequence of the phytoene desaturase gene (*pds*) of *H. verticillata* (Accession number AY639658) has three putative start codons: ATG1, ATG2 and ATG3 (Figure 1). The region between ATG1 and ATG3 is rich in alanine (Ala) (11%)

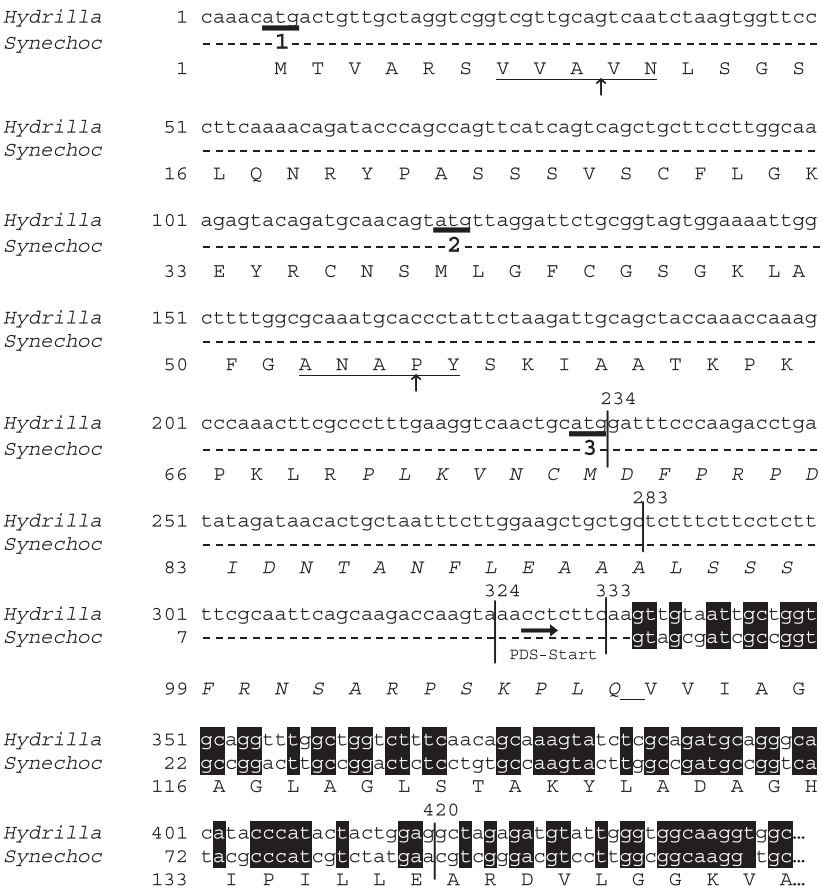

Figure 1 Partial DNA sequence alignment at the 5′ end of the phytoene desaturase gene (*pds*) of *Hydrilla* and *Synechococcus*. Underlined ATG codons with numbers 1, 2 and 3 correspond to the three putative start codons in *Hydrilla verticillata*. Horizontal arrow indicates the location of the primer PDS-Start. Shaded areas indicate homology. The amino acid sequence corresponds to the *Hydrilla* phytoene desaturase (PDS) protein. Underlined amino acids with a vertical arrow indicate potential recognition and cleavage sites in Gram-negative bacteria. Italicized amino acids represent the region of the *Hydrilla* PDS protein that is highly conserved in plants and is not present in *Synechococcus*. Numbers associated with vertical lines correspond to relevant nucleotides.

and serine (Ser) (13%) and has an isoelectric point of 10.38. A search for motifs within the amino terminus identified a t-SNARE coiled-coil domain [nucleotides (nt) 234–419] and a von Willebrand factor C (VWFC) domain (nt 283–333) (Figure 1). A computer analysis using the SignalP algorithm developed by Schein *et al*. (2001) predicted that the ATG1 to ATG3 region encoded for a chloroplast transit peptide, with a prediction level of 0.786.

In the DNA sequence alignment of *H. verticillata pds* with *Synechococcus elongatus pds* (Accession number X55289), the start codon of the cyanobacterial sequence corresponded to position nt 330 of *H. verticillata pds*. Using the primer PDS-Start (Figure 1), a deletion clone starting at nt 327 of *H. verticillata pds* was generated fusing the open reading frame (ORF) to a histidine-tag (His-tag) in a bacterial expression vector. Although this sequence encoded for what appeared to be the mature protein, the resulting protein had no PDS activity. Therefore, a similar construct was made with the complete *pds* sequence starting at ATG1 that included the putative sequence for a transit peptide and was expressed in *E. coli* for enzymatic assays. This construct produced an active PDS enzyme, but N-terminal His-tag purification by affinity chromatography was unsuccessful, suggesting possible cleavage in *E. coli*. Analysis of the 110 amino acids at the N-terminus of PDS using the program SignalP (Nielsen *et al*., 1997; Bendtsen *et al*., 2004) predicted two cleavage site positions in Gram-negative bacteria. The first was VVA-VN and the second was ANA-PY (Figure 1). A new construct was made using the deletion clone ATG3 without codon 76 (3ORF clones). These constructs expressed a His-tagged protein that was easily purified and, unlike the clone starting at amino acid 330 (aa330), the protein had enzymatic activity.

