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ORIGINAL PAPER

Chlamydomonas reinhardtii genetic variants as probes for fluorescence sensing system in detection of pollutants

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Abstract The unicellular green alga *Chlamydomonas reinhardtii* is employed here for the setup of a biosensor demonstrator based on multibiomediators for the detection of herbicides. The detection is based on the activity of photosystem II, the multienzymatic chlorophyll–protein complex located in the thylakoid membrane that catalyzes the light-dependent photosynthetic primary charge separation and the electron transfer chain in cyanobacteria, algae, and higher plants. Several *C. reinhardtii* mutants modified on the D1 photosystem II protein are generated by site-directed mutagenesis and experimentally tested for the development of a biosensor revealing the modification of the fluorescence parameter $(1-V_J)$ in the presence of herbicides. The A250R, A250L, A251C, and I163N mutants are highly sensitive to the urea and triazine herbicide classes; the newly generated F255N mutant is shown to be especially resistant to the class of urea. It follows that the response of the multi-biomediators is associated to a particular herbicide subclass and can be useful to monitor several species of pollutants.

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Introduction

There is growing interest for the generation of rapid inexpensive assays to screen for the presence of herbicides in the environment. The European Union (EU), in the “European Water Act of 1980” document, states that the concentration of herbicides in water must be lower than 0.1 or 0.5 μg/L of any individual or total herbicide class, respectively [1]. The most recent EU Commission Regulations regarding pesticides, N. 149/2008 of 29 January 2008, fix the maximum levels of pesticide residues on foodstuffs of animal origin and on certain products of plant origin, including fruit and vegetables. Therefore, to answer the concern of the European Union on this issue, extremely sensitive and reliable analytical methods to detect low residual concentrations are required. Nowadays, the data on herbicides pollution are still quite scarce since monitoring data are based on a few investigations carried out by methods able to detect relatively high concentrations of herbicides. Chromatographic methods (high-performance liquid chromatography, gas chromatography, gas chromatography–mass spectrometry, and capillary electrophoresis) are the most commonly reliable and sensitive methods used to monitor the presence of herbicides. Nevertheless, the requirement for expensive equipments, organic solvents, and laborious sample preparation prevents the use of chromatography for rapid screening and pre-screening of a large number of samples.

The multienzymatic chlorophyll–protein complex photosystem II (PSII) contributes to the electron transport chain

that starts the photosynthetic primary charge separation. In the reaction center (RC), when the primary donor chlorophyll dimer P680 absorbs a photon, an electron jumps from the ground state to the excited state, from which it can pass to the primary acceptor Q_A [2]. Biosensing applications are based on the total or partial inhibition of this electron transfer due to the presence of chemical or physicochemical environmental conditions reacting either by direct binding to the RC complexes or by changing the equilibrium of the local environmental chemistry. In particular, the PSII complex contains the target site of the most widely used photosynthetic herbicides, the Q_B site of D1 protein. When these pollutants are present in the sample solution and come into contact with the photosystem, they inhibit the transport of electrons from the primary acceptor Q_A to the secondary quinone Q_B along the photosynthetic chain and partially or fully block the electron transfer. This inhibition results in a variation of PSII fluorescence emission that can be monitored by fluorescence analysis in a pesticide concentration-dependent manner. Herbicides targeting PSII belong to a variety of chemical classes such as triazines, triazinones, ureas, biscarbamates, dinitrophenols, and cyanophenols, to name only a few [1, 3].

Since the binding affinity of chemicals depends on the structural conformation of the quinone binding site Q_B, where they are bound on the D1/D2 proteins of RC, molecular biology studies have succeeded in verifying the effect of a single amino acid modification of D1 protein on chemical binding affinity [3, 4]. The unicellular alga *Chlamydomonas reinhardtii* is a biologic photosynthetic organism that offers multiple opportunities for D1 protein bioengineering. Consequently, several mutants from *C. reinhardtii* are constructed by site-directed mutagenesis in order to obtain a wide range of sensitivity and resistance to herbicides according to Johanningmeier et al. [5].

