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Optical biosensors for environmental monitoring based on computational and biotechnological tools for engineering the photosynthetic D1 protein of *Chlamydomonas reinhardtii*

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ABSTRACT

Homology-based protein modelling and computational screening followed by virtual mutagenesis analyses were used to identify functional amino acids in the D1 protein of the photosynthetic electron transfer chain interacting with herbicides. A library of functional mutations in the unicellular green alga *Chlamydomonas reinhardtii* for preparing biomediators was built and their interactions with herbicides were calculated. D1 proteins giving the lowest and highest binding energy with herbicides were considered as suitable for preparing the environmental biosensors for detecting specific herbicide classes. Arising from the results of theoretical calculations, three mutants were prepared by site-directed mutagenesis and characterized by fluorescence analysis. Their adsorption and selective recognition ability were studied by an equilibrium-adsorption method. The S268C and S264K biomediators showed high sensitivity and resistance, respectively, to both triazine and urea classes of herbicides. When immobilized on a silicon septum, the biomediators were found to be highly stable, remaining so for at least 1-month at room temperature. The fluorescence properties were exploited and a reusable and portable multiarray optical biosensor for environmental monitoring was developed with limits of detection between 0.8×10^{-11} and 3.0×10^{-9}, depending on the target analyte. In addition, biomediator regeneration without obvious deterioration in performance was demonstrated.

1. Introduction

Photosystem II (PSII) is a light-driven water-plastoquinone oxidoreductase that catalyses the oxidation of water to molecular oxygen, thereby contributing to the maintenance of an aerobic atmosphere on earth. In PSII, light is absorbed by chlorophyll–protein complexes and then channelled into the photochemically active reaction centre chlorophyll (P680). Excited P680 (P680*) becomes photo-oxidized and donates an electron to the primary pheophytin acceptor. This charge separation is stabilized by the transfer of an electron to quinone Q_A, a firmly bound plastoquinone molecule located in the D2 subunit of PSII, and subsequently to Q_B, a mobile plastoquinone located within the D1 subunit of PSII. Electron transfer from Q_A^- to the secondary plastoquinone Q_B occurs via non-heme iron. Q_B is a two-electron carrier and while the partially reduced Q_B^- form is tightly bound to the D1 protein (the Q_B pocket), the fully reduced and protonated Q_BH_2 form has a relatively low affinity for the hydrophobic pocket within D1. Consequently, another molecule of the plastoquinone pool displaces Q_BH_2 occupying the D1 binding pocket (Giardi and Pace, 2005; Barber, 2006).

Traditionally, photosynthesis is regarded as a light-dependent system for producing oxygen and biomass from water and carbon dioxide. However, with our increasing knowledge about photosynthetic reaction mechanisms and structural details of the reaction centres (Keddy et al., 1995; Barber, 2006), new technological applications that utilize the unique activities of PSII have become possible. These applications include the construction of biosensors (Giardi and Pace, 2005; Campàs et al., 2008). The advantage of PSII-based biosensors is that this enzyme complex recognizes certain environmentally important classes of analytes such as triazines, phenylurea, diazines and phenolic compounds, which are still widely used today for weed control in agriculture. These

Abbreviations: LED, light emitting diode; PSII, photosystem II; TAP, Tris–acetate–phosphate medium; LOD, limit of detection; PDB, protein data bank; PCR, polymerase chain reaction.

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herbicides are the most common pollutants found on the surface and in ground waters at concentrations that exceed the maximum residue level (MRL) imposed by EU directive 91/414/CEE. For example, of 1000 sites analysed in Italy, 50% show the presence of terbuthylazine with a MRL ≥ 0.1 μg/L; atrazine, banned in Europe in 1980, is still present in over 20% of the 3000 analysed sites (Paris et al., 2009). Regulatory limits for pesticide residues have been imposed by the United States, Australia, New Zealand, Japan, Canada, the European Union and Taiwan (Hamilton et al., 2003). As an example, the "European Water Act of 1980" stated that the concentration of an individual or herbicide class in water must be lower than 0.1 or 0.5 μg/L, respectively. New limits were imposed in 2008 for herbicide residues in food (Commission Regulation EC No. 149/2008): from 0.01 to 0.05 mg/kg. It follows that extremely sensitive and reliable analytical methods are required to detect such low residue concentrations.

These herbicide compounds can bind reversibly to the D1 subunit of PSII within its Q_B binding pocket, also called the herbicide-binding niche. Upon binding, the compounds alter or inhibit electron transfer by displacing the plastoquinone Q_B, thus blocking electron flow, oxygen evolution and changing the fluorescence properties of PSII (Giardi and Pace, 2006; Chaplen et al., 2007; Campàs et al., 2008). The recognition of PSII photosynthetic herbicides is not highly specific, because other classes of compounds, such as heavy metals, are also detected, albeit with lower sensitivity (Chaplen et al., 2007). The lack of specificity for a class of chemical reagents can limit the range of application of PSII-based biosensors.