Resistance of the ATG1 series to fluridone

The *Hydrilla pds* was subjected to site-directed mutagenesis to create a series of expression constructs with all possible natural amino acids at position 304 of the PDS sequence. *In vitro* assays on wild-type and mutant PDS allowed the assessment of the amino acid substitutions. In general, replacement of arginine 304 (Arg304) by any amino acid resulted in higher resistance to fluridone than obtained with the wild-type enzyme, with the exception of the proline (Pro) and tryptophan (Trp) substitutions, which resulted in inactive PDS proteins (Table 1). The highest levels of resistance to fluridone tested *in vitro* were obtained with threonine (Thr), cysteine (Cys), alanine (Ala) and glutamine (Gln) mutations, which resulted in resistance to sensitivity (R/S) values of 40, 30, 23 and 19, respectively (Table 1).

Table 1 Inhibition of *Hydrilla verticillata* phytoene desaturase (PDS) mutant proteins in the presence of fluridone.

Amino acid*	Symbol	I_{50} (nM)	R/S
Non-polar			
Glycine	Gly	640	3.2
Alanine	Ala	4500	22.5
Valine	Val	2200	11.0
Leucine	Leu	3200	16.0
Isoleucine	Ile	2000	10.0
Methionine	Met	2300	11.5
Proline	Pro	Not active	–
Phenylalanine	Phe	530	2.6
Tryptophan	Trp	Not active	–
Uncharged polar			
Serine	Ser	3200	16.0
Threonine	Thr	10000	40.0
Asparagine	Asn	630	3.2
Glutamine	Gln	3800	19.0
Tyrosine	Tyr	810	4.4
Cysteine	Cys	6000	30.0
Charged polar			
Lysine	Lys	1000	5.0
Arginine	Arg	200	1.0
Histidine	His	1500	7.5
Aspartic acid	Asp	2000	10.0
Glutamic acid	Glu	310	1.5

I_{50}, concentration of herbicide that inhibits 50% of the activity of PDS; R/S, resistance to sensitivity ratio (I_{50} mutation/I_{50} wild-type PDSArg) in the presence of fluridone.
*Amino acid at position 304 of PDS.

Cross-resistance of the Thr, Cys, Ser and His mutants to seven phytoene desaturase inhibitors

PDS aa304 mutants (Thr, Cys, Ser and His), starting at either ATG1 (complete clones) or ATG3 (3ORF constructs) (Figure 1), were selected for a cross-resistance study against seven PDS-inhibiting herbicides in *in vitro* assays (Figure 2). All of these PDS mutant enzymes were more resistant than the wild-type to fluridone (R/S values up to 10) (Table 2). Their resistance to norflurazon was even greater (R/S values up to 52) (Table 2). Interestingly, all four mutations caused the enzymes to be generally more sensitive (negative cross-resistance) to beflubutamid, diflufenican and picolinafen, with R/S values between 0.2 and 0.8 (bold values in Table 2). Values of R/S < 1 indicate a higher susceptibility than the wild-type.

Analysis of the double mutants

Three mutation sites known to increase the herbicide resistance of *Synechococcus pds*, different from that

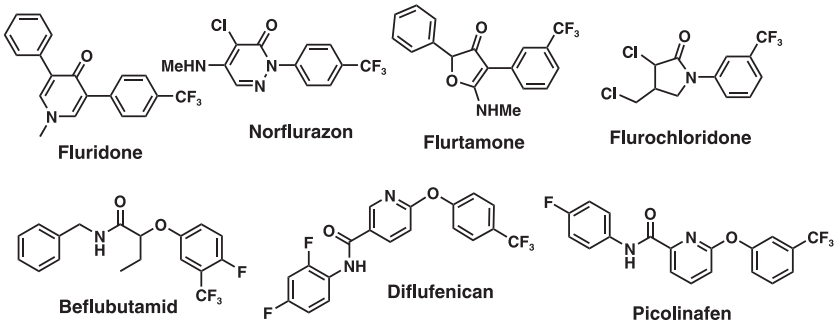

Figure 2 Chemical structures of the seven phytoene desaturase (PDS)-inhibiting herbicides used in this study.

Table 2 *In vitro* activity of phytoene desaturase (PDS) inhibitor herbicides on wild-type (Arg304) and mutated *Hydrilla verticillata* enzymes. Bold values show negative cross-resistance (R/S < 1).

		Arginine		Cysteine		Histidine		Serine		Threonine	
Herbicide	Clone	I_{50}	R/S	I_{50}	R/S	I_{50}	R/S	I_{50}	R/S	I_{50}	R/S
Fluridone	ATG1	1.8	1.00	5.7	3.20	5.0	2.80	9.9	5.50	18.1	10.00
	ATG3	305	1.00	904	2.96	1470	4.82	819	2.68	2373	7.78
Norflurazon	ATG1	3.1	1.00	89.4	28.80	5	1.60	54.9	17.70	161.2	52.00
	ATG3	113	1.00	1231	10.89	315	2.79	2591	22.93	1421	12.58
Flurtamone	ATG1	2.8	1.00	5.3	1.90	3.3	1.20	5.9	2.10	8.1	2.90
	ATG3	286	1.00	782	2.73	651	2.77	443	1.55	1729	6.05
Flurochloridone	ATG1	12.2	1.00	44.3	3.60	14.0	1.10	23.0	1.90	52.0	4.30
	ATG3	211	1.00	321	1.52	256	1.21	425	2.01	**162**	**0.77**
Beflubutamid	ATG1	4.3	1.00	**3.5**	**0.80**	**3.0**	**0.70**	**2.6**	**0.60**	**1.2**	**0.30**
	ATG3	103	1.00	196	1.90	159	1.54	321	3.12	111	1.08
Diflufenican	ATG1	5.8	1.00	**2.1**	**0.40**	**2.0**	**0.40**	**1.1**	**0.20**	**1.0**	**0.20**
	ATG3	132	1.00	163	1.23	**62**	**0.47**	153	1.16	**67**	**0.51**
Picolinafen	ATG1	5.6	1.00	**2.4**	**0.40**	**3.0**	**0.50**	**2.3**	**0.40**	**2.7**	**0.50**
	ATG3	215	1.00	**73**	**0.34**	**71**	**0.33**	**43**	**0.20**	**47**	**0.22**