The objective of the research work described in this paper is the experimental validation of the applicability of *C. reinhardtii* mutant strains as an array of fluorescent biomediators featuring different sensitivity and selectivity to herbicides and low limits of detection: those algae are used for the construction of a multibiomediator optical biosensing system that can become a good candidate for monitoring environmental pollutants. This work aims at further extending the results previously reported by some of the authors [6] through the test of newly produced sensitive mutants and at engineering a multifluorimeter instrument able to determine the fluorescence parameter $V_J = (F_{2ms} - F_0)/(F_M - F_0)$, where F_0, F_M, and $F_{2\ ms}$ are respectively the initial fluorescence, the maximum fluorescence, and the fluorescence measured after 2 ms. The variable $(1-V_j)$ takes into account all parameters involved in the fluorescence emission profile and provides a more specific response being intimately related to the analyte–bioreceptor binding, correlated to the presence of the herbicide [7]. All engineered mutants exhibit a relatively long lifetime activity preserving 100% of stability for about 1 month after immobilization.

Materials and methods

Strain culture conditions, site-directed mutagenesis, and immobilization method

Wild-type and mutant strains of *C. reinhardtii* are grown in liquid Tris–acetate–phosphate (TAP [8]) with white-light neon lamps at 50 μmol m^{-2} s^{-1}, 25°C temperature, and 150-rpm agitation. Cells are harvested in mid-exponential growth phase (up to $2–3\times10^7$ cells per milliliter). The *C. reinhardtii* IL strain represents the control reference strain and its derivative mutant Del1 is used as a recipient strain to obtain the following D1 site-directed mutants: F255N, A251C, A250R, A250L, and I163N. Site-directed mutagenesis and polymerase chain reaction (PCR) are performed as described by Johanningmeier et al. [9] and Preiss et al. [10]. PCR fragments are amplified from the plasmid pSH5 containing the complete intronless *psbA* gene encoding the D1 protein and the 3′-flanking regions. The introduction of the mutation is confirmed by sequence analysis. After transformation, photosynthetically growing colonies are selected and analyzed. Wild type and mutants are immobilized in an agar–TAP medium deposed on a silicon septum of 1-cm diameter (Pyrex, Bibby Sterelin Ltd., UK). The so-prepared supports are assembled into the 24 measurement cells of the instrument, in the absence and in the presence of different concentrations of herbicides.

Fluorescence analyses and calculation of the LODs

Chlorophyll fluorescence is a physiological parameter routinely used to measure the photochemical efficiency of PSII. Measurements of chlorophyll fluorescence is a reliable and non-invasive method to determine a set of parameters linked to various stress factors, including the activity of the herbicides [11, 12]. From the shape and the form of the fluorescence transition curve (Kautsky curve) of PSII, it is possible to derive various fluorescence parameters linked to PSII photochemical efficiency.

Based on the typical shape of the fluorescence rise, Strasser and Strasser [7] developed a test providing adequate information about the structure and function of photosynthetic apparatus. Among the measurable parameters, the relative variable fluorescence is calculated as $V_J = (F_{2ms} - F_0)/(F_M - F_0)$, where F_0, F_M, and $F_{2\ ms}$ are the initial fluorescence, the maximum fluorescence, and the fluorescence after 2 ms, respectively.

