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Evaluation of three herbicide resistance genes for use in genetic transformations and for potential crop protection in algae production

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Summary

Genes conferring resistance to the herbicides glyphosate, oxyfluorfen and norflurazon were developed and tested for use as dominant selectable markers in genetic transformation of *Chlamydomonas reinhardtii* and as potential tools for the protection of commercial-scale algal production facilities against contamination by organisms sensitive to these broad-spectrum herbicides. A synthetic glyphosate acetyltransferase (GAT) gene, when fitted with a strong Chlamydomonas promoter, conferred a 2.7×-fold increase in tolerance to the EPSPS inhibitor, glyphosate, in transgenic cells compared with progenitor WT cells. A mutant Chlamydomonas protoporphyrinogen oxidase (protox, PPO) gene previously shown to produce an enzyme insensitive to PPO-inhibiting herbicides, when genetically engineered, generated transgenic cells able to tolerate up to 136× higher levels of the PPO inhibitor, oxyfluorfen, than nontransformed cells. Genetic modification of the Chlamydomonas phytoene desaturase (PDS) gene-based gene sequences found in various norflurazon-resistant organisms allowed production of transgenic cells tolerant to 40× higher levels of norflurazon than nontransgenic cells. The high efficiency of all three herbicide resistance genes in producing transgenic cells demonstrated their suitability as dominant selectable markers for genetic transformation of Chlamydomonas and, potentially, other eukaryotic algae. However, the requirement for high concentrations of glyphosate and its associated negative effects on cell growth rates preclude its consideration for use in large-scale production facilities. In contrast, only low doses of norflurazon and oxyfluorfen (~1.5 μM and ~0.1 μM, respectively) are required for inhibition of cell growth, suggesting that these two herbicides may prove effective in large-scale algal production facilities in suppressing growth of organisms sensitive to these herbicides.

Introduction

Interest in utilizing algae as a source of biofuels has led to intensified research with these organisms, ranging from studies of the basic biology of algae to design and engineering of industrial production facilities. Numerous algal strains have been identified as candidates for biofuel production based on their lipid production, growth rates and suitability for genetic manipulation. The importance of maintaining axenic or near-axenic algal cultures is critical, as contaminating organisms will reduce the production potential of algal bioreactors or raceways. In parallel, genetic engineering of algae requires a means of selecting for transgenic events. For both purposes, the availability of new genes that confer a selective advantage to the growth of desired algae over nondesired algae and other organisms is critical. With this dual goal in mind, we initiated this study to potentially exploit herbicide resistance genes that had not previously been widely used in algal systems as dominant selectable markers either for genetic transformation or for 'crop protection' in large-scale algal cultures.

One of the most widely used herbicides in agriculture today is glyphosate. Introduced for use as a broad-spectrum herbicide in 1974 (Malik *et al.*, 1989), glyphosate has become one of the best-selling herbicides worldwide. This is largely due to the development of transgenic crop plants resistant to glyphosate, beginning with the introduction of glyphosate-resistant soybean in 1996 (Dill *et al.*, 2008; Padgette *et al.*, 1995). Glyphosate's mode of action involves inhibition of enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the biosynthesis of aromatic amino acids. Glyphosate has been adopted for use in both rural and urban environments, as it exhibits minimal human and environmental toxicity (Smith and Oehme, 1992; Williams *et al.*, 2000). Over the years, substantial research has been dedicated to the discovery and development of genes that can provide resistance to glyphosate. Early efforts focused on mutant and natural forms of EPSPS that could maintain catalytic function in the presence of glyphosate. Two forms predominated. The first was an EPSPS composed of mutant domains from *E. coli* and *Zea mays* genes, and the second was a natively glyphosate-insensitive EPSPS from *Agrobacterium* CP4. Both forms were used in the development of glyphosate-resistant crops, with the *Agrobacterium* EPSPS favoured, as it showed higher tolerance to glyphosate than the *E. coli*/*Zea mays* double mutants (Barry *et al.*, 1997). An alternative mode of glyphosate resistance can be achieved by detoxification of glyphosate. This has been achieved either using enzymes capable of oxidizing glyphosate or using enzymes capable of acetylating glyphosate. An example of the former type of enzyme is glyphosate oxidase (Barry *et al.*, 1992). An example of the latter is an acetyltransferase from *Bacillus* that was modified via DNA shuffling to enhance specificity for glyphosate to yield an enzyme that was able to confer glyphosate resistance to a variety of organisms (Castle *et al.*, 2004).

Another family of herbicides that is attractive for potential use in algal cultures is the PPO inhibitors. This class, which includes compounds such as oxadiazon, oxyfluorfen and acifluorfen, functions by inhibiting protoporphyrinogen oxidase (PPO, protox). Protox, the last common enzyme in the biosynthetic pathway for heme and chlorophyll production, catalyses the oxidation of protoporphyrinogen IX to protoporphyrin IX (Beale and Weinstein, 1990). Inhibition of protox leads to an accumulation of the substrate, protoporphyrinogen IX, which is exported from the chloroplast to the cytoplasm, where it is oxidized by a nonspecific plasma membrane-bound peroxidase (Ha *et al.*, 2004). Accumulation of the oxidized product, protoporphyrin IX, in the cytoplasm leads to the formation of singlet oxygen, resulting in membrane peroxidation (Duke *et al.*, 1991). Compounds that inhibit protox have been used as herbicides in crop systems for many years. Certain crop species, including rice, soybean and tobacco, have been engineered to tolerate normally lethal doses of PPO inhibitors (Ha *et al.*, 2004; Jung *et al.*, 2004; Lermontova and Grimm, 2000; Warabi *et al.*, 2001).

The third class of herbicides addressed in this work targets the enzyme activity of phytoene desaturase (PDS). PDS functions in the carotenoid biosynthesis pathway, and inhibition of this enzyme causes degradation of chlorophyll and the chloroplast membrane, resulting in photobleaching of green tissues (Böger and Sandmann, 1998). To date, three mutant forms of PDS have been identified from various organisms that provide tolerance to PDS-inhibiting herbicides. Two of these mutants were discovered in the photosynthetic cyanobacteria *Synechococcus* and *Synechocystis* (Chamovitz *et al.*, 1991; Martinez-Ferez and Vioque, 1992), while the most recently discovered norflurazon-resistant PDS mutant was found in *Hydrilla verticillata* (Michel *et al.*, 2004). Three independent norflurazon-resistant *Hydrilla* strains were characterized, and surprisingly, all three showed different amino acid substitutions at the same location in PDS (histidine, serine or cysteine in place of arginine at position 304). Engineered substitutions at this site showed that a fourth amino acid (threonine) conferred even higher resistance to norflurazon than the three naturally occurring substitutions (Arias *et al.*, 2006).