I_{50}, concentration of herbicide that inhibits 50% of the activity of PDS; R/S, resistance to sensitivity ratio (I_{50} mutation/I_{50} wild-type PDSArg).
I_{50} values for ATG1 and ATG3 were obtained by different methods; however, R/S values are comparable.

corresponding to aa304 of *H. verticillata*, were introduced into *H. verticillata pds*. The resulting enzymes exhibited herbicide resistance, with R/S ratios of 1.6, 4.5 and 14.5 for Pro320, Arg436 and glycine 403 (Gly403), respectively. Double mutants of *Hydrilla pds* were made as a combination of the three mutations reported in *Synechococcus* and either Thr304 or Ser304. All six double mutants resulted in inactive proteins, showing no production of ζ-carotene *in vitro*.

Kill curve of phytoene desaturase inhibitor herbicides tested in *Arabidopsis* Col 0

The dose–response curves of the seven PDS-inhibiting herbicides on *A. thaliana* Col 0 were obtained. The 50% effective concentration (EC_{50}) values determined for *A. thaliana* were as follows: fluridone, 13.5 nM; norflurazon, 26.0 nM; flurochloridone, << 5 nM; flurtamone, 8.0 nM; diflufenican, 66.7 nM; picolinafen, ~4.5 nM; beflubutamid, 78.9 nM.

Arabidopsis transformation with *Hydrilla pds* constructs

A. thaliana was transformed with the wild-type *Hydrilla pds*, *pds* mutated at aa304 for Thr, Cys, Ser and His, and the empty vector as control. Seedlings harbouring mutations of *Hydrilla pds* showed resistance to norflurazon in T1, T2 and T3 generations. The use of 25 mg/L hygromycin for selection in the first generation showed a clear distinction of resistant vs. non-resistant seedlings. At this concentration, the seedlings had to be removed from selection in 5 days or they remained stunted. Despite the high concentration of hygromycin used, the selection of resistant from non-resistant seedlings in the second and third generations was problematic as a gradient of seedling height and root length was observed. A clear distinction between resistant and non-resistant seedlings was much easier in all generations using selection on norflurazon. Resistant plants exhibited normal growth and development even after weeks of continuous

Table 3 Percentage survival of T3 generation of segregating *Arabidopsis thaliana* lines transformed with empty vector, wild-type phytoene desaturase gene (*pds*) (arginine) or mutant (cysteine, serine, histidine and threonine) *Hydrilla verticillata pds*. Plants tested against seven phytoene desaturase (PDS)-inhibiting herbicides. Bold values show negative cross-resistance (R/S < 1).

		Survival (%)					
Compound	(nM)	Empty	Arginine	Serine	Threonine	Histidine	Cysteine
Fluridone	100	0	0	72 ± 27	60 ± 30	71 ± 51	25 ± 21
Norflurazon	300	0	0	49 ± 23	37 ± 24	70 ± 51	34 ± 26
Flurochloridone	100	0	0	39 ± 40	8 ± 12	36 ± 55	18 ± 17
Flurtamone	100	0	0	46 ± 34	24 ± 17	0	0
Diflufenican	300	35	**18 ± 2**	66 ± 48	54 ± 37	53 ± 50	**18 ± 6**
Picolinafen	100	80	**55 ± 1**	**68 ± 28**	**64 ± 17**	**16 ± 14**	**5 ± 8**
Beflubutamid	100	11	15	81 ± 18	30 ± 20	**8 ± 7**	**0**

R/S, resistance to sensitivity ratio.

Table 4 Highest sublethal concentrations of fluridone and norflurazon effective on selected transgenic plant lines harbouring the wild-type (WT) (Arg304) or mutant (Ser304, Thr304, His304 or Cys304) *Hydrilla verticillata* phytoene desaturase gene (*pds*). Copy number of *Hydrilla pds* is indicated.

	Vector													
	Ser304			Thr304			His304			Cys304			Arg304	WT
Plant line	36	54	57	17	20	30	87	89	90	83	84	85	4	
Gene copy number	1	1	1	1	2	3	3	1	nd	2	1	1	2	na
Highest sublethal concentration														
Fluridone (nM)	150	150	150	150	200	100	100	125	150	100	100	100	25	< 10
Norflurazon (nM)	1000	1000	1000	1000	3000	1000	500	3000	1000	1000	1000	3000	30	< 20
Hygromycin (mg/L)	25	25	25	25	25	25	25	25	25	25	25	25	25	<< 25

na, not applicable; nd, not determined.

selective pressure, whereas non-resistant plants were achlorophyllous (purple instead of green).

The T3 generation of transgenic lines Thr 17, His 87, 89 and 90, and Ser 36, 54 and 57 did not show segregation, whereas the lines Cys 83, 84 and 85 and Thr 20 and 30 showed segregation for hygromycin resistance. According to the number of copies of *Hydrilla pds* in non-segregating transgenic lines, these were generally homozygous, except for His 87 which had three copies. The copy number for His 90 was not determined.