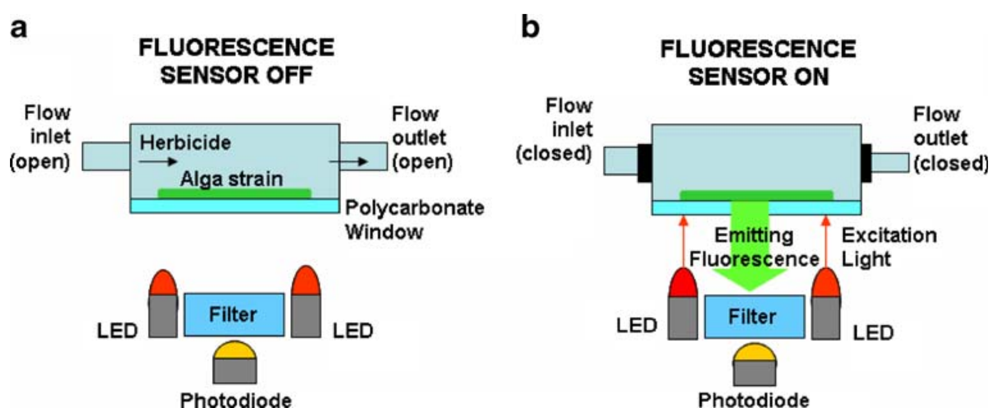

Fig. 1 Schematic concept of the fluorescence measurement: the biological container and the optical compartment are represented at the *top* and the *bottom*, respectively; **a** the sample solution containing the herbicide under test flows into the biological cell; **b** the alga strain biomediator is photoactivated and the emitting fluorescence captured by the photodetector (in static condition)

Measurements are carried out according to the scheme of Fig. 1 by using the OPTICBIO-Multicell instrument (from Biosensor Srl, Italy, www.biosensor.it, Fig. 2). The fluorescence excitation is carried out for 5 s at 650-nm wavelength by LED sources (1,500-mcd intensity) and the fluorescence emission is recorded at 720 nm by a photodiode. Chlorophyll fluorescence is measured on *C. reinhardtii* wild-type and mutant strains after 10 min of incubation with white light at 50 µmol m^{-2} s^{-1} (measured by Quantitherm Lightmeter Thermometer—Hansatec), 25°C temperature, and 150-rpm agitation, in the absence and in the presence of herbicide. Prior to each measurement, samples are kept in the dark for 10 min to reach the steady dark adapted state of PSII. The total fluorescence measurement is completed in 20 s, resulting significantly faster than the laboratory chromatographic methods.

The limits of detection (LODs) of *C. reinhardtii* wild type and mutants are 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, $\mathrm{LOD} = 2.6 \times \sigma \times \mathrm{I50}/(100 - 2.6 \times \sigma)$ as reported by Koblizek et al. [13].

Bioinstrument description

The multibiomediator fluorescence biosensor is based on a new versatile portable instrument, called OPTICBIO-Multicell, which combines the cell array configuration with the modular concept. Table 1 shows the main operational characteristics of the instrument. The instrument is equipped with 96 LEDs with different emission peak wavelengths for fluorescence excitation and 24 silicon photodiodes and optical filters for the measurement of the fluorescence emission. Each photodiode provides a spectral response in the range of 300–1,100 nm and different band-pass optical filters are used to select the set fluorescence peak: in the case of *C. reinhardtii* organisms, a bandwidth centered at 720±5.0 nm is selected. The instrument features a range of wavelengths for fluorescence excitation and emission and a unique function of luminous intensity regulation. The instrument automatically calculates the

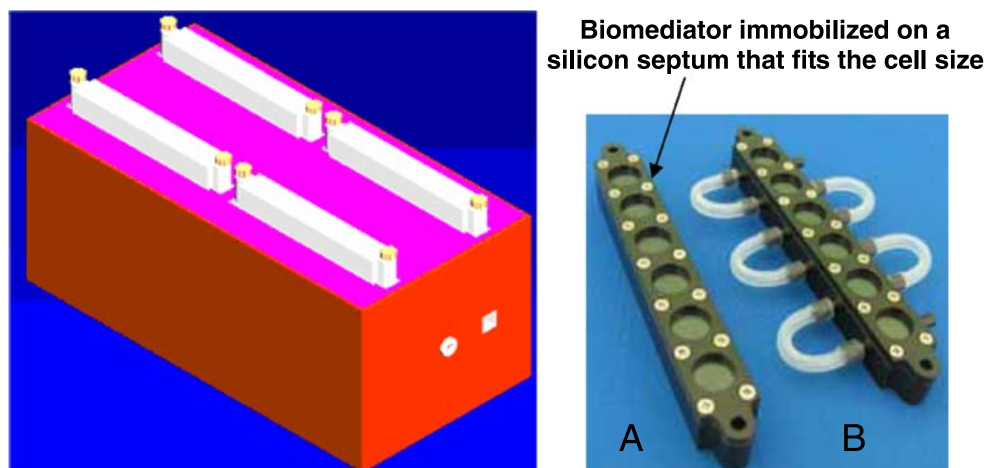

Fig. 2 *Left*: 3D schematic view of the instrument with four measurement cell arrays mounted on top; *Right*: two biological arrays made up by six containers (10 mm $\varnothing_{int}$×10 mm H, 785 µl each) for static (*A*) and flow (*B*) measurement mode