Recent advances in the molecular biology of PSII and in algal chloroplast transformation have produced a number of site-directed mutants characterized by amino acid substitutions in the sequence of the reaction centre D1 protein (Newman et al., 1990; Johanningmeier and Heiss, 1993; Oettmeier, 1999). The herbicide-binding site consists of about 65 amino acids. Notably, modifications to only one amino acid within the Q_B binding pocket can change photosynthetic activity and herbicide-binding considerably. Depending on the position and type of amino acid substitution, chemically different inhibitors show differential affinity for the D1 binding niche. Interestingly, a mutation that causes resistance towards one class of inhibitors can lead to hypersensitivity toward another class (Oettmeier, 1999).

In this study, with the intent to obtain algal-based biosensors for detecting environmental pollutants, D1 protein–herbicide interactions were investigated by bioinformatic modelling in the unicellular green alga *Chlamydomonas reinhardtii*. Using a multidisciplinary approach, *C. reinhardtii* strains with amino acid modifications in the D1 protein Q_B pocket were produced. These mutants were then used to develop a reusable and portable optical biosensor that utilizes mutant algal biomediators with high resistance and enhanced sensitivity toward different herbicide classes, thereby allowing the specific discrimination of these herbicides.

2. Materials and methods

2.1. Chemicals

Atrazine [(1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine)]; diuron [(3-(3,4-dichlorophenyl)-1,1-dimethylurea)]; linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea]; prometryne [(N,N′-diisopropyl-6-methylthio-1,3,5-triazine-2,4-diamine)]; and terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine) were purchased from Sigma (Aldrich Corporation, USA).

2.2. Bioinformatic modelling

D1 protein–herbicide interactions were investigated by a combination of homology modelling and virtual mutagenesis. Computational analysis was used to predict the specific mutations to the D1 protein that would increase its herbicide-binding affinity while maintaining its electron transport capacity and stability (Moult, 1999; Xiong et al., 1998; Camacho and Vajda, 2002). The three-dimensional structure of *C. reinhardtii* D1 protein was homology modelled by Basic Local Alignment Search Tool (Schaffer et al., 2001) using as template the crystal structure of the corresponding protein of *T. elongatus* (PDB code: 2AXT7). *T. elongatus* D1 protein displays 87% amino acid sequence identity with *C. reinhardtii* D1. The template structure was cleaned with respect to missing residues and the overall geometry optimized to 0.1 kcal/(mol × Å) using the MMFF94 force-field (Halgren, 1999). Homology modelling was performed using VLifeMDS 3.0 (Barve et al., 2006). Bad contacts in the resulting model were removed and the energy optimized up to 0.1 kcal/(mol × Å).

Simulation of herbicide docking in the D1 binding pocket was performed using the global range molecular modelling program (GRAMM; Vakser et al., 1999) which locates the area of the global minimum of intermolecular energy for structures of different accuracy. Using GRAMM the lowest energy structure obtained for D1 with atrazine docked was used as a model for the triazine class of herbicides (Xiong et al., 1998; Camacho and Vajda, 2002). A protein region spanning an 8 Å radius from the Q_B site was selected. Four different orientations of atrazine were evaluated and the resulting structures were energy minimized. The lowest energy structure was used to estimate and compare energy changes resulting from amino acid substitutions within the Q_B site. Residues within the Q_B binding sites of D1 protein were substituted into all the 19 possible amino acids modelled in different conformations through a rotamer generation procedure. The rotamers were generated with dihedral angles of 60°, 180° and −60°, for sp3-sp2 hybridized bonds, +90° and −90° for aromatics and 0° and 180° for carboxylates and amides. However, for torsions around Cα–Cβ bonds, six rotamers with torsion angles incremented with a step of 60° were generated. Binding energy calculations were performed by energy minimization of the ligand-free (apo)protein, of the ligand and of the protein–ligand complex to a gradient RMS of 0.1 kcal/(mol × Å), using the MMFF94 force-field as implemented in VLifeMDS 3.0. The binding energy was then calculated as: $E_{binding} = E_{complex} - (E_{apo} + E_{ligand})$.

2.3. Strains, culture conditions, and production of mutant

C. reinhardtii strains were grown in liquid Tris–acetate– phosphate medium (TAP) (Harris, 1989) at 50 μmol m^{-2} s^{-1} light intensity, 25 °C temperature, and 150 rpm agitation. The *C. reinhardtii* IL strain contains the wild type *psb*A gene without introns and represents the control reference strain. Its derivative Del1 mutant has a defined deletion in the *psbA* gene and was used as a recipient strain to obtain D1 site-directed mutants by particle gun transformation, as previously reported (Johanningmeier and Heiss, 1993). Site-directed mutagenesis and PCR were performed as described (Xiong et al., 1998; Johanningmeier et al., 2000). PCR fragments were amplified from the plasmid pSH5, which contained the complete intronless *psbA* gene and the 3′-flanking region.