Cross-resistance of the T3 generation of transgenic plants

The T3 generation of transgenic plants expressing any of the herbicide-resistant forms of *H. verticillata pds* were resistant to 100 nM fluridone (Table 3), whereas plants harbouring the empty vector did not survive concentrations of fluridone as low as 20 nM. Fifty per cent of the plants carrying the wild-type *H. verticillata pds* (Arg304) survived at 20 nM fluridone, probably because of the presence of additional copies of *pds* (Table 4). Plants carrying any of the mutations at aa304 were resistant up to 300 nM norflurazon and 100 nM flurochloridone (Table 3). In general, plants with the Thr and Ser constructs showed some level of resistance to flurtamone and beflubutamid. Plants harbouring the mutated *pds* were more sensitive (negative cross-resistance) to diflufenican, picolinafen and beflubutamid than those transformed with the empty vector or the wild-type *H. verticillata pds* (bold values in Table 3).

Non-segregating T3 lines transformed with the empty vector or the wild-type *H. verticillata pds* showed no resistance to the herbicides fluridone, flurochloridone and flurtamone at 100 nM, or norflurazon at 300 nM. Lines transformed with either the Thr or Ser mutation showed nearly 100% survival to the same herbicide treatments. A high level of resistance to fluridone, norflurazon and flurtamone was also observed in plants transformed with the His mutation (Table 5). On the other hand, the non-segregating T3 lines transformed with any of the *pds* mutations also exhibited negative cross-resistance to the herbicides diflufenican, picolinafen and beflubutamid, showing lower levels of survival in these

Table 5 Percentage survival of T3 generation of non-segregating *Arabidopsis thaliana* lines transformed with empty vector, wild-type (arginine) or mutant (serine, threonine or histidine) *Hydrilla verticillata* phytoene desaturase gene (*pds*). Plants tested against seven phytoene desaturase (PDS)-inhibiting herbicides. Bold values show negative cross-resistance (R/S < 1).

		Survival (%)				
Compound	(nM)	Empty	Arginine	Serine	Threonine	Histidine
Fluridone	100	0	0	100	100	100
Norflurazon	300	0	0	99 ± 1	96	100
Flurochloridone	100	0	0	67 ± 28	32	100
Flurtamone	100	0	0	11 ± 17	94	0
Diflufenican	300	100	89	**52 ± 27**	**56**	**80 ± 28**
Picolinafen	100	100	100	**72 ± 7**	**48**	**59 ± 21**
Beflubutamid	100	81	100	**45 ± 13**	**26**	**27 ± 12**

R/S, resistance to sensitivity ratio.

herbicides than plants transformed with the empty vector or the wild-type *pds* (bold values in Table 5). In the T3 generation, all selected lines carrying the Cys mutation were segregating; therefore, they were not included in Table 5.

Maximum resistance to fluridone and norflurazon

Although all plants transformed with *H. verticillata pds* mutations exhibited resistance to fluridone and norflurazon, the Thr 20 line was the only line that survived in 200 nM fluridone and 3000 nM norflurazon (Table 4). These concentrations represent R/S values of 57 and 14 for norflurazon and fluridone, respectively. Overall, the copy number of *H. verticillata pds* encoding for herbicide resistance did not appear to affect the level of resistance to these two herbicides (Table 4).

Evaluation of the biometric parameters of plant lines

Under low light intensity and non-competitive laboratory conditions, the transgenic lines carrying either *pds* mutations or the wild-type *H. verticillata pds* had a normal appearance when compared with the wild-type *A. thaliana*. Chlorophyll levels and coloured carotenoid contents were analysed in three plant lines for each construct. The mean values are shown in Table 6. In general, the chlorophyll and carotenoid contents of plants carrying *pds* mutations were of the same order of magnitude as those of wild-type plants (Table 6). Similar results have been reported in tobacco plants expressing a mutated cyanobacterial *pds* providing resistance to bleaching herbicides (Wagner *et al*., 2002). Transfers from culture conditions to soil and seed harvesting from transgenic plants were performed simultaneously with the wild-type in every generation. No differences were observed in the growth rate and development of plants carrying *pds* mutations.

Table 6 Chlorophyll and carotenoid content of wild-type and T3 generation of non-segregating lines of *Arabidopsis thaliana* transformed with various forms of the *Hydrilla verticillata* phytoene desaturase gene (*pds*).

Plant line	Chlorophyll (mg/g FW)	Carotenoids (μg carotenoid/g FW)
Wild-type *A. thaliana*	1.8 ± 0.3	3238 ± 453
Empty vector	1.6 ± 0.2	2844 ± 448
Arg304 *pds*	2.2 ± 0.2	3760 ± 473
Cys304 *pds*	1.8 ± 0.4	2162 ± 481
His304 *pds*	2.1 ± 0.3	2137 ± 1067
Ser304 *pds*	1.7 ± 0.3	2331 ± 168
Thr304 *pds*	2.1 ± 0.4	3091 ± 578