Table 1 Main technical characteristics of the OPTICBIO-Multicell fluorimeter

Operation mode	Emitting fluorescence measurement; automatic, static, and dynamic
Results	Kautsky curve real-time displayed on PC, with output parameters: F_0, F_V, F_M, $F_{2\ ms}$, F_V/F_M, $(1-V_J)$
Settings	Photoperiod (LED operation), time-controlled with 1-µs precision
	LED luminous intensity (128 levels)
	Flow speed
	Measurement cycle: (1) baseline recording, (2) lighting, (3) system relaxation
	Number of measurement cycles
	Operating LED (red, blue)
	Dynamic or static measurement mode (flow *on*/*off*)

The instrument is able to calculate the parameters: V_J the relative variable fluorescence, the initial fluorescence F_0, the maximum fluorescence F_M, and the fluorescence after 2 ms $F_{2\ ms}$

fluorescence parameter (1-V_j). It is equipped with 24 measurement cells (four arrays of six cells each), consisting of two physically and functionally different compartments, separated by a polycarbonate window. The optical module at the bottom is mounted inside the instrument case and provides fluorescence excitation and detection. The biological module, perfectly sealed, hosts the biosample under test. The biological cell arrays can be easily and quickly removed to replace the samples, while keeping the optical components fixed. Both static and dynamic operations are allowed thanks to an automatically controlled fluidic system (Fig. 2).

Results and discussion

The *C. reinhardtii* mutant strains A250R, A251C, and A250L, modified in the Q_B pocket of D1 which accommodates the electron transfer plastoquinone, were previously produced according to Johanningmeier et al. [9]. The mutant F255N and I163N are newly generated and represent positions in the D1 protein that can modify the herbicide binding pocket [14]. The selection of amino acid modifications, based on a newly developed model able to suggest modifications in D1 protein affecting herbicide binding, will be reported in a specific paper (Rea et al., submitted manuscript).

Measurements in the biosensor are carried out according to the scheme of Fig. 1 by using the multicell fluorimeter instrument. The light energy absorbed by chlorophyll molecules in photosystem II is used for the photochemistry of photosynthesis, while energy in excess is dissipated as heat or is re-emitted as longer wavelength red/far-red light energy; this re-emission of light is called chlorophyll fluorescence. These routes of energy pattern compete with each other in such a way that any increase in the efficiency of one will result in a decrease in the yield of the others. Therefore, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of