The mutagenic primers used to produce the S268C mutant were 5′-GCTTCTTTCAACAACTGTCGTTC-3′ and 5′-GAACGACACAGTTGTTGAAAGAAGC-3′. The specific PCR-generated *psbA* fragments were directly precipitated onto tungsten particles and delivered into the host by particle gun. Following transformation, photosynthetically active growing colonies were selected and analysed. The introduction of the mutation was confirmed by sequence analysis.

2.4. Fluorescence analysis

For fluorescence measurements, Chlamydomonas cultures containing equal amounts of chlorophyll (20 μg) were incubated with herbicide in a range of concentrations (10^{-10} to 10^{-5} M) with mild shaking for 10 min. The incubation was performed under white light of 50 μmol m^{-2} s^{-1} in order to maintain the metabolic activity of PSII and to maximize the transport and the activity of herbicides inside the chloroplast, as suggested by Mattoo et al., 1999. Fluorescence measurements were carried out after 10 min of incubation in the dark.

Fluorescence induction was measured using a fluorimeter purchased from Biosensor (Rome, Italy, www.biosensor.it). The fluorimeter provided inputs of 500 μmol photons m^{-2} s^{-1} red light for 5 s at 650 nm and measured the fluorescence emission by photodiode at 680 nm. The red light covered a surface of 1 cm^2, resulting in highly reproducible measurements averaged over 1000 points. The sensor allowed determination of the main chlorophyll fluorescence parameters: F_0 was calculated using an algorithm that determines the line of best fit for the initial data points recorded at the onset of illumination, this line was extrapolated to time zero to determine F_0; F_M was the maximum value achieved during recording; F_v was the variable component of fluorescence. Measuring fluorescence at 2 ms gives F_{2ms} and the corresponding $V_J = (F_{2ms} - F_0)/(F_M - F_0)$ that refers to the rate of reoxidation of Q_A^- with respect to its reduction. Comparisons among different samples were enabled by normalizing the chlorophyll fluorescence and calculating the transient as relative variable fluorescence $[1 - V_J]$ at any given time. This procedure simplified the analysis by considering only the dynamic accumulation of Q_A in its reduced form, Q_A^- (Loll et al., 2005). The limits of detection (LODs) of *C. reinhardtii* wild type and mutants were determined on the basis of 99% confidence interval, which, assuming the normal distribution corresponds to 2.6 × standard error of the measurements (σ). Then, using the modified relationship for the Langmuir absorption isotherm, $LOD = 2.6 \times \sigma \times I50/(100 - 2.6 \times \sigma)$ as previously reported (Koblizek et al., 2002; Touloupakis et al., 2005).

All statistical tests were performed by analysis of variance (ANOVA) and the statistical significance of differences was evaluated by P-level ≤ 0.05 (Meier and Zund, 1993). P-values were calculated by comparing fluorescence values in a mutant incubated with or without herbicides with respect to the control.

2.5. Biosensor experimental set-up and immobilization of photosynthetic cells

Fig. 1 depicts the configuration of the newly developed biosensor. A custom-made flow cell containing the biomediator immobilized into a silicon frit is mounted above the fluorescence induction analyzer and continuously washed with measuring buffer. Chlamydomonas cultures containing 5 μg of chlorophyll were immobilized by physical adsorption on a silicon septum