Previous work has shown Chlamydomonas to be susceptible to all three of the above-mentioned herbicide classes (Reboud, 2002). To date, no information is publically available detailing efforts to develop glyphosate resistance genes for algae. In 1990, a mutant strain of Chlamydomonas was isolated and characterized that showed elevated levels of tolerance to PPO inhibitor compounds (Kataoka *et al.*, 1990). This strain, *rs-3*, showed a 15-fold increase in the effective dose $(ED)_{90}$ for killing Chlamydomonas with oxadiazon (90 μM) and a 10.7-fold increase in the ED_{90} for oxyfluorfen (3.2 μM). Later characterization of this mutant showed a single nucleotide alteration in the Protox gene, causing a valine to methionine amino acid change (Randolph-Anderson *et al.*, 1998). Upon discovery of this gene, the Sumitomo Chemical Company (involved in the discovery and characterization of this mutant gene) filed a patent on the gene and its use. This resulted in the lack of availability of the gene for academic purposes. Moreover, no transgenic plants carrying a genetically engineered version of *rs-3* were developed for the marketplace. Expiration of the Sumitomo patent provided an opportunity for the development of the *rs-3* gene as a selectable marker and as a potential source of herbicide resistance for algae grown in commercial settings.

Finally, analysis of the Chlamydomonas PDS gene showed significant conservation in amino acid content with the PDS proteins from Hydrilla and the two cyanobacteria noted above. As detailed below, this included the location of the mutation that conferred norflurazon resistance in Hydrilla and Synechocystis. This suggested that a similar mutation in the Chlamydomonas PDS might also be successful in conferring resistance to norflurazon.

Here, we report the develop a series of gene constructs conferring resistance to the herbicides glyphosate, oxadiazon/oxyfluorfen and norflurazon that can be used as selectable markers for the genetic transformation of Chlamydomonas and, potentially, other eukaryotic algae. We also provide evidence that the oxyfluorfen and norflurazon-resistant genes provide high levels of resistance to these two respective herbicides in transgenic Chlamydomonas cells and allow for nearly normal cell growth rates in the presence of normally lethal concentrations of the herbicides. As such, these herbicide resistance genes appear promising as potential tools for helping to maintain axenic or near-axenic algal cultures in commercial settings.

Results and discussion

Analysis of herbicide tolerance in wild-type and mutant Chlamydomonas strains

As a test of the effectiveness of various herbicides to kill eukaryotic algae, we subjected four strains of Chlamydomonas to various growth tests in the presence of three different classes of herbicides represented by glyphosate, oxadiazon/oxyfluorfen and norflurazon. The four strains used for this study, CC124, CC1010, CC503, and CC3491 (Chlamydomonas Resource Center), were selected based on their different mating types (mt+ and mt−) and their cell wall composition (walled or wall-less). Both CC124 and CC1010 possess intact cell walls and are mt− and mt+, respectively. The other two strains, CC3491 (mt−) and CC503 (mt+), are deficient in producing intact cell walls. Such wall-less strains are used in laboratory research because there is no need to use autolysin (Buchanan *et al.*, 1989; Harris, 1989) to remove cell walls prior to genetic transformation using either electroporation (Shimogawara *et al.* (1998) or glass bead vortexing (Kindle, 1990) techniques. Each algal strain was grown in the presence of multiple concentrations of herbicides in either liquid TAP media or as colonies on Petri dishes containing 1% agar and TAP medium. Results of these tests are presented in Table 1.

All four algal strains showed sensitivity to glyphosate. When pH was controlled (glyphosate can significantly impact media pH), the dose required for complete inhibition of growth ranged from 15 to 30 mM in liquid cultures and 7.5 to 15 mM on plates. An approximate twofold increase in glyphosate sensitivity was noted in wall-less compared with walled cells. Mating type, conversely, had no significant effect in regard to glyphosate sensitivity. Growth condition also appeared to have an effect on toxicity, as cells showed an increased sensitivity to glyphosate on plates compared with growth in liquid culture.

Two PPO inhibitors, oxadiazon and oxyfluorfen, were also effective in preventing growth of all four algal strains, at levels much lower than glyphosate (Table 1). Lethal doses of oxadiazon were identical for all strains (4 μM) in liquid media and ranged from 2.5 to 5 μM in solid media. Oxyfluorfen showed a higher level of toxicity to Chlamydomonas cells than oxadiazon. Oxyfluorfen was effective at roughly 30-fold lower concentrations

Table 1 Determination of minimal concentrations of four herbicides required to kill walled and wall-less strains of Chlamydomonas. Glyphosate, two PPO-inhibiting compounds, oxadiazon and oxyfluorfen, and norflurazon were assayed for toxicity to Chlamydomonas. Four strains of Chlamydomonas were assayed for comparison of herbicide sensitivities: CC3491 (wall-less, mt−), CC503 (wall-less, mt+), CC124 (walled, mt−), CC1010 (walled, mt+)

	Glyphosate (mM)		Oxadiazon (μM)		Oxyfluorfen (μM)		Norflurazon (μM)	
Strain	Liquid	Agar	Liquid	Agar	Liquid	Agar	Liquid	Agar
CC3491	15	7.5	4	2.5	0.11	0.06	1.5	3
CC503	15	7.5	4	2.5	0.10	0.12	1.5	4
CC124	30	15	4	2.5	0.08	0.12	3.5	4
CC1010	30	15	4	5	0.07	0.12	2.5	4
Range	15–30	7.5–15	4	2.5–5	0.07–0.11	0.06–0.12	1.5–3.5	3–4

(lethal doses: 0.07–0.11 μM in liquid media, 0.06–0.12 μM in solid media) than oxadiazon. Unlike glyphosate, neither the cell wall nor the growth condition significantly affected the level toxicity of oxadiazon and oxyfluorfen on cell growth. Mating type again showed little effect in influencing toxicity levels.

Of several available phytoene desaturase-inhibiting herbicides (Hao *et al.*, 2011), we chose norflurazon for our studies. All four Chlamydomonas strains displayed sensitivity to norflurazon, producing a characteristic white phenotype when grown in complete darkness. When grown in the presence of light, the four strains failed to grow on elevated levels of norflurazon in both liquid (1.5–3.5 μM) and solid (3–4 μM) media. When each strain was compared in relation to its cell wall composition or mating type, it was noted that in liquid media, wall-less strains were more susceptible to significantly lower concentrations of norflurazon than walled cells.

Herbicide resistance constructs and transformation efficiencies

The first gene tested for generating glyphosate resistance in Chlamydomonas was the class II EPSPS gene from *Agrobacterium* strain CP4, as it has been used to generate glyphosate resistance in multiple organisms. However, despite numerous attempts and modifications, this gene was found unsuitable for generating glyphosate-resistant lines of Chlamydomonas.