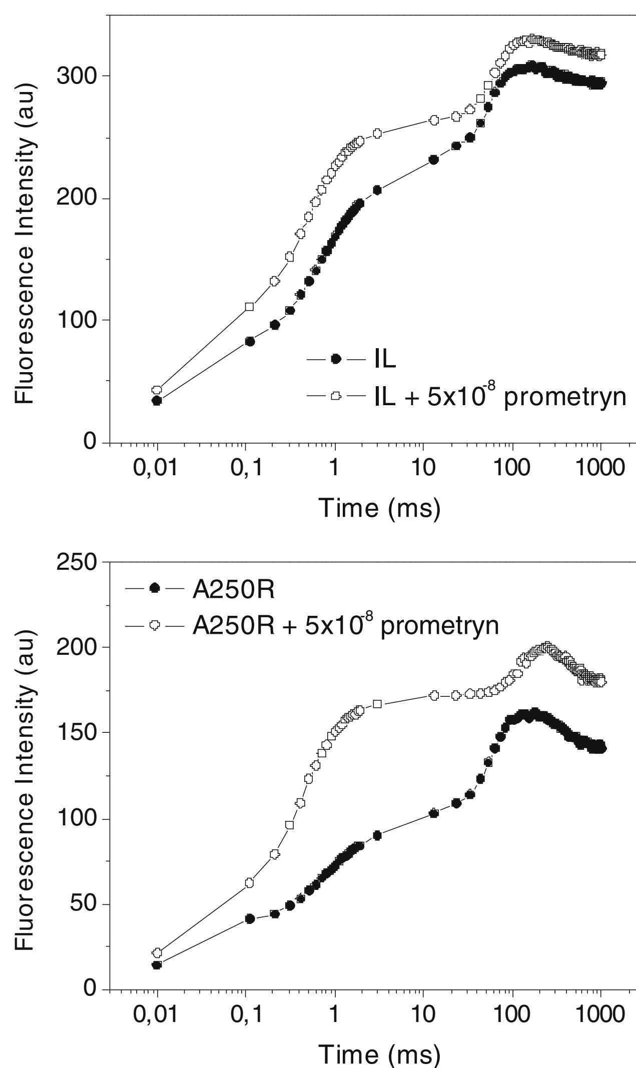

Fig. 3 Effect of prometryn on the fluorescence emission profile of *C. reinhardtii* in wild type and A250R, a mutant strain highly sensitive to the herbicide. The relative variable fluorescence $V_J = (F_{2ms} - F_0)/(F_M - F_0)$ is calculated on the basis of the fluorescence parameters F_0, F_M, $F_{2\ ms}$, the initial fluorescence, the maximum fluorescence, and the fluorescence after 2 ms, respectively