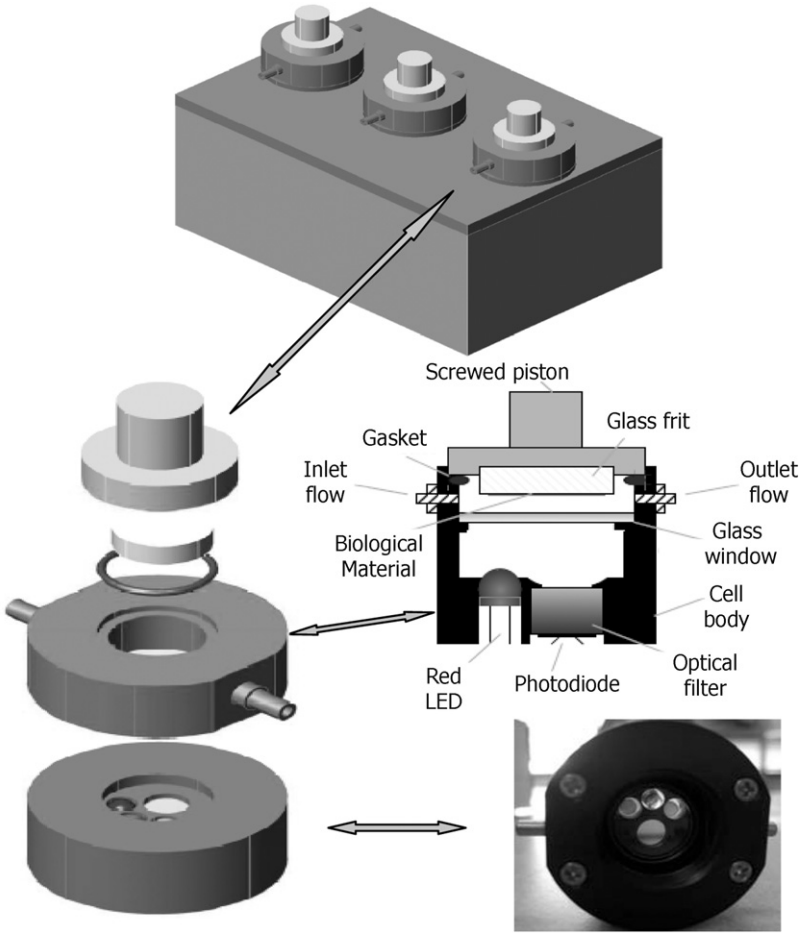

Fig. 1. (A) Diagram of the biosensor. The cells are composed of an interchangeable cylinder equipped with a silicon septum. (B) Details of the optical excitation module that provides the excitation red light of 500 μmol photons m^{-2} s^{-1} for 5 s at 650 nm and detects the fluorescence emission at 680 nm by the photodiode. The red light covered a surface of 1 cm^2, resulting in highly reproducible measurements averaged over 1000 fluorescence points in a range of microseconds to seconds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

Table 1
Energies of the structures of intronless and selected mutated D1 proteins docked with atrazine and the energy lowering with respect to the intronless control protein.

Mutants	Binding energy (kcal/mol)	Calculated energy decrease
IL	50.71	
S268C	38.34	12.37
F211N	40.74	9.97
L271I	42.35	8.36
S264K	90.89	−40.08

Simulated herbicide docking in the D1 binding pocket was performed using the GRAMM program (Vakser et al., 1999); each mutation generated a set of rotamers with different dihedral angles, all of which were energy minimized using MMFF94 force-field as implemented in VLifeMDS 3.0 (Halgren, 1999; Barve et al., 2006). The model was used to predict the lowest energy structure for the D1-atrazine complex (Xiong et al., 1998; Camacho and Vajda, 2002).

(Pyrex, Bibby Sterelin Ltd., UK) in the presence of liquid TAP medium or alternatively in a solid agar-TAP medium with a modification of the method by Scognamiglio et al., 2009. Using a micropipette tip, the samples were distributed to fit in a circle of 1 cm diameter over the frit; this corresponds to the diameter of the measuring chamber in the flow cell. Then, measuring buffer (20 mM Tricine pH 7.8, 70 sucrose, 100 mM NaCl, and 5 mM $MgCl_2$) was allowed to flow through the filter of the flow cell until a green layer was generated. During measurements, the flux of herbicide solution was sent both through and over the biomediator layer (Giardi et al., 2006 EU Patent, unpublished results).

The biosensor performed the measurements automatically using an electronic microcontroller (architecture 8051, Atmel AT89C51ED2) to supply and measure micro-pump flow, read LEDs at 650 nm excitation and performs data conversion for the PC. The flow of buffer (0.2 ml/min) was driven by a small peristaltic pump (Watson Marlow 101U/R). All fluorescence measurements were carried out at room temperature. The mechanical part of the biosensor (Fig. 1) was composed of a tube system that enabled flow transport through the micro-pump. Delrin® (polyoxymethylene), that was proved to be biologically inert and not to adsorb herbicides during the duration of the measurement (data not shown), was used to construct the cell.

The principle of herbicide detection was based on a modification of fluorescence induction in the biosensor signal. First, the fluorescence activity in the absence of herbicide was recorded, then the herbicide-containing sample was loaded onto the cell and the resid-