The second route explored for creating glyphosate resistance in Chlamydomonas was detoxification of glyphosate via acetylation. The glyphosate acetyltransferase selected, originally recovered from *Bacillus lichenformis* and optimized for activity via DNA shuffling (Castle *et al.*, 2004), was placed under control of the Chlamydomonas PsaD promoter and terminator. In addition, the first intron from Rbcs2 was inserted to improve gene expression (Lumbreras *et al.*, 1998). This construct was designated as pGly1 (Figure 1). Multiple tests confirmed this gene as able to produce glyphosate resistance in Chlamydomonas.

Construct pGlyR1 showed variable transformation efficiency over a range of glyphosate ratios. When pH of the selection media was controlled (an important prerequisite when the acid form of glyphosate was employed), pGlyR1 achieved a median transformation efficiency of >4000 cfu/μg DNA when cells were selected in the presence of 10 mM glyphosate. Of concern, however, was the ability of a portion of wild-type cells to also

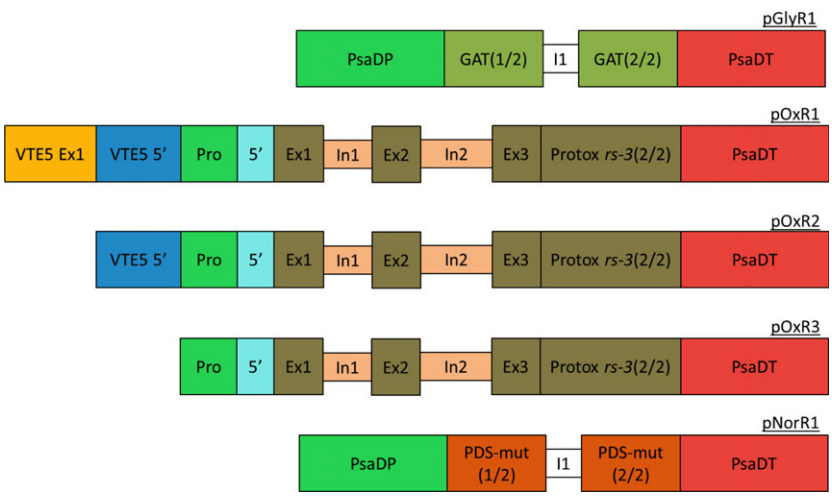

Figure 1 Herbicide resistance constructs. GlyR, OxR and NorR constructs were used to generate resistance to glyphosate, oxadiazon/oxyfluorfen and norflurazon, respectively. The following gene components are labelled as follows: PsaDP/PsaDT(promoter and terminator regions of Chlamydomonas PsaD), I1(first intron from Chlamydomonas Rbcs2), GAT(synthetic glyphosate acetyltransferase cDNA, divided into parts 1/2 and 2/2), PDS-mut(Chlamydomonas PDS cDNA, containing R268T mutation, divided into parts 1/2 and 2/2), 5′/Ex1/In1/Ex2/In2/Ex3(5′ UTR, exon 1, intron 1, exon 2, intron 2 and exon 3 of Chlamydomonas protox gene), Protox *rs-3*(2/2)(second half of Chlamydomonas protox cDNA, containing *rs-3* mutation), VTE5 Ex1/VTE5 5′/Pro(exon 1 and 5′ UTR for Chlamydomonas VTE5 gene, and shared promoter sequence separating VTE5 and protox genes in Chlamydomonas.

survive at this concentration of glyphosate after a mock transformation (i.e. electroporation with plasmid DNA lacking the glyphosate resistance gene). At 10 mM glyphosate, background transformation rates reached a level of 3000 cfu/μg DNA, although these colonies were easy to differentiate from pGlyR1-transformed cells, as they took several days longer to become visible on plates. 40 mM glyphosate was sufficient to eliminate growth of any wild-type cells, but pGlyR1 transformation recovery was reduced to 420 cfu/μg DNA. A suitable level of glyphosate was determined to be 20 mM. At this concentration, transformation with pGlyR1 generated a large number of transformants (1899 cfu/μg DNA) while growth of cells subjected to mock transformations was significantly reduced (10 cfu/μg DNA) (Table 2).

Three constructs were analysed for generating resistance to PPO inhibitors: pOxR1, pOxR2 and pOxR3 (Figure 1). All three constructs contained a fusion of part of the wild-type protox gene (including promoters and introns) with a cDNA containing the latter half of the protox gene, which contains the *rs-3* mutation. The three constructs differed only in the length of the promoter (complete details of the constructs can be found in Methods).

All three constructs successfully generated PPO inhibitor-resistant lines of Chlamydomonas. Similar to the results with pGlyR1 and glyphosate resistance, the OxR constructs showed a gradual decrease in transformation efficiency as the level of herbicide was increased. But, as expected, this was accompanied with a decrease in the 'background' of colonies recovered following transformations with plasmid DNA lacking the oxadiazon/oxyfluorfen resistance gene. A level of 15 μM oxadiazon was sufficient to eliminate survival of nontransformed cells, while still allowing survival of newly transformed cells. With oxyfluorfen, however, the amount necessary to prevent survival of nontransformed cells (0.20 μM) also prevented the survival of cells transformed with any of the three OxR constructs. A concentration of 0.17 μM oxyfluorfen eliminated most nontransformed cells (8 cfu/μg DNA) while still allowing survival of numerous transformed cells (Table 2).

To ascertain which of the three *rs-3* constructs, pOxR1, pOxR2 or pOxR3, possessed the highest efficiency in generating PPO inhibitor-resistant cells, the three plasmids were compared in regard to their abilities to generate transformants able to survive in the presence of 15 μM oxadiazon and 0.17 μM oxyfluorfen. Each construct was used for transformation of Chlamydomonas CC3491 cells in triplicate experiments, and a median transformation efficiency for each construct was ascertained (Table 2). The number of PPO inhibitor-resistant constructs generated from initial selection on oxadiazon was comparable with the number recovered on oxyfluorfen. All constructs successfully generated PPO inhibitor-resistant cells, and analysis of variance indicated no statistically significant difference in transformation efficiency between the three constructs. Median transformation rates, however, suggest that pOxR3, which contains the shortest of the three promoter lengths (139 bp), may be less efficient in generating oxadiazon- and oxyfluorfen-resistant cells than the other two constructs. In the presence of oxyfluorfen, pOxR3 generated 338 cfu/μg DNA, whereas pOxR1 and pOxR2 generated 556 and 858 cfu/μg DNA, respectively. This pattern of transformation efficiency was also seen in the presence oxadiazon. Electroporation of Chlamydomonas often results in fragmentation and partial degradation of the foreign DNA prior to its integration into the genome. It is possible, then, that the additional promoter length found in pOxR1 and pOxR2 acts as a buffer during the electroporation event, resulting in a higher number of integrated transgenes containing the requisite minimal promoter length.

