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Review

Herbicide resistance and supersensitivity in photosystem II

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Dedicated to Prof. Dr. Dr. hc mult. Achim Trebst on the occasion of his 70th birthday

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Abstract. Resistance to triazine herbicides in higher plants was first observed in 1970. A mutation in the photosystem II reaction center D1 protein at position $Ser_{264} \rightarrow Gly$ is responsible for this resistance. So far, 37 single mutants, 16 double mutants, 5 triple mutants and 5 deletion/insertion mutants in the D1 protein have been obtained by randomly induced and site-directed mutagenesis in cyanobacteria and algae. The influence of these mutations on the binding affinities of different classes of herbicides will be discussed. Because a sufficiently high resolution X-ray structure of photosystem II does not yet exist, the reaction center of purple photosynthetic bacteria, which is homologeous to photosystem II, served as a model. In the bacterial reaction center a total of 25 single and 3 double herbicide-resistant mutants have been generated.

Key words. L-subunit; D1 protein; Q_B-site; herbicide binding affinities; molecular modeling.

Introduction

Modern agriculture cannot exist without the use of herbicides for the efficient control of weeds. Herbicides improve the amount and quality of crops. Herbicides can be directed against a variety of targets, like biosynthesis of amino acids, chlorophylls, carotenoids, and plastoquinone. Furthermore, tubulin formation, mitosis and photosynthesis can be inhibited (for reviews, see [1, 2]). Plants have developed resistance against all major groups of herbicides. This review will restrict itself exclusively to herbicides which target photosystem II.

Herbicides that target photosystem II inhibit at the reducing side of photosystem II, where they compete with the native plastoquinone at the Q_B-site. They have a higher affinity for the Q_B-site than plastoquinone itself. Consequently, linear electron transport to the cytochrome b_6/f-complex is interrupted, and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), which is essential for the reduction of carbon dioxide, cannot be synthesized. However, herbicides do not block cyclic electron transport around photosystem II. Herbicides targeting photosystem II can belong to a variety of chemical classes such as triazines, triazinones, ureas, biscarbamates, dinitrophenols and cyanophenols, to name only a few (for review, see [3]).

Resistance against xenobiotics is a widespread phenomenon in nature. Especially bacteria with their fast duplication times are very apt to become resistant. In 1941, when penicillin was available for the first time, virtually all strains of *Staphylococcus aureus* were susceptible to this antibiotic. However, only three years later the first resistant strains were observed, which could inactivate penicillin by β-lactamase. Today, more than 95% of all *S. aureus* strains are resistant to penicillin-type antibiotics [4].

It took plants a longer time than bacteria to develop resistance against photosystem II herbicides. *s*-Triazine herbicides, such as atrazine, simazine and terbutryn,

were introduced into the market in 1955. The first report on a weed resistant to atrazine and simazine appeared in 1970. Common groundsel (*Senecio vulgaris* L.) in a nursery was found to be resistant against simazine and atrazine in doses up to 6.72 kg/ha [5]. It was soon recognized that herbicide resistance in the weed species was passed down maternally and attributed to a change in the properties of photosystem II. However, the molecular basis of resistance, an amino acid change in the sequence of one of the reaction center proteins of photosystem II (see below), was not discovered until 1983 [6].

The term 'resistance' (also sometimes called tolerance) indicates the fact that, in order to impair the growth of a herbicide-resistant plant, a higher dose of herbicide relative to the susceptible wild type is required. Resistance means that the I_{50}-value (the I_{50}-value indicates the concentration necessary to inhibit the rate of electron transport by 50%) for inhibition of photosynthetic electron transport is higher in chloroplasts isolated from mutant plants as compared with the susceptible wild type. In common use is also the pI_{50}-value, which denotes the negative decadic logarithm of the I_{50}-value. In this context, the 'R/S-value' is of importance because it is a quantitative measure of the degree of resistance. The R/S-value is the ratio of the I_{50}-value in electron transport in chloroplasts isolated from resistant versus susceptible plants. In case of resistance, the R/S-value is always > 1.

Resistance against all types of photosystem II herbicides is never observed. It will be restricted to certain classes, like *s*-triazines, as mentioned above. The triazine-resistant weeds may not be resistant, for instance, against ureas. On the other hand, triazine-resistant weeds may be even more sensitive to other types of herbicides. This 'supersensitivity' or 'negative cross-resistance' has indeed been observed for phenolic herbicides in triazine-resistant weeds. It should be noted that, in this case, the R/S-value is < 1.

Herbicide-resistant or -supersensitive organisms are worthy of interest for two reasons:

(1) Herbicide-resistant crops will play an increasing role in agriculture because they permit weeds to be much more efficiently controlled. In Canada, for example, atrazine-resistant oilseed rape is used as a crop and was obtained by a sexual cross from the atrazine-resistant weed *Brassica campestris*. Although this cultivar carries a 20% yield penalty due to resistant cytoplasm, it has proven suitable in areas where weed densities