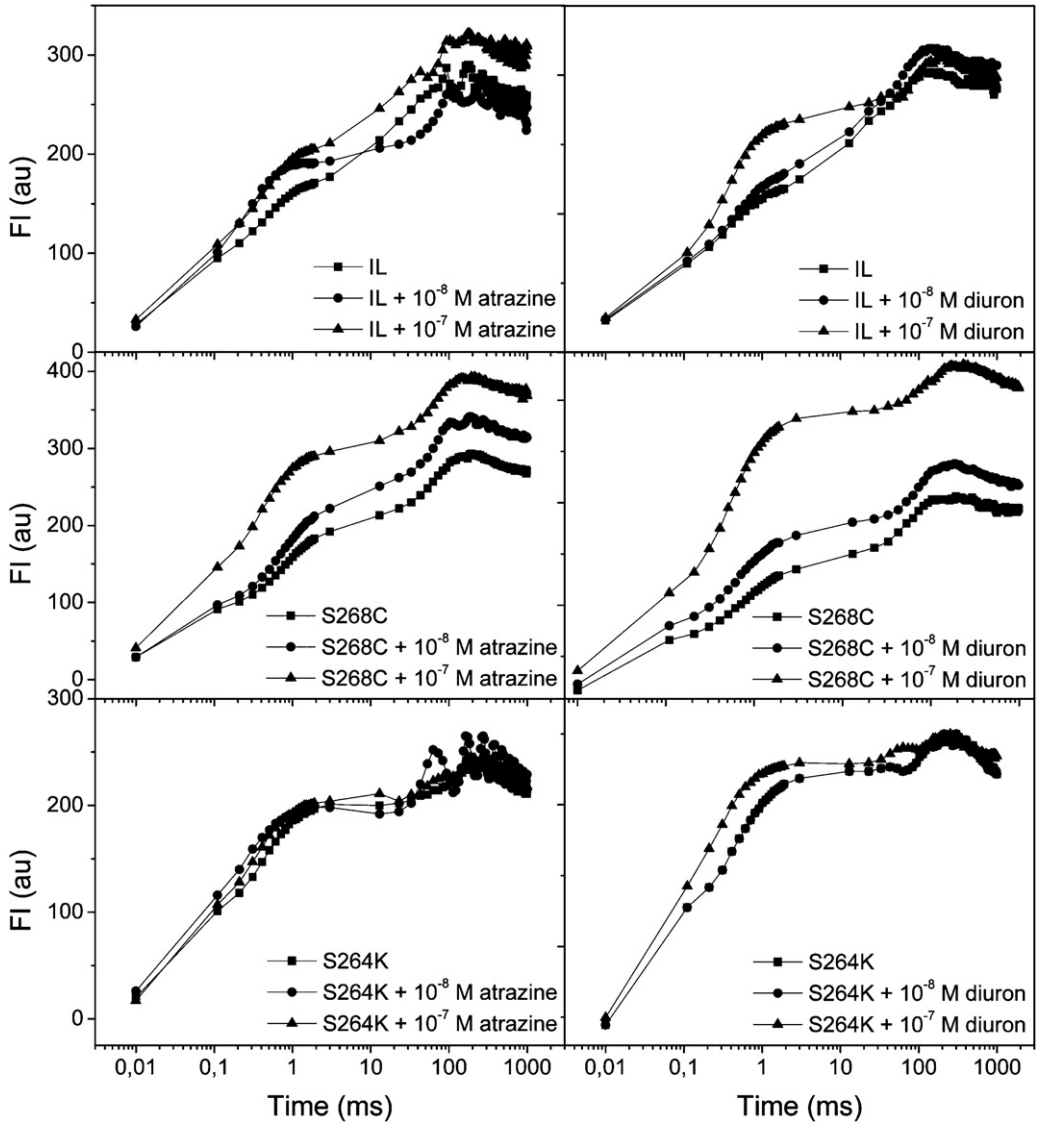

Fig. 2. Fluorescence induction pattern for immobilized IL, S268C and S264k strains with and without herbicides at various molar concentrations, as measured by the biosensor system. From the fluorescence profiles, $V_J = (F_{2ms} - F_0)/(F_M - F_0)$ was extrapolated and I_{30} and LODs for the three strains in the presence of herbicides were determined and reported in Table 2.

Table 2
Inhibition of fluorescence by various herbicides for the IL and S268C strains immobilized on a silicon layer.

Chemical Class	Herbicides	IL (I_{30} M)	S268C (I_{30} M)	C (M)	S264K F_v/F_M stimulation (%) at herbicide concentration (M)
Triazine	Atrazine	$(9.4 \pm 0.4) \times 10^{-8}$	$(2.6 \pm 0.5) \times 10^{-8}$	3.1 ± 0.9	30 at 10^{-10} M
Triazine	Prometryn	$(5.4 \pm 0.3) \times 10^{-8}$	$(2.2 \pm 0.2) \times 10^{-8}$	2.4 ± 0.5	18 at 10^{-9} M
Triazine	Terbuthylazine	$(7.3 \pm 0.4) \times 10^{-8}$	$(2.3 \pm 0.4) \times 10^{-8}$	3.2 ± 0.8	20 at 10^{-10} M
Urea	Diuron	$(3.6 \pm 0.3) \times 10^{-8}$	$(3.1 \pm 0.2) \times 10^{-8}$	2.1 ± 0.5	30 at 10^{-10} M
Urea	Linuron	$(4.4 \pm 0.4) \times 10^{-8}$	$(1.2 \pm 0.3) \times 10^{-8}$	3.7 ± 0.7	20 at 10^{-9} M

The I_{30} values represent the molar concentrations (M) of herbicide that induced 30% inhibition of the relative variable fluorescence $[1 - V_J]$. The ratios of IL control I_{30} to the S268C I_{30} (C (M)) are reported. The relative standard deviations are reported. P-values ≤ 0.05 were calculated as reported in Section 2. For S264K, the herbicide concentrations that stimulated the F_v/F_M ratio are reported.

ual activity was registered after 10 min. The ratio of the signals in the presence and in the absence of herbicide was plotted against the herbicide content in the sample. Three to five independent measurements were performed for each herbicide concentration. Then, the herbicide was removed by flushing with measuring buffer and the biosensor was ready for a new measurement.