Chlamydomonas phytoene desaturase shares significant amino acid sequence identity with PDSs from several organisms, including *Hydrilla*, *Synechococcus elongatus* PCC7942 and *Synechocystis* sp. PCC6803 (Figure 2)—all of which can be rendered insensitive to norflurazon by specific single amino acid substitutions. The location of two of these mutations, *Synechocystis* R195 and *Hydrilla* R304, occurs in a highly conserved region of the protein. When aligned with Chlamydomonas PDS (Figure 2), significant sequence similarity can be seen in this particular region, including the aforementioned arginine that was mutated in *Synechocystis* and *Hydrilla* (highlighted in red, Figure 2). To test whether a similar substitution would confer norflurazon resistance to Chlamydomonas, we modified the cDNA of PDS to encode Thr at position 268 in place of the conserved Arg. The modified cDNA was then placed under the control of the Chlamydomonas PsaD promoter and terminator, and the first intron of Rbcs2 was inserted into the cDNA sequence to enhance expression of the transgene (Lumbreras *et al.*, 1998). The resultant plasmid was designated as pNorR1 (Figure 1).

When delivered into Chlamydomonas cells by electroporation, pNorR1 generated numerous norflurazon-resistant colonies (Table 2). However, in initial experiments, it was noted that Chlamydomonas cells undergoing transformations with plasmid DNA lacking the mutant PDS gene often produced several colonies on norflurazon-containing plates. We determined a stepped lighting pattern to be most effective in generating the highest number of transformed cells while limiting growth of wild-type cells. Following a 24-h recovery in light (80 μmol photons/s/m^2), transformed cells were plated onto TAP media containing 4 μM norflurazon and incubated at 25 °C under reduced light (5 μmol photons/s/m^2) for 7 days. Following this incubation, the plates were placed under moderate light intensity

Table 2 Transformation efficiency of herbicide resistance genes. Transformation efficiency was reported as the minimum, median and maximum number of colony-forming units (cfu) per μg of plasmid DNA used. Background indicates the numbers of cfu/μg that were false positives (i.e. nontransformed)

			Transformation efficiency			
Plasmid	Gene	Herbicide selection	Min.	Median	Max.	Backgr. (%)
pGlyR1	GAT	Glyphosate (20 mM)	440	1899	4160	10 (0.5)
pOxR2	protox rs-3	Oxyfluorfen (0.17 μM)	212	858	2120	8 (0.9)
pNorR1	PDS (R268T)	Norflurazon (4 μM)	500	550	700	55 (10.0)
pSP124s	Bleomycin	Zeocin (7.5 mg/L)	20	796	1764	–

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PCC7942         LLPAMIRGQSYVEEMDQYSWTEWLRKQNIPERVNDEVFIAMAKALNFIDPDEISATVVLT 191
PCC6803         LLPAIVQGQSYVEEMDKYTWSEWMAKQNIPPRIEKEVFIAMSKALNFIDPDEISATILLT 191
Hydrilla        LLPAMIGGQPYVEAQDGLTVQEWMRKQGVPDRVNDEVFIAMSKALNFINPDELSMQCILI 300
Cre             LLPAIIFGQKYVEEQDHLSVTQWMRQQGVPDRVNEEVFIAMAKALAFIDPDRLSMTVVLT 264
                    ****:: ** ***  *  :  :*: :*.:* *::.******:*** **:**.:*   :*

PCC7942         ALNRFLQEKKGSMMAFLDGAPPERLCQPIVEHVQARGGDVLLNAPLKEFVLNDDSSVQAF 251
PCC6803         ALNRFLQEKNGSKMAFLDGAPPERLCQPLVDYITERGGEVHINKPLKEILLNEDGSVKGY 251
Hydrilla        ALNRFLQEKHGSKMAFLDGNPPERLCKPIADHIESLGGQVILNSRIQKIELNADKSVKHF 360
Cre             ALNRFLQERHGSKMAFLDGAPPERLCQPMVDHFTARGGELKMNARVKDIVLNDDGSVKHY 324
                ********::** ****** ******:*:.::.   **:: :*  ::.: ** * **: :

PCC7942         RIAGIKGQEEQLIEADAYVSALPVDPLKLLLPDAWKAMPYFQQLDGLQGVPVINIHLWFD 311
PCC6803         LIRGLDGAPDEVITADLYVSAMPVDPLKTMVPAPWREYPEFKQIQGLEGVPVINLHLWFD 311
Hydrilla        VLT-----NGNIITGDAYVFATPVDILKLLLPEDWKEISYFKKLDKLVGVPVINVHIWFD 415
Cre             KLT-----TGEVVEGDLYMSAMPVDILKLLVPDQWKPNPYFSQLKELEGVPVINIHIWFD 379
                     :        ::: .* *: * *** ** ::*  *:  . *.::. * ******:*:***

PCC7942         RKLT-DIDHLLFSRSPLLSVYADMSNTCREYEDPDRSMLELVFAPAKDWIGRSDEDILAA 370
PCC6803         RKLT-DIDHLLFSRSPLLSVYADMSNTCREYSDPDKSMLELVLAPAQDWIGKSDEEIVAA 370
Hydrilla        RKLKNTYDHLLFSRSPLLSVYADMSVTCKEYYNPNQSMLELVFAPAEKWISCSDSEIINA 475
Cre             RKLT-TVDHLLFSRSPLLSVYADMSTTCKEYYDTEKSMLELVFAPAKDWIGRSDEDIIAA 438
                    ***.   ****************** **:** :.::******:***:.**. **.:*: *

PCC7942         TMAEIEKLFPQHFSGEN-PARLRKYKIVKTPLSVYKATPGRQQYRPDQASPIANFFLTGD 429
PCC6803         TMAEIKQLFPQHFNGDN-PARLLKSHVVKTPRSVYKATPGRQACRPDQRTSVPNFYLAGD 429
Hydrilla        TMQELAKLFPDEISADQSKAKILKYHVVKTPRSVYKTVPDCEPCRPLQRSPIEGFYLAGD 535
Cre             TMTELERLFPTEIKADQSLAKIRKYKVIKTPLSVYESRAGREAFRPSQRTPIKNFFLAGD 498
                    ** *: :*** .:..::  *:: * :::*** ***:: .. :  ** * :.: .*:*:**

PCC7942         YTMQRYLASMEGAVLSGKLTAQAIIARQDELQRRSSGR-----PLAASQA---------- 474
PCC6803         FTMQKYLGSMEGAVLSGKQCAQAIAADFN-PQTVPPTR-----EIVTVG---------- 472
Hydrilla        YTKQKYLASMEGAVLSGKLCAQAIVQDCSLLASRVQKS----PQTLTIA---------- 580
Cre             FTKQKYLASMEGAIFSGKLAAEQIVNDYNYKGVAPPARSSSSPELVAASALLAVAAVGAG 558
                    :* *:**.*****::***  *: *    .                :
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Figure 2 Partial alignment of PDS gene sequences across species. Labels correspond to phytoene desaturase associated with each species. PCC7942, *Synechococcus elongatus* PCC7942; PCC6803, *Synechocystis* sp. PCC6803; Hydrilla, *Hydrilla verticillata*; Cre, *Chlamydomonas reinhardtii*. Locations of amino acid mutations that confer herbicide resistance are marked. Substitutions that confer herbicide resistance are as follows; Synechococcus: V403G, Synechocystis: R195C, Hydrilla: R304H, R304S, R304C or R304T.