Discussion

An alignment made with the PDS protein sequences of *H. verticillata* and *Synechococcus* suggests that the mature PDS protein of *Synechococcus* begins at amino acid residue 111 of *H. verticillata* PDS. Analysis of the sequence upstream from nt 335 provides strong evidence that it encodes for a chloroplast transit peptide. In general, transit peptides are 30–80 residues in length, deficient in acidic amino acids, contain 12%–13% Ala and 16%–17% Ser, and have an average isoelectric point of 10.9 (Zhang and Glaser, 2002). The region between ATG1 and ATG3 of *H. verticillata* PDS consists of 76 amino acids containing 11% Ala and 13% Ser, and an isoelectric point of 10.38. In contrast, the isoelectric point for the rest of the PDS protein, without the ATG1 to ATG3 fragment, is only 5.75 and contains 9% Ala and 6% Ser. Furthermore, computer analysis using the algorithm developed by Schein *et al*. (2001) also predicts that the ATG1 to ATG3 region of *Hydrilla* PDS encodes for a chloroplast transit peptide, with a prediction level of 0.786. A minimum threshold of 0.420 is sufficient for a sequence to be classified as encoding a chloroplast transit peptide.

The expression of a PDS protein without the putative transit peptide (starting at nt 326, PDS-Start in Figure 1) in *E. coli* resulted in inactive PDS. However, a PDS protein that included 33 amino acids upstream of PDS-Start was functional, indicating that the stretch of amino acid residues 77–107 is required to express the active protein. The alignment of 17 plant PDS protein sequences from GenBank revealed a highly conserved stretch of 39 amino acids upstream of the start codon of PDS in *Synechococcus*. A search for motifs within this region of *Hydrilla* PDS, using the software MOTIF (http://

motif.genome.jp/), identified two interesting domains, a t-SNARE coiled-coil homology domain profile (nt 234–419) and a VWFC domain (nt 283–333) (Figure 1). SNAREs provide specificity for protein docking, where the v-SNARE carried by a vesicle binds to the t-SNARE on the plasma membrane to form a four-helix bundle SNAREpin. At least one helix is predicted for *H. verticillata* PDS (aa 88–97) within the 33 amino acid residues mentioned above. The homology with the VWFC domain is particularly interesting, as this domain is thought to participate in oligomerization, although not in the initial dimerization step of a protein (Voorberg *et al*., 1991; Bork, 1993). In the case of PDS, this domain could be necessary for the correct assembly of the protein complex to work in carotenoid synthesis.

It has been demonstrated that PDS exists as a monomeric and oligomeric complex assembled after import of the protein in the stroma of the thylakoid (Bonk *et al*., 1997). From a physiological perspective, protein–protein interaction between the thykaloid membrane-localized phytoene synthase and the stroma-localized PDS would be highly advantageous, if not required, to enable the channelling of phytoene. Indeed, phytoene is a highly hydrophobic molecule and will be compartmentalized into the thylakoid membrane rather than the hydrophilic environment of the stroma. The association of PDS with the thylakoid membrane would ensure access to phytoene.

It has been proposed that carotenoid desaturases assemble as dimers, in addition to forming part of larger protein complexes in the membrane (Bartley *et al*., 1990; Pecker *et al*., 1992). Analysis using motif search software (SCL, 2003) indicated that there was 92% homology between the nt 324–432 region of *pds* and a dimerization domain of dehydrogenase (Figure 1). Analysis of *Hydrilla* PDS protein by size exclusion chromatography also supports the formation of a dimer and an even larger oligomer (Figure 3). Analysis of the fractions containing peaks 1, 2 and 3 by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) yielded single bands of 61.3 kDa corresponding to the size of the PDS unit.

A. thaliana transformed with the Thr304 *H. verticillata* PDS had R/S values up to 57 and 14 for norflurazon and fluridone, respectively. Some *A. thaliana* lines transformed with His and Cys mutations of *Hydrilla* PDS also survived 3 μM norflurazon treatments. A similar R/S ratio (58 for norflurazon) has been reported for tobacco plants transformed with mutant forms of *Synechococcus* PDS. In the same study, the R/S value for fluridone was only 3 (Wagner *et al*., 2002). Tobacco plants transformed with the *Erwinia uredovora* (*crtI*) *pds* have been shown to survive up to 3 μM norflurazon and 12 μM fluridone (Misawa *et al*., 1993). In our study, one non-segregating line, carrying *H. verticillata* PDS with the Thr mutation, was resistant to 3 μM norflurazon and 200 nM fluridone.

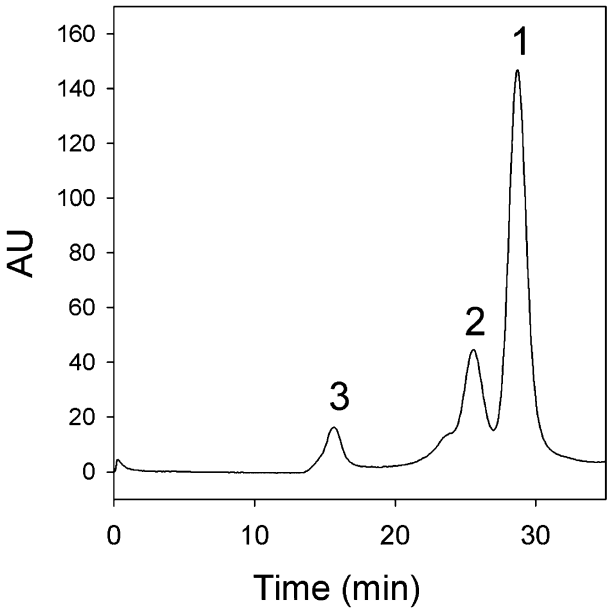

Figure 3 Size exclusion chromatography of ATG3 Arg304 *Hydrilla verticillata* phytoene desaturase (PDS) with the histidine (His)-tag removed. Peak 1 represents the monomer (61.3 kDa), peak 2 is the dimer (122.6 kDa) and peak 3 is an oligomeric complex (greater than 300 kDa). The fractions containing peaks 1, 2 and 3 yielded single protein bands of 61.3 kDa corresponding to the size of the PDS protein by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