3. Results and discussion

In this study the bioinformatic model suggested that modification of the D1 protein can increase or decrease its sensitivity, selectivity and stability toward different classes of herbicides. Selected mutants were generated by site-directed mutagenesis. Their photosynthetic activity was characterized and they were tested for use as immobilized biomediators in a newly constructed optical biosensor suitable for detection of herbicides.

3.1. Bioinformatic-mediated development of D1 mutants

In the eukaryotic PSII complex, the D1 protein binds the cofactor plastoquinone in the Q_B pocket, which is also the target of photosynthetic herbicides, atrazine in particular (Mattoo et al., 1999). For our study, the D1 protein–herbicide interactions were investigated by a combination of homology modelling and virtual mutagenesis. Computational analysis predicted mutations of the D1 protein that would increase its herbicide-binding affinity while maintaining its electron transport activity. The lowest energy structure was used to estimate and compare the energy changes resulting from amino acid substitutions to the Q_B site (Landt et al., 1990; Moult, 1999; Johanningmeier et al., 2000; Wilski et al., 2006; Yi et al., 2007).

Molecular modelling procedures indicated several mutations exhibiting comparable or lower energy values than those calculated for the wild type D1 protein bound with atrazine. However, some mutations (e.g., Tyr246), although energetically stable, were excluded because of their potential to hinder the correct entrance of atrazine into the binding pocket. Other mutations were excluded (e.g., mutations to Gly256) because our model predicted that they would cause steric hindrance. Mutation targets did not include some key residues (e.g., His215 and His272) for which substitution is known to alter specific features of the D1 protein indispensable for electron transport (Trebst, 1987; Oettmeier, 1999). The four specific D1 mutants F211N, S268C, L271I and S264K were compared to the IL control strain for their atrazine binding energy values (Table 1). Among these four virtual mutants, S268C was deemed to be particularly useful for biomediation purposes and selected for production because (a) this mutation allowed better binding with His272, which is important for electron transport, (b) the substituted Cys268 residue binds D2-Thr231, thus reinforcing the interaction of the D2 residues around the D1 binding pocket, and (c) this mutant protein was predicted to bind atrazine with a significantly lower energy compared to the other strains F211N and L271I (approx 12 kcal/mol lower binding energy against 8.0 and 9.0 kcal/mol for the other mutants; see Table 1).

The mutant S264K, previously described by Tibuzzi et al., 2007, was also selected for further evaluation because position 264 is known to be important for resistance to atrazine herbicide and may be useful to discriminate classes of herbicides (Landt et al., 1990; Oettmeier, 1999). Docking analysis indicated that this mutation almost doubles the D1-atrazine binding energy compared to the IL control strain (Table 1). This tremendous increase in energy can be attributed to the large numbers of bumps caused by the replacement of the small serine residue with the very large lysine residue. Although favourable interactions with atrazine were also present, they were not sufficient to outweigh the energy increase caused by the steric hindrance. Hence, S264K was predicted to display a very low affinity for atrazine.

Therefore, on the basis of the model results, the IL control strain, S268C, and S264K were generated by site-directed mutagenesis and their interaction with herbicides were investigated by the fluorescence induction technique useful as optical transducer for biosensors.

3.2. Fluorescence-mediated characterization of mutant D1 response to herbicides

Light energy absorbed by chlorophyll molecules in PSII is used for photosynthesis, while the excess energy is dissipated as heat or re-emitted as fluorescence at longer wavelength in the red/far-red. By measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and energy dissipation can be obtained.

Therefore, the mutants were characterized by fluorescence induction, achieved by measuring emission in the range of 0.001–0.1 s, which corresponded to an accumulation of the electron acceptor Q_A^- on the reducing side of PSII when illumination was begun after a dark period (Johanningmeier et al., 2000). Fig. 2 shows the fluorescence induction pattern for the immobilized IL, S268C and S264K mutants with and without herbicides (atrazine and diuron) at various molar concentrations. The results indicated that IL and the S268C mutant were dramatically affected by all tested herbicides, even at the lowest concentration of 10^{-8} M and that a greater increase in Q_A^- accumulation occurred for S268C than

Table 3
The limits of detection in molar concentration (M) of IL and S268C strains for various herbicides were calculated on the basis of 99% confidence interval, which, assuming the normal distribution, corresponds to 2.6 × standard error of the measurements (σ) as previously reported (Koblizek et al., 2002; Touloupakis et al., 2005).