(80 μmol photons/s/m^2) for an additional 11 days. Under these conditions, pNorR1 generated 550 cfu/μg DNA (Table 2). Screening of these colonies via PCR confirmed that 90% harboured the PDS transgene. The 10% of colonies that did not show an intact transgene were then assayed to determine whether the norflurazonR phenotype was permanent and heritable. These colonies were resuspended in TAP media and spotted to TAP plates containing 0 and 4 μM norflurazon. None of the nontransformed lines assayed showed continued viability in the presence of 4 μM norflurazon. In contrast, colonies containing the pNorR1 transgene showed robust growth when spotted to TAP containing 4 μM norflurazon. These results indicate that the growth of nontransformed cells following electroporation can be managed with a stepped lighting pattern, and any nontransformed cells can be eliminated with a secondary screen on TAP plates containing 4 μM norflurazon.

Level of herbicide resistance imparted by transgenes

After generating various herbicide-resistant lines of Chlamydomonas, we analysed these lines to determine the maximum levels of herbicide to which they are tolerant. Tests were conducted both in liquid media and on agar plates. For liquid media, cell growth was monitored daily by counting cells with a haemocytometer. For growth on agar, standardized numbers of cells were spotted in serial dilutions onto agar plates containing various levels of herbicide, and plates were monitored visually for growth.

Glyphosate resistance

For tests on solid media, multiple transgenic lines were spotted onto agar plates containing various levels of glyphosate and assayed for their growth. We found that all pGlyR1-transformed lines tested were able to survive plating on levels of glyphosate up to 40 mM in both liquid and agar media (maximum solubility of glyphosate in water is 50 mM) (Table 3). However, several lines showed reduced rates of growth on agar media as glyphosate levels were increased. Liquid tests of transgenic lines showed similar results. Notably, while the transgene appeared to have only a minimal effect on growth of the algae in liquid TAP media (0.48% increase in doubling time compared with wild type), the cells show a 51% increase in required doubling time at the levels of glyphosate necessary to prevent growth of the WT progenitor strains (CC3491). At the level required to eliminate all growth of wild-type algae (30 mM), transgenic lines show a 180% increase in doubling time, illustrating the ability of the GAT gene to impart glyphosate resistance to Chlamydomonas cells, but not eliminate deleterious effects in growth.

Oxyfluorfen and oxadiazon resistance

All three OxR constructs, although displaying differential transformation efficiencies, behaved similarly when tested for maximum herbicide tolerances. Tests of the transgenic lines on agar plates were conducted with both oxadiazon and oxyfluorfen. For oxadiazon, transgenic lines produced with pOxR1, pOxR2 and pOxR3 were able to survive on levels up to 750 μM in liquid media and 1.5 mM on agar, representing a 188- to 600-fold increase compared with wild-type cells. These lines were also able to survive on levels of oxyfluorfen up to 5.0 μM on agar and 15 μM in liquid media, an approximately 83- to 136-fold increase compared with wild-type cells. Quantitative growth tests in liquid media were limited to oxyfluorfen (Table 3). All lines showed similar levels of tolerance to oxyfluorfen. Because it showed the highest transformation efficiency of the three constructs, only pOxR2 will be discussed in this section. Compared with the WT

Table 3 Growth analysis of transgenic lines. Transgenic lines were assayed for their ability to survive in increasing levels of herbicide in both liquid and solid (agar) media. Lethal dose 'increase' indicates the tolerance of transgenic lines to their respective herbicides in comparison to nontransformed CC3491. Transgenic lines were also assayed for their rate of growth in the presence of increasing levels of herbicide. The rate of growth of transgenic lines was analysed by comparing the doubling time of cells in TAP to that in the presence of herbicide necessary to kill WT cells. Doubling time 'increase' is the per cent increase in the amount of time required for cell density to double in the presence of herbicide compared with growth in TAP medium alone

		Lethal dose—Liquid media		
Plasmid	Herbicide resistance	Wild type	With transgene	Increase
pGlyR1	Gly^R	15 mM	40 mM	2.7×
pOxR2	Oxy^R	0.11 μM	15 μM	136×
pNorR1	Nor^R	1.5 μM	60 μM	40×

		Lethal dose—Solid media		
Plasmid	Herbicide resistance	Wild type	With transgene	Increase
pGlyR1	Gly^R	7.5 mM	40 mM	5.3×
pOxR2	Oxy^R	0.06 μM	15 μM	83×
pNorR1	Nor^R	3.0 μM	120 μM	40×

		Doubling time—Liquid media (Hrs)		
Plasmid	Herbicide resistance	TAP	At WT lethal dose	Increase, %
pGlyR1	Gly^R	9.17	13.89	51.5
pOxR2	Oxy^R	8.66	9.54	10.3
pNorR1	Nor^R	8.98	9.21	2.6

progenitor (CC3491), pOxR2 transgenic lines showed no significant differences in their rate of growth in nonselective media. There was, however, a reduction in growth rate when oxyfluorfen was present, although this reduction was substantially less than the reduction observed with pGlyR1-transformed cells grown in the presence of glyphosate. At 1.5 μM oxyfluorfen, lines showed 24% increases in doubling times, and at 5.0 mM, growth of transgenic lines was nearly blocked. However, at 0.11 μM oxyfluorfen (the maximum lethal dose for all Chlamydomonas strains tested), transgenic lines showed only moderate increases in doubling times (~10%), illustrating the potential for oxyfluorfen to maintain axenic cultures while only slightly impacting growth of the transgenic line.

Norflurazon resistance

Cells transformed with the pNorR1 plasmid showed a 40× increase in tolerance to norflurazon (lethal doses of 60 μM in liquid media and 120 μM on solid media) compared with WT cells (lethal doses of 1.5 μM and 3 μM on liquid and solid medium, respectively) (Table 3). When compared to CC3491 growth rates, cells carry the transgene showed only minor decreases in growth rate (Table 3). At the level of herbicide necessary to kill the WT progenitor cell line (CC3491), transgenic cells showed only a 2.6% increase in doubling time compared with transgenic cells in TAP media. At 3.5 μM, the level of norflurazon necessary to kill off all strains of Chlamydomonas transgenic cells exhibited a 12.9% increase in doubling time.

Large-scale growth of oxyfluorfen-resistant cells

To examine the potential application of herbicides in maintaining axenic cultures in large-scale algal production, we analysed the growth of wild-type and transgenic lines of Chlamydomonas in a bioreactor setting. Analysis of pOxR2-transformed cells was selected for two reasons. First, these transformants exhibited nearly normal growth rates when cultured in the presence of oxyfluorfen at concentrations sufficient to kill wild-type algae, and second, the low micromolar concentrations of oxyfluorfen needed to suppress growth of contaminating cells make this herbicide the most economical for use.