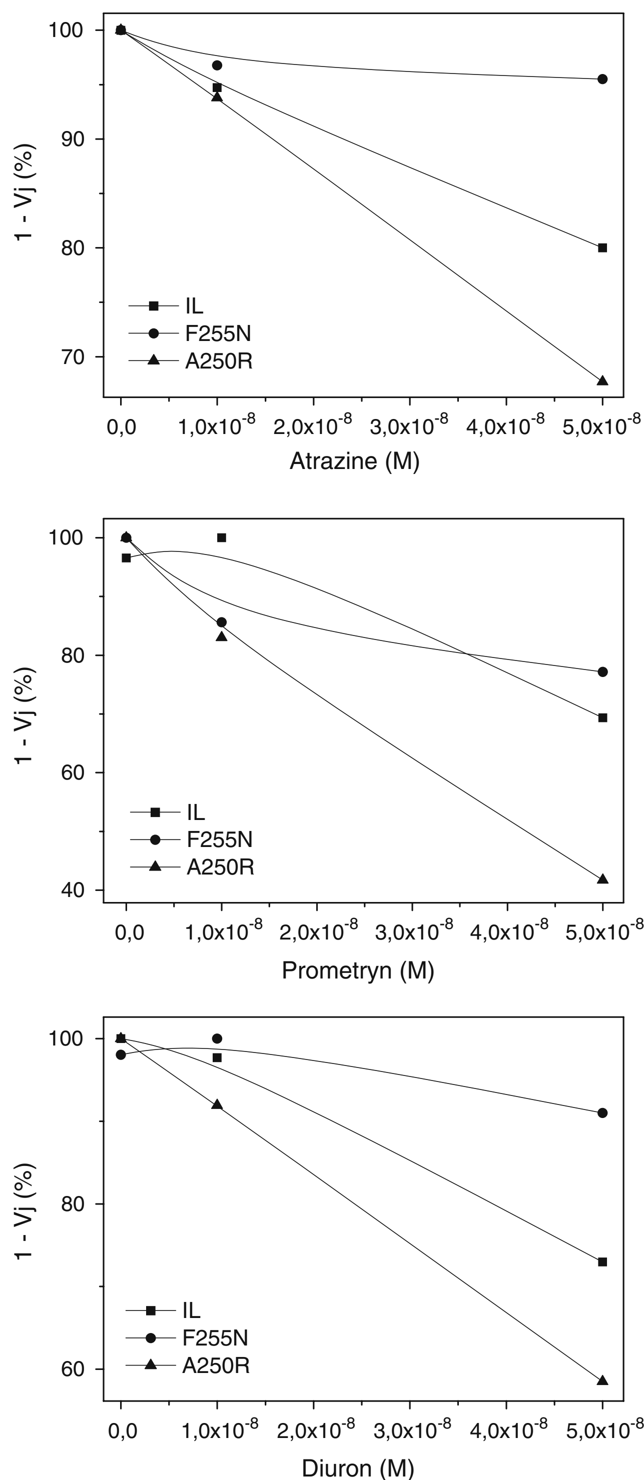

Fig. 4 Relative variable fluorescence ($1-V_J$) plot of IL, F255N, and A250R PSII activity in the presence of increasing amounts of atrazine, prometryn, and diuron

photochemistry and energy dissipation can be obtained. The most widely used technique is fluorescence induction, achieved by measuring changes in fluorescence yield when a light is switched on after a dark period; under exciting light, the fluorescence yield rapidly rises and then slowly decreases by picturing the so-called Kautsky curve [15–17]. The fluorescence emission profiles of wild-type and mutant strains were recorded in the absence and in presence of increasing amounts of several herbicides. From the fluores-

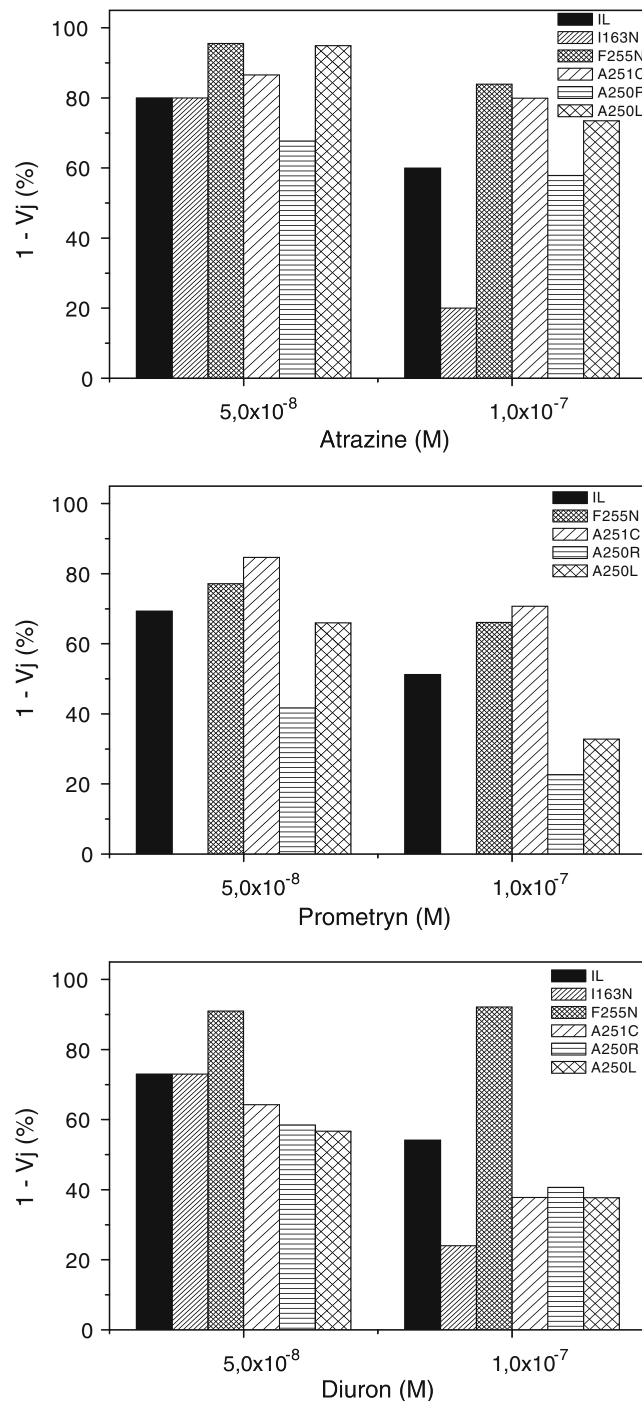

Fig. 5 Inhibition effect of atrazine, prometryn, and diuron on the PSII activity of *C. reinhardtii* wild-type and five mutant strains, highly sensitive or resistant to the herbicides. The histograms are generated by using the parameter ($1-V_J$) against herbicides