Limits of detection (M)			
Chemical Class	Herbicide	IL	S268C
Triazine	Atrazine	$2.4 \pm 1.2 \times 10^{-9}$	$6.8 \pm 2.0 \times 10^{-10}$
Triazine	Prometryne	$1.4 \pm 1.0 \times 10^{-9}$	$6.5 \pm 1.9 \times 10^{-10}$
Triazine	Terbuthylazine	$3.0 \pm 1.1 \times 10^{-9}$	$2.5 \pm 0.7 \times 10^{-10}$
Urea	Diuron	$1.0 \pm 0.8 \times 10^{-9}$	$0.8 \pm 0.2 \times 10^{-11}$
Urea	Linuron	$2.2 \pm 1.4 \times 10^{-9}$	$0.9 \pm 0.2 \times 10^{-11}$

The relative standard deviations are reported; P-values ≤ 0.05 were calculated as described in Section 2.

Table 4
(a) Regeneration of the mutants adsorbed over the silicon septum, wetted with TAP medium and kept at room temperature under operational conditions. Regeneration was calculated as percent of the initial value activity by washing with a flow of 1 ml/min of TAP medium for 10 min. The experiments were repeated three times and a typical result is presented. *P*-values ≤0.05 were calculated as reported in Section 2. (b) $[1 - V_J]$ parameter variation under various chlorophyll quantities in the range of 14–70 μg/ml in the presence of 10^{-7} M herbicide.

(a) Biomediator regeneration after incubation with atrazine			(b) Chlorophyll quantity (μg/ml)	$[1 - Vj]$ parameter variation under different chlorophyll quantity + herbicide 10^{-7} M
	$[1 - Vj]$ (%)	s.e.		
Initial activity + 10^{-7} M atrazine regeneration	100	±1.2	14	0.41
	78	±3.3	35	0.41
	98	±2.8	49	0.40
			70	0.40
			14	0.23
			35	0.24
			49	0.24
Initial activity + 10^{-10} M atrazine regeneration	100	±0.7	70	0.22
	83	±1.3	14	0.16
	97	±1.9	35	0.16
			49	0.16
			70	0.16

for the IL control. Electron transfer was decreased in mutant S264K compared to IL control as indicated by the high level of Q_A^- accumulation and only a slight effect was observed after the addition of herbicide (Fig. 2). It is known that reoxidation of Q_A^- is inhibited in presence of herbicides and since the fluorescence value $[1 - V_J]$ refers to the rate of reoxidation of Q_A^- with respect to its reduction $[1 - V_J]$ changes in a way that is strongly correlated with the herbicide concentration (Wilski et al., 2006). Therefore, the $[1 - V_J]$ parameter was selected for use in the biosensor system (Table 2).

3.3. Development of a biosensor for herbicide detection

The biosensor was composed of (i) a flow cell with an interchangeable cylinder equipped with a filter septum, where the biomediator was immobilized; (ii) a peristaltic pump that enabled the flow of the herbicide-containing buffer through the flow cell and then through the biomediator; and (iii) a fluorimeter that provided the excitation light and detected the induced fluorescence signal (Fig. 1). The wild type IL and the S264K and S268C mutant strains (resistant and sensitive to herbicides, respectively) were evaluated as biomediators in the biosensor.

A prime consideration in biomediator preparation is that preparation of PSII particles from microorganisms is time consuming; however, the immobilization of whole cells can be relatively straightforward and was selected for our purposes. The V_J chlorophyll fluorescence parameter was calculated automatically by the sensor. Table 2 displays the molar concentrations (I_{30}) of herbicide that induced 30% inhibition of the relative fluorescence percent $[1 - V_J]$. I_{30} values based on $[1 - V_J]$ for the three strains in the presence of five herbicides owing to triazine class (atrazine, prometryn and terbuthylazine) and urea class (diuron and linuron) were determined. The fluorescence measurements obtained with the biosensor confirmed the greater resistance of the S264K mutant to triazines relative to the IL strain: for this mutant, I_{30} values could not be calculated within the range of herbicide concentrations tested. In addition, contrary to what is found in higher plants, this D1 mutation resulted in elevated resistance also to the urea class of herbicides, according to findings reported by Wilski et al., 2006. Moreover, stimulation of the fluorescence parameter occurred at the lowest concentrations of herbicide (Table 2). As suggested by the bioinformatic model, the experiments demonstrated that the S268C mutant was much more sensitive toward atrazine than the IL strain (LODs: IL, $2.4 \pm 1.2 \times 10^{-9}$; S268C, $6.8 \pm 2.0 \times 10^{-10}$); interestingly, S268C was also notably more sensitive to the urea-type herbicides diuron and linuron. The limits of detection of the various herbicides measured with the biosensor ranged from 0.9×10^{-11} to 3.0×10^{-9} molar (M), depending on the biomediator and herbicide pairing tested (Table 3). The lowest calculated limit of detection of 0.8×10^{-11} M was revealed by the S268C mutant for diuron.