Levels of 0.0, 0.1 and 1.0 μM oxyfluorfen were tested in the bioreactors. At 0.1 μM oxyfluorfen, transgenic cells grew at rates nearly identical to that in nonselective media (after 120 h, cells in nonselective media had reached stationary phase growth, and culture density of cells grown in 0.1 μM oxyfluorfen was 89% of those in nonselective media) (Figure 3). Transgenic cells grew at 1.0 μM, as expected from earlier tests, but growth was visibly reduced—with cell counts indicating a nearly 80% reduction in cell density compared with herbicide-free media. It may be noted that CC3491 was unable to grow in the presence of 0.1 μM oxyfluorfen during the bioreactor tests, suggesting 0.1 μM oxyfluorfen is completely adequate to maintain axenic cultures while avoiding the deleterious effects of higher herbicide concentrations on growth of the transgenic line.

Conclusion

Use of GAT, protox *rs-3* and mutant PDS genes for genetic transformation of Chlamydomonas resulted in successful recovery of herbicide resistance cells. The GAT gene (construct pGlyR1) displayed the highest efficiency in generating transgenic Chlamydomonas cells generating 1899 cfu/μg DNA compared with 858–940 cfu/μg DNA with the protox *rs-3* gene (construct pOxR2) and 550 cfu/μg DNA with the mutant form of PDS (construct pNorR1). However, because cells transformed with the GAT gene displayed markedly reduced growth rates when grown in high concentrations of glyphosate-containing media needed to suppress growth of wild-type cells, this gene appears unlikely to be of use for producing algae in commercial settings.

Overall, the genetically engineered protox *rs-3* mutant gene appears to be the most favourable for selecting genetically transformed Chlamydomonas and for growing axenic cultures of transgenic Chlamydomonas in bioreactors. Transformation efficiency with this gene was comparable with that of other antibiotic-based selectable markers routinely used Chlamydomonas research laboratories. While the number of oxadiazon/oxyfluorfen-resistant colonies generated from this transgene was slightly less than the number generated by GAT gene, the resultant transformants displayed remarkably high levels of resistance to oxyfluorfen treatment (i.e. resistance levels 136× above the dose lethal to wild-type cells) with only minor effects of oxyfluorfen treatments on growth rates of transformed cells. Small- and mid-scale tests also suggested the potential for this gene to serve as a useful tool in maintenance of axenic algal cultures in large-scale production facilities. Indeed, the exceptionally low dosages (~0.1 μM) of oxyfluorfen needed to inhibit cell growth and, thus its relatively low cost, make it a potentially

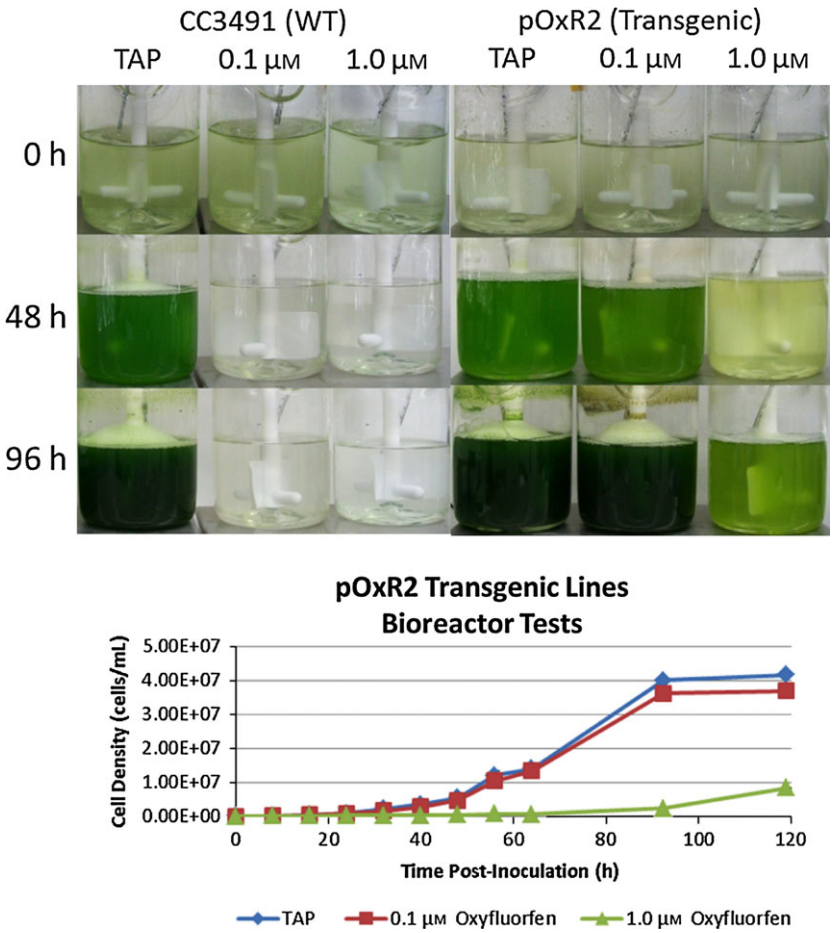

Figure 3 Largescale growth tests with pOxR2-transformed oxyfluorfen-resistant Chlamydomonas. pOxR2-transformed lines were grown alongside their WT progenitor strain (CC3491) to examine large-scale growth. All lines were grown in TAP as well as in the presence of two levels (0.1 μM and 1.0 μM) of oxyfluorfen.

attractive option for use in large-volume algal ponds or raceways.

Methods and materials

Chemicals

All algal cell growth experiments were performed in Tris acetate phosphate (TAP) media (Harris, 1989). Glyphosate was procured from Shanghai Majin (China). Additional herbicides (oxadiazon, oxyfluorfen and norflurazon) were procured from Chem-Service, Inc. (West Chester, PA).

Chlamydomonas culture growth and maintenance

Four strains for Chlamydomonas were used for experiments: CC3491, CC503 (both lacking a cell wall), CC124 and CC1010 (both possessing a cell wall). Strains were maintained on TAP media (Harris, 1989) containing 20 g/L agar at 50 μmoles photons/s/m^2. For most experimental procedures, strains were inoculated into liquid cultures of TAP medium and maintained with shaking at 100 RPM under light at 200 μmoles photons/s/m^2.

Algal growth tests

Transgenic and wild-type lines of Chlamydomonas were inoculated in 100 μL volumes of TAP media (or TAP media supplemented with herbicide for transgenic lines) and allowed to grow for 48 hours at 200 μmoles photos/s/m^2 lighting. For spot testing on agar plates, cells were washed in TAP, and a 3 μL aliquot of three dilutions (333 cells/μL, 33 cells/μL and 3 cells/μL) was spotted onto plates containing various levels of herbicide. Each line was also spotted onto TAP plates containing no herbicide as a measurement of maximum growth rate. Plates were incubated at 25 °C under 200 μmoles photons/s/m^2 lighting until colonies could be observed, usually in 4–8 days. Growth of cell lines on herbicide-containing plates was compared with their growth on herbicide-free plates to estimate growth rate. For liquid tests, cell densities were normalized, and an equivalent amount of cells were dispensed into 100 μL or 3 mL TAP media volumes containing various levels of herbicide or herbicide-free TAP media as a control. Cultures were grown with shaking under identical growth conditions as plates. Cell growth rates were calculated by comparing cell density of lines in herbicide-containing media to cell densities in herbicide-free media. Cell density was determined with a haemocytometer.

Construct designs—GAT and EPSPS gene constructs

Two genes, the synthetic glyphosate acyltransferase (GAT) (PMID: 15155947) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 (PMID: 8598558), were synthesized by GenScript (NJ) to be codon-optimized for expression in Chlamydomonas. The first intron of the Chlamydo-

monas Rbcs2 gene was incorporated into both genes to act as an enhancer element for expression (Lumbreras *et al.*, 1998). The genes were then placed individually under the control of the Chlamydomonas PsaD promoter and terminator. This final construction of the GAT gene was named pGlyR1. The EPSPS gene was further modified by being fused to two different 36nt chloroplast-targeting transit peptides. The two transit peptides were isolated from the Chlamydomonas genes, Rubisco activase and ferredoxin (Leon *et al.*, 2007), leading to the final two constructs, pHRTP20 and pHRTP22, respectively.

Construct designs—protox gene constructs

For expression of the *rs-3* mutant form of protox, a hybrid expression cassette was constructed using portions of the wild-type and *rs-3* mutant protox gene. Chlamydomonas protox (Cre09.g396300) cDNA was synthesized by GenScript (NJ) containing the 1-bp mutation specific to the *rs-3* mutant. The last 1124 nucleotides of this cDNA, which include the point mutation conferring resistance to PPO inhibitors, were ligated to a 5′ portion of the wild-type protox gene. This portion included the first two exons and introns of the protox gene and a portion of the third exon. Introns were included in this construct to enhance expression of the transgene. The gene fusion was placed under the control of the PsaD terminator and the endogenous protox promoter. In the Chlamydomonas genome, protox is arranged in a head-to-head orientation with the gene VTE5, with the 5′ UTRs of the genes separated by 139nt, presumably functioning as a transcriptional promoter for both genes. Upstream segments of 139, 304 and 452nt were isolated, representing the promoter, VTE5 5′ UTR and 1st VTE5 exon, respectively. These fragments were incorporated into three constructs, each representing a variable promoter length. The final constructs contained the following components: 'pOxR3'—139nt promoter, 'pOxR2'—443nt promoter, containing the VTE5 5′ UTR, 'pOxR1'—895nt promoter, containing the 1st exon and 5′ UTR of VTE5.

Construct designs—PDS gene constructs

The cDNA encoding PDS from Chlamydomonas (Cre12.g509650) was amplified from a cDNA pool using primers designed from the cDNA sequence, adding NdeI and EcoRI sites to the 5′ and 3′ ends, respectively. The resultant PCR product was further modified. First, a 2-bp change was made to the nucleotides 802 and 803 (CG to AC), resulting in an amino acid substitution at position 268 (Arg to Thr). NdeI and EcoRI sites allowed for directional ligation of the cDNA into a plasmid containing the promoter and terminator of the Chlamydomonas PsaD gene. In a similar fashion to the GAT gene, the first intron from the Chlamydomonas Rbcs2 gene was integrated into the GAT gene to enhance gene expression (Lumbreras *et al.*, 1998). The resultant plasmid was labelled 'pNorR1'.

Transformations

Chlamydomonas cells were transformed with DNA following the electroporation protocol outlined by Shimogawara *et al.* (1998). Briefly, cells were grown in TAP medium to a density of $1–3 \times 10^6$ cells/mL. Cells were harvested via centrifugation and resuspended in TAP media containing 60 mM sucrose to a density of 4×10^8 cells/mL. An aliquot of 1×10^8 cells was combined with 1 μg of the plasmid DNA of interest in a 0.4 cm electroporation cuvette (BioExpress, www.BioRad.com). Following incubation of the cuvette in a 16 °C water bath for 5 min, each sample was pulsed at 0.75 kv, 25uF, and no resistance using a Gene Pulser II (www.BioRad.com). Cells were recovered in 10 mL of TAP+60 mM sucrose for 24 h with low light (100 μmol photons/m^2/s) and minimal shaking. Transformants were then plated on various TAP plates containing the desired types and levels of herbicide. Plates were incubated for 6–10 days to allow herbicide-resistant colonies to develop.

Bioreactor growth tests

Transgenic and wild-type Chlamydomonas were grown as seed cultures in 500-mL Erlenmeyer flasks to mid-log phase ($\sim 1 \times 10^6$ cells/mL). To prevent transgene silencing, transgenic strains were grown in the presence of the minimal amount of herbicide necessary to kill nontransgenic wild-type cells. Upon reaching the desired culture density, cells were pelleted via centrifugation (2000 g, 5 min). The cell pellet (containing $\sim 2 \times 10^8$ cells) was washed with TAP and repelleted, and the final pellet resuspended in a 1L bioreactor (BellCo, #1965-81005) containing TAP media and a preselected amount of herbicide. Algal cultures in the bioreactors were then illuminated at 200 μmol photons/m^2/s. Cultures were agitated by a combination of stirring (100 rpm) and bubbling with filtered air (6.5 units monitored using a flow metre—Cole Palmer PMR1-010285). Cell density was measured every 8 h using a haemocytometer.

Determinations of lethal doses and doubling time increases

Lethal dosage of each herbicide was determined for each strain (wild type and transgenic) tested. Strains were grown in various ranges of herbicide and monitored visually for growth. The lethal dose was determined as the median concentration of herbicide that completely prevented growth of the algal strain. For the quantification of the herbicidal effects on growth rate, cell growth was measured via cell counting with a haemocytometer until the cells reached late log-phase growth ($\sim 1 \times 10^7$ cells/mL). An average doubling time was then determined for these cells, using the following equation: $T_D = \Delta T*(\log(2)/\log(C_2/C_1))$, where ΔT = the amount of time between cell counts, and C_1 and C_2 are the cell densities for the 1st and 2nd counts, respectively. Doubling times for cells in each level of herbicide were compared with determine the impact of herbicide on cell growth rate.

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Conflicts of interest

The authors declare no conflict of interests.

References

Arias, R.S., Dayan, F.E., Michel, A., Howell, J. and Scheffler, B.E. (2006) Characterization of a higher plant herbicide-resistant phytoene desaturase and its use as a selectable marker. *Plant Biotechnol. J.* **4**, 263–273.