In previous work, all plants expressing bacterial or cyanobacterial PDS exhibited positive cross-resistance to PDS-inhibiting herbicides (Misawa *et al*., 1994; Wagner *et al*., 2002). Expression of the bacterial *crtI* gene in tobacco plants conferred resistance to norflurazon, fluridone, flurtamone, flurochloridone and diflufenican (Misawa *et al*., 1994). Here, we report that transgenic plants carrying a higher plant *pds* with various mutations at aa304 show negative cross-resistance (or supersensitivity) to some PDS-inhibiting herbicides (i.e. diflufenican, beflubutamid and picolinafen). Negative cross-resistance was observed *in vitro* in the enzyme assays and *in planta*, in both segregating and non-segregating transgenic *A. thaliana* plant lines. Herbicide supersensitivity, or negative cross-resistance, has been described for several other classes of herbicide, such as inhibitors of photosystem II (Oettmeier, 1999; Gressel, 2002) and mitotic disruptors (Vaughn *et al*., 1987), but has never been observed for herbicides that inhibit carotenoid biosynthesis.

Use as a selectable marker

Plants transformed with the mutated higher plant PDS showed an interesting herbicide resistance profile. Any of the

Figure 4 Use of Thr304 mutation of *Hydrilla verticillata* phytoene desaturase gene (*pds*) as a selectable marker in *Arabidopsis thaliana*. Plants transformed with empty vector pCambia 1303 (left), *pds* Arg304 (right), *pds* Thr304 segregating line (top) and *pds* Thr304 non-segregating line (bottom) were grown in the presence of 300 nM norflurazon. The phenotype of untransformed *A. thaliana* plants exposed to 300 nM norflurazon was similar to that seen with plants transformed with empty vector.

pds mutations conferred resistance to fluridone in transgenic *Arabidopsis*, with a minimum R/S value of 7, whereas plant lines harbouring the Thr304 PDS reached an R/S value of 14. Plants carrying *Hydrilla pds* mutations were able to stand the selective pressure of fluridone and norflurazon *in vitro* for the entire life cycle. This represents an advantage relative to selectable markers such as hygromycin, which require removal of selective pressure after a few days (Nazakawa and Matsui, 2003). Indeed, the distinctive green colour shown by plants harbouring *pds* mutations, compared with the bleached non-transformed plants, makes this gene an excellent tool for selection (Figure 4). The use of norflurazon was highly effective for selection, with a maximum R/S ratio of 57. This herbicide is the most water soluble of the compounds tested and is highly stable in solution or culture medium. Probably the most interesting aspect of the use of *Hydrilla pds* mutations as a selectable marker is the higher plant origin. Given the negative public perception to the use of antibiotic resistance genes or other selectable markers of bacterial origin, a plant gene that is not found in mammalian systems is a better option as a selectable marker. Furthermore, this system also benefits from the negative cross-resistance conferred by *pds* mutations to some of the PDS inhibitors. Indeed, this may represent an interesting tool to eliminate transformed plants if they were to appear in the wild.

Conclusion

We have reported the first case of evolution of resistance to PDS inhibitors in higher plants through somatic mutations (Michel *et al*., 2004). In the present study, we determined that *Arabidopsis* plants transformed with *Hydrilla pds* containing mutations for Cys, Ser, His and Thr at position 304 resulted in high resistance to fluridone and norflurazon. In particular, plants harbouring the Thr304 mutation had a fluridone R/S ratio of 14. *Hydrilla* PDS with Thr304 is proposed here as a selectable marker based on two main advantages. First, consumers may better accept a genetically modified food if transformed with a plant gene. Second, *Hydrilla* PDS Thr304 confers high resistance to a limited spectrum of herbicides (fluridone, norflurazon and flurtamone), whereas it renders the genetically modified plants more susceptible than the wild-type to other PDS inhibitors, such as diflufenican, picolinafen and beflubutamid. This feature could be beneficial to eliminate undesirable transgenic plants in the wild, rather than creating highly resistant weeds.

Herbicide-resistant crops have dramatically altered modern agricultural practices; they have resulted in a greater use of soil conservation practices and a decrease in the number of chemicals released into the environment. Our constructs clearly confer resistance to PDS inhibitors in *Arabidopsis* and may be used to develop resistant crops. The unique aspect of the natural mutations of *Hydrilla pds* is their negative cross-resistance that could result in the development of designer herbicides.

Experimental procedures

Materials

Fluridone and norflurazon were purchased from Chem-Service, Inc. (West Chester, PA, USA). Diflufenican and flurtamone were kindly provided by Aventis Crop Sciences (Frankfurt, Germany). The herbicides beflubutamid, picolinafen and flurochloridone were kindly provided by Ube Industries Ltd. (Ube City, Japan), BASF (Limburgerhof, Germany) and Syngenta (Bracknell, Berkshire, UK), respectively.