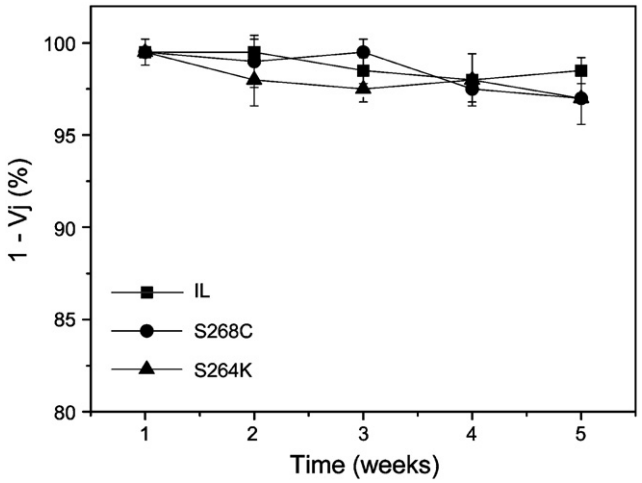

Fig. 3. Biomediator stability after immobilization on a silicon septum in the presence of agar-TAP medium as nutrient source.

Fig. 3 shows that the mutants IL, S268C and S264K are highly stable in our conditions of immobilization. We found that the biomediators remained completely stable on a silicon septum in the presence of agar-TAP medium as nutrient source for at least 1-month after immobilization (Fig. 3).

Moreover, the fluorescence parameter $[1 - V_J]$ is scarcely influenced either by the amount of chlorophyll present or by regeneration by washing with TAP medium. This indicates a high reproducibility of the biomediator measurements (Table 4).

4. Conclusion

Optical biosensors for the monitoring of environmental pollutants such as heavy metals, pesticides, chemical species or toxic microorganisms have received great attention in recent years (Rodriguez-Mozaz et al., 2006; Diercks et al., 2008; Scognamiglio et al., 2009). Among these the development of a new generation of rapid and inexpensive biosensors able to screen for the presence of herbicides in the environment is currently a promising research field.

Progress in chloroplast transformation techniques and increased resolution of the crystal structure of PSII has improved our understanding of structure–function relationships in PSII. Here, unicellular microalgae were exploited for the construction of a whole-cell-based biosensor capable of providing real time information about herbicide contamination. We produced *C. reinhardtii* mutant cells using a computational approach followed by site-directed mutagenesis. The exploitation of bioinformatics indicated the great relevance of the S268 amino acid in the binding of atrazine and guided the production of the S268C mutant which resulted in a net increase in LOD's sensitivity for triazines and urea herbicides. Exploiting bioinformatics to design the S268C mutant resulted in a net increase in LOD's sensitivity for both triazines and urea herbicides, indicating the importance of this amino acidic site in the binding of herbicides. Thus, the modelling approach used in this study may be an important tool for acquiring fundamental knowledge about the structure and function of the D1 protein that could be used in designing other mutant proteins as sensitive and specific recognition elements for biomediator purposes.

For biosensing purposes, the most important characteristics of the biomediator are stability under operating conditions and sensitivity and specificity toward herbicide classes. The biosensor developed in this work was able to distinguish among classes of photosynthetic herbicides such as triazine and phenylurea compounds.

The large number of active ingredients approved for use in agricultural applications (approximately 600) makes it difficult to obtain accurate information on herbicide application in different countries (Guzzella et al., 2006). Therefore, we evaluated the effect of real samples composed of a complex mixture of several herbicides (Guzzella et al., 2006) on the photosynthetic activity of wild type, S268C and S264K mutant strains of *C. reinhardtii*. We found that a real water matrix (control without herbicide) did not inhibit PSII activity and in some cases even induced activation by 5–21%, measured as oxygen evolution (data not shown). Our results indicate that even low concentrations of pollutants affect living algal mutants by altering physiological processes. The biosensor developed herein can be applied for the analysis of real samples and for evaluation of the real physiological impact of active herbicide compounds. An in-depth study using actual environmental samples is in progress, the results of which will be presented in a future publication. In conclusion, by a multidisciplinary approach we optimized the sensitivity, selectivity and stability of algal photosynthetic D1 protein toward herbicide classes thereby enabling its use in an innovative biosensor.

Disclosure statement

Due to the potential direct commercialisation of the device described in the paper, all the authors agree with publication of the manuscript and declare that there are no actual or potential future conflicts of interest with any person, corporation or other business entity or any external associations or relationships.

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