Table 2 Limits of detection (LOD) of *C. reinhardtii* wild-type and mutant strains for atrazine, prometryn, and diuron

Strains	I_{20}	I_{20} standard error	LOD I_{20}	Herbicides
IL	6.03×10^{-8}	2.66×10^{-9}	1.60×10^{-9}	Atrazine
	3.75×10^{-8}	3.17×10^{-9}	1.00×10^{-9}	Prometryn
	2.46×10^{-8}	1.93×10^{-9}	6.50×10^{-10}	Diuron
I163N	5.0×10^{-8}	1.20×10^{-10}	1.33×10^{-9}	Atrazine
	nd	nd	nd	Prometryn
	8.8×10^{-9}	3.76×10^{-10}	2.35×10^{-10}	Diuron
F255N	7.12×10^{-8}	1.38×10^{-8}	1.90×10^{-9}	Atrazine
	1.92×10^{-8}	4.86×10^{-9}	5.12×10^{-10}	Prometryn
	nr	nr	nr	Diuron
A251C	2.73×10^{-8}	6.20×10^{-9}	7.29×10^{-10}	Atrazine
	4.04×10^{-8}	2.70×10^{-9}	1.08×10^{-9}	Prometryn
	2.2×10^{-8}	3.91×10^{-9}	5.87×10^{-10}	Diuron
A250R	3.14×10^{-8}	3.06×10^{-9}	8.38×10^{-10}	Atrazine
	1.32×10^{-8}	3.18×10^{-9}	3.52×10^{-10}	Prometryn
	2.4×10^{-8}	4.15×10^{-9}	6.40×10^{-10}	Diuron
A250L	3.73×10^{-8}	1.18×10^{-9}	9.95×10^{-10}	Atrazine
	1.17×10^{-8}	2.12×10^{-9}	3.12×10^{-10}	Prometryn
	2.40×10^{-8}	3.49×10^{-9}	6.40×10^{-10}	Diuron

LODs are 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 = $2.6 \times \sigma \times \mathrm{I50}/(100 - 2.6 \times \sigma)$ [13]

nd not determined, *nr* not revealed

cence profiles, a number of parameters correlated to the photosynthetic activity of the PSII, like F_0, F_M, and F_{2ms}, were extrapolated. Among the significant parameters, the relative variable fluorescence $V_J = (F_{2ms} - F_0)/(F_M - F_0)$ that depends on the redox state of Q_A is calculated; in particular, the value $(1-V_J)$ refers to the rate of reoxidation of Q_A^- with respect to its reduction. If the reoxidation of Q_A^- is inhibited in the presence of the herbicide, the value $(1-V_J)$ changes in a way that is correlated to the herbicide concentration by calculating it from the Kautsky curve (Fig. 3). Figure 2 shows the schematic view of the instrument and the biological cell arrays. The 24 cells of the fluorimeter sensor hosts the six mutants, controls, and samples treated with three herbicides (atrazine, prometryn, and diuron) at various concentrations. The cells contain the biomediator immobilized on agar–TAP medium deposited on a silicon layer. The silicon septum is of the proper size to fit the cell. The so-prepared supports are assembled into the 24 measurement cells of the instrument, in the absence and in the presence of different concentrations of herbicides. Chlorophyll fluorescence is measured on *C. reinhardtii* wild-type and mutant strains after 10 min of incubation with herbicides. Prior to each measurement, samples are kept in the dark for 10 min to reach the steady dark adapted state of PSII. The PSII efficiency dose–response curves are determined here for the three herbicides atrazine, prometryn, and diuron. The relative variable fluorescence V_J obtained for wild-type and for the mutant strains in the absence and in the presence of herbicides were plotted; then, I_{30} and LODs were calculated (Fig. 4).