Bacterial expression vectors

H. verticillata pds has three in-frame putative start codons (Figure 1). The complete *pds* sequence that includes codon ATG1 was cloned in the vector TOPO4 (Invitrogen, Carlsbad, CA) and the plasmid was named pHy4ATG5 (Michel *et al*., 2004). This plasmid was used as a template to substitute Arg304 of PDS with all the natural amino acids using a QuickChange

Site-directed Mutagenesis Kit (Stratagene, CA, USA; no. 2000518), and the plasmids were named the ATG1 series. The primers used to replace Arg304 by Cys, Ser and His have been reported previously (Michel *et al*., 2004). The forward primer used to change Arg to the remaining amino acids was Hyd-Ala-For [5′-gcatcctgattgccttaaa(cgc)tttccttcaggaaaagc-3′] for Ala, where the three nucleotides in parentheses were replaced by (aat) for aspartic acid (Asn), (gag) for glutamic acid (Glu), (gat) for asparagine (Asp), (cag) for Gln, (att) for isoleucine (Ile), (aag) for lysine (Lys), (atg) for methionine (Met), (ttc) for phenylalanine (Phe), (act) for Thr, (tat) for tyrosine (Tyr), (tgg) for Trp, (gtt) for valine (Val), (ggt) for Gly, (ctt) for leucine (Leu) and (cct) for Pro. Reverse primers were the complementary sequences of the forward primers. After each site-directed mutagenesis, each new *pds* was completely sequenced to confirm that the clone contained no additional mutations. The 19 resulting plasmids were named pPDS(aa), i.e. for the Ser mutation, the plasmid was pPDSSer. Plasmids pPDS(aa) were transformed into bacterial Top10 cells (Invitrogen) for expression and analysis of PDS.

Expression plasmids starting at nt 234 (Figure 1), named the ATG3 series, were made using pRSETb (Invitrogen) as a backbone. p3ORF-ATGSet, p3ORF-ATGSerSet, p3ORF-ATGCysSet and p3ORF-ATGHisSet have been described previously (Michel *et al*., 2004). The same procedure was followed to make plasmid p3ORF-ATGThrSet for the Thr mutation.

Four mutations in other regions of the *pds* of *Synechococcus* have been reported to confer resistance to norflurazon and fluridone (Chamovitz *et al*., 1991). Using p3ORF-ATG (Michel *et al*., 2004) as template plasmid, the mutations reported for *Synechococcus* were added to *H. verticillata pds*. The following forward primers were used for these mutations, and their corresponding complementary sequences were used as reverse primers: Hyd-320-Pro-For: 5′-ggaagttgaagaacacatacgatcatcctcttttcagcagg-3′ to change Leu425 to Pro425; Hyd-403-Gly-For: 5′-gttgtaaagaccccgaggtcaggttacaagacggtcc-3′ to change Val509 to Gly509; Hyd-436-Arg-For: 5′-ggtgactacacaaagcagaagtatagggcctcaatggaagg-3′ to change Leu542 to Arg542; Hyd-913-Phe-For: 5′-cttcataaaccctgatgaattttccatgcaatgcatcc-3′ to change Leu293 to Phe293. After mutagenesis, *pds* was subcloned using the *Eco*RI site into pRSETb to add the His-tag.

Double mutants

Plasmids p3ORF-ATG with Ser and Thr mutations, p3ORFSer and p3ORFThr, respectively, were used to make eight double mutants by adding each of the four mutations described in *Synechococcus* sp. employing the primers indicated above. Subcloning the double mutants into vector pRSETb resulted in the plasmids: p3ORF-ATGThr320ProSet, p3ORF-ATGThr403Gly, p3ORF-ATGThr436Arg, p3ORF-ATGThr913Phe, p3ORF-ATGSer320Pro, p3ORF-ATGSer403Gly, p3ORF-ATGSer436Arg and p3ORF-ATGSer913Phe. The PDS proteins containing the double mutations were expressed in BL21(DE3)pLysS cells, purified using nickel columns and tested for *in vitro* PDS activity as indicated by Michel *et al*. (2004).

PDS-Start

A deletion clone from the original *H. verticillata pds* was generated using the primer PDS-Start (5′-cctcttcaagttgtaattgctggtg-3′), located at position –3 of the corresponding start codon of the *pds* sequence of *Synechococcus* (Figure 1), and subcloned into TOPO4 vector. The PDS protein was expressed in Top10 *E. coli* cells and tested for activity.

Biochemical analysis of phytoene desaturase

Separate Top10 cultures harbouring the pPDS(aa) plasmids, ATG1 series, were grown, and their cells were harvested as described previously (Michel *et al*., 2004). Lysed cell extracts were adjusted to 10 mg/mL total protein. Crude extracts containing phytoene (EB) were prepared according to Misawa *et al*. (1995). To determine the PDS activity of pPDS(aa), 500 μL of the cell extracts containing the mutated PDS were mixed with 500 μL of EB extract and 5 μL of a 10 mM solution of decylplastoquinone (cat. D7786, Sigma, St Louis, MO), and incubated with agitation at 350 r.p.m. at 30 °C for 3 h in the dark. The carotene produced was determined by high-performance liquid chromatography (HPLC) (C18 column μBondapack; Waters, Milford, MA), and the results are expressed as units of activity relative to the activity of the control without herbicide.

As the ATG1 PDS clones could not be purified by affinity chromatography, the evaluation of fluridone resistance in these clones was performed using total protein and quantified by HPLC. As a result, these enzyme assays were carried out on equivalent total protein extracts, but the exact amount of PDS in each assay probably varied.