Barry, G.F., Kishore, G.M., Padgette, S.R., Taylor, M.L., Kolacz, K.H., Weldon, M., Hallas, L. (1992) Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. In *Biosynthesis and Molecular Regulation of Amino Acids in Plants*(Singh, B.K., Flores, H.E. and Shannon, J.C., eds), pp. 139–145. Mockville, MD: American Society of Plant Physiologists.

Barry, G.F., Kishore, G.M., Padgette, S.R. and Stallings, W.C. (1997). *Glyphosate-Tolerant 5-Enolpyruvylshikimate-3-Phosphate Synthases*. United States Patent US5633435A

Beale, S.I. and Weinstein, J.D. (1990) Tetrapyrrole metabolism in photosynthetic organisms. In *Biosynthesis of Heme and Chlorophylls* (Dailey, H.A., ed.), pp. 287–391. New York: McGraw-Hill.

Böger, P. and Sandmann, G. (1998) Carotenoid biosynthesis inhibitor herbicides - mode of action and resistance mechanisms. *Pestic. Outlook* **9**, 29–35.

Buchanan, M.J., Imam, S.H., Eskue, W.A. and Snell, W.J. (1989) Activation of the cell wall degrading protease, lysin, during sexual signalling in Chlamydomonas: the enzyme is stored as an inactive, higher relative molecular mass precursor in the periplasm. *J. Cell Biol.* **108**, 199–207.

Castle, L.A., Siehl, D.L., Gorton, R., Patten, P.A., Chen, Y.H., Bertain, S., Cho, H.J., Duck, N., Wong, J., Liu, D. and Lassner, M.W. (2004) Discovery and directed evolution of a glyphosate tolerance gene. *Science*, **304**, 1151–1154.

Chamovitz, D., Pecker, I. and Hirschberg, J. (1991) The molecular basis of resistance to the herbicide norflurazon. *Plant Mol. Biol.* **16**, 967–974.

Dill, G.M., Cajacob, C.A. and Padgette, S.R. (2008) Glyphosate-resistant crops: adoption, use and future considerations. *Pest Manag. Sci.* **64**, 326–331.

Duke, S.O., Lydon, J., Becerril, J.M., Sherman, T.D., Lehnen, L.P. and Matsumoto, H. (1991) Protoporphyrinogen oxidase-inhibiting herbicides. *Weed Sci.* **39**, 465–473.

Ha, S.B., Lee, S.B., Lee, Y., Yang, K., Lee, N., Jang, S.M., Chung, J.S., Kim, Y.S., WI, S.G. and Back, K. (2004) The plastidic Arabidopsis protoporphyrinogen IX oxidase gene, with or without the transit sequence, confers resistance to the diphenyl ether herbicide in rice. *Plant, Cell Environ.* **27**, 79–88.

Hao, G.F., Zuo, Y., Yang, S.G. and Yang, G.F. (2011) Protoporphyrinogen oxidase inhibitor: an ideal target for herbicide discovery. *Chimia*, **65**, 961–969.

Harris, E.H. (1989) *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory use*. San Diego: Academic Press.

Jung, S., Lee, Y., Yang, K., Lee, S.B., Jang, S.M., Ha, S.B. and Back, K. (2004) Dual targeting of *Myxococcus xanthus* protoporphyrinogen oxidase into chloroplasts and mitochondria and high level oxyfluorfen resistance. *Plant, Cell Environ.* **27**, 1436–1446.

Kataoka, M., Sato, R. and Oshio, H. (1990) Isolation and partial characterization of mutant *Chlamydomonas reinhardtii* resistant to herbicide S-23142. *J. Pestic. Sci.* **15**, 449–451.

Kindle, K.L. (1990) High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc. Natl Acad. Sci. USA*, **87**, 1228–1232.

Leon, R., Couso, I. and Fernandez, E. (2007) Metabolic engineering of ketocarotenoids biosynthesis in the unicelullar microalga *Chlamydomonas reinhardtii*. *J. Biotechnol.* **130**, 143–152.

Lermontova, I. and Grimm, B. (2000) Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. *Plant Physiol.* **122**, 75–84.

Lumbreras, V., Stevens, D.R. and Purton, S. (1998) Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. *Plant J.* **14**, 441–447.

Martinez-Ferez, I.M. and Vioque, A. (1992) Nucleotide sequence of the phytoene desaturase gene from *Synechocystis* sp. PCC 6803 and characterization of a new mutation which confers resistance to the herbicide norflurazon. *Plant Mol. Biol.* **18**, 981–983.

Michel, A., Arias, R.S., Scheffler, B.E., Duke, S.O., Netherland, M. and Dayan, F.E. (2004) Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). *Mol. Ecol.* **13**, 3229–3237.

Malik, J., Barry, G. and Kishore, G. (1989) The herbicide glyphosate. *Biofactors.* **2**, 17–25.

Padgette, S.R., Kolacz, K.H., Delannay, X., Re, D.B., LaVallee, B.J., Tinius, C.N., Rhodes, K., Otero, Y.I., Barry, G.F., Eicholtz, D.A., Peschke, V.M., Nida, D.L., Taylor, N.B. and Kishore, G.M. (1995) Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci.* **35**, 1451–1461.

Randolph-Anderson, B.L., Sato, R., Johnson, A.M., Harris, E.H., Hauser, C.R., Oeda, K., Ishige, F., Nishio, S., Gillham, N.W. and Boynton, J.E. (1998) Isolation and characterization of a mutant protoporphyrinogen oxidase gene from *Chlamydomonas reinhardtii* conferring resistance to porphyric herbicides. *Plant Mol. Biol.* **38**, 839–859.

Reboud, X. (2002) Response of *Chlamydomonas reinhardtii* to herbicides: negative relationship between toxicity and water solubility across several herbicide families. *Bull. Environ. Contam. Toxicol.* **69**, 554–561.

Shimogawara, K., Fujiwara, S., Grossman, A. and Usuda, H. (1998) High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics*, **148**, 1821–1828.

Smith, E.A. and Oehme, F.W. (1992) The biological activity of glyphosate to plants and animals: a literature review. *Vet. Hum. Toxicol.* **34**, 531–543.

Warabi, E., Usui, K., Tanaka, Y. and Matsumoto, H. (2001) Resistance of a soybean cell line to oxyfluorfen by overproduction of mitochondrial protoporphyrinogen oxidase. *Pest Manag. Sci.* **57**, 743–748.

Williams, G.M., Kroes, R. and Munro, I.C. (2000) Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regul. Toxicol. Pharmacol.* **31**, 117–165.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 pGlyR1 Description: Glyphosate acetyltransferase cDNA (GAT), codon-optimized for expression in Chlamydomonas.
Figure S2 pOxR2 Description: Protoporphyrinogen oxidase gene (Chlamydomonas).
Figure S3 pNorR1 Description: cDNA for Chlamydomonas phytoene desaturase.