As shown in Fig. 5, each strain exhibits a different PSII activity inhibition in the presence of herbicides showing different sensitivity or resistance to various herbicide concentrations. A250L, A250R, A251C, and I163N mutants are highly sensitive to classes of triazinic and phenylurea compounds. In particular, the mutant strain I163N shows the highest sensitivity in the presence of 10^{-7} M atrazine,

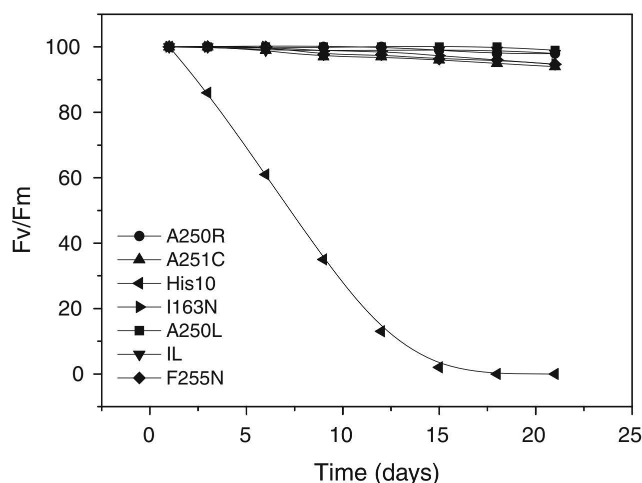

Fig. 6 Stability curves plotted as F_v/F_M against time, where F_v/F_M indicates the photochemical efficiency of PSII, for *C. reinhardtii* genetic variants immobilized on agar–TAP medium on a silicon septum. The mutant strain His10, in which ten histidines are inserted in various positions of D1 protein, is plotted to show a behavior of a typical mutant with low stability during time that is discarded as biomediator

while A250R in the presence of 5.0×10^{-8} M prometryn, and A250L and A250R mutants in the presence of 10^{-7} M prometryn. On exposure to diuron, most mutant strains feature a high sensitivity, with only F255N mutant showing a high resistance to both diuron and atrazine. It follows that a response in the inhibition of fluorescence is associated to a particular herbicide subclass as suggested by Giardi et al. [18].

The results of the LOD calculations, according to the model mentioned above, are shown in Table 2. These results show that the mutant strains exhibit LOD values of 10^{-10} M, enhanced by one to two orders with respect to IL wild type. Moreover, their variable sensitivity allows us to discriminate among different classes and subclasses of herbicides. All engineered mutants exhibit a relatively long activity lifetime, as observed from the stability curves in Fig. 6, preserving 100% of stability for about 1 month after immobilization. For this biosensoristic purpose, only the most stable mutants have been considered among all produced ones (Fig. 6).

Conclusions

Herbicides are the second most important class of pesticides used in the European Union. A major difficulty in estimating environmental quality related to herbicides contamination is due to seasonal change of field application and the extremely low levels of the maximum admissible concentrations set by the European policy [19].

These premises induced us to develop new alga mutants able to detect herbicides and herbicide subclasses. Progress in chloroplast engineering and in crystal structure has substantially increased the possibility of manipulating and of gaining insight regarding structure–function relationships in PSII. Here, the unicellular microalga *C. reinhardtii* is exploited for the production of fluorescence probes to be implemented in the construction of a multibiomediator algal sensor system [20–22]. By the use of the proposed biosensor, only environmental samples giving positive results would require more detailed laboratory analyses; thus, the overall cost and the time required for each sample could be significantly reduced by this multiarray pre-screening technique. Since the engineered biomediators show different sensitivity/resistance, selectivity, and low detection limits, they can be considered good candidate for the development of a biosensor for the detection of complex environmental samples containing multiple pollutant species; a mixture of ten or more triazines, urea, and phenolic compounds are generally present in water river, as shown by Touloupakis et al. [23]. In conclusion, an appropriate selection of the mutants with the highest sensitivity and lowest limit of detection according to the herbicide to be detected, possibly joined with a data analysis automation program, can ensure a fine herbicide monitoring, when coupled to the proper fluorimeter sensor like the OPTIC-BIO-Multicell used by the authors.

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