The assays for the ATG3 PDS clones against fluridone were performed as described in Michel *et al*. (2004). The Arg (wild-type) and Ser, His, Cys and Thr mutations of the pPDS(aa) series and p3ORF-ATG(aa)SetB series were selected for the cross-resistance study with the seven PDS-inhibiting herbicides at six different concentrations. The concentrations of herbicide that inhibited 50% of the activity of PDS (I_{50} values) in biochemical assays were calculated using a four-parameter logistic function, as described by Michel *et al*. (2004). The R/S values calculated for the enzymes are the ratio of I_{50} of the herbicide relative to I_{50} of the same herbicide on the wild-type protein PDS with Arg304.

Size exclusion chromatography of *Hydrilla* PDS

Purified *Hydrilla* PDS was loaded on a high-resolution Superdex 75 size exclusion column (Amersham Biosciences, Piscataway, NJ) mounted on a ÄKTA purifier (Amersham Biosciences). The solvent system consisted of the assay buffer (200 mM sodium phosphate, pH 7.2), and the flow rate was 1 mL/min. The protein eluting from the column was visualized with the UV detector set at A = 280 nm. To ensure that the peaks with shorter retention times corresponded to larger proteins, and were in fact PDS oligomers, these fractions were run on SDS-PAGE.

Herbicide dose–response curves on *A. thaliana*

A. thaliana seeds Columbia (Col-0, wild-type A) were surface sterilized and plated on half-strength Murashige and Skoog medium (MS) containing between 5 and 200 nM of herbicide. The medium was prepared with half-concentration MS salts, 1 × Gamborg's vitamin solution, 15 g/L sucrose and 2 g/L phytagel. Solutions of the herbicides were added to the medium after autoclaving and cooling at 50 °C. Plates were observed after a 5-day incubation in continuous light at 25 °C and the number of live seedlings was counted and expressed as the percentage survival.

Plant expression vectors

Plasmid pHy4ATG5 was used as a template for site-directed mutagenesis of codon Arg304 to Cys, Ser, His and Thr, as indicated previously. The resulting plasmids were pPDSCys, pPDSSer, pPDSHis and pPDSThr, respectively. The wild-type (Arg304) *pds* and each of the mutations were subcloned as a 1813-bp *Spe*I-*Ssp*I fragment into the *Spe*I-*Pml*I sites of pCambia 1303 vector (Cambia, Canberra, Australia). The resulting plasmids were pPDArg1303, pPDCys1303, pPDHis 1303, pPDSer1303 and pPDThr1303 for wild-type, Cys, His, Ser and Thr mutations, respectively. The plasmids were transformed into *Agrobacterium* strain C58C1 (Sciaky *et al*., 1978), and used to transform *A. thaliana* by the dip floral method (Clough and Bent, 1998). *Agrobacterium* harbouring the plasmid pCambia1303 without *pds* was used as a control of the transformations and later as a control for herbicide screening.

Plant transformation and screening

Seeds from T1, T2 and T3 generations were screened on hygromycin (25 mg/L) and norflurazon (200 nM) separately. Seedlings that were resistant to hygromycin in T1 were selected in either hygromycin or norflurazon in T2 and T3 and screened with multiple PDS inhibitors (Tables 3–5). The presence of *H. verticillata pds* was confirmed by polymerase chain reaction (PCR) in the putative transgenic plants. T3 seeds were screened for resistance to the PDS-inhibiting herbicides in triplicate using three different concentrations. Norflurazon, diflufenican and beflubutamid were tested at 100, 200 and 300 nM, whereas picolinafen, fluridone, flurtamone and flurochloridone were tested at 20, 50 and 100 nM. Seeds were plated on half-strength MS medium supplemented with the corresponding herbicides and incubated under continuous light at 25 °C. Seedlings were observed after 5 days using a dissecting scope, the number of green, yellow and purple seedlings was recorded, and the number of green seedlings was expressed as the percentage survival. The copy number of *H. verticillata pds* in the T3 generation of *A. thaliana* was determined by Southern blot using a DIG luminescent detection kit (Roche, Indianapolis, IN) (Table 4). The probe used for detection was a 700-bp fragment amplified using primers PDS-Start and RPDS942 (5′-TTGGCTTACATAATCTTTCAGGTG-3′).

The EC_{50} values, or the concentrations of herbicide that resulted in 50% survival of the seedlings, were calculated using sigmoidal and negative exponential equations fitted to the data. Within this context, the R/S ratio represents the ratio of EC_{50} of a herbicide relative to EC_{50} of that herbicide on the wild-type.

Maximum resistance to fluridone and norflurazon

Seeds from the T3 generation of all mutant plant lines that did not show segregation on hygromycin were additionally tested in higher concentrations of fluridone and norflurazon to determine the maximum resistance to these two herbicides. The concentrations tested were 100, 125, 150, 175 and 200 nM fluridone and 300, 500, 750, 1000 and 3000 nM norflurazon.

Chlorophyll and carotenoid determination

Leaf chlorophyll was extracted from 10 mg in 3 mL of dimethyl sulphoxide for 2 h according to Hiscox and Israelstam (1979). The absorbance of the extracts was measured at 645 and 663 nm, and total chlorophyll concentrations were determined according to Arnon (1949). Carotenoids were extracted from 50 mg of tissue, and total carotenoid concentrations were determined spectrophotometrically according to Sandmann and Böger (1983) with an extinction coefficient of E_{445} = 2500 (% w/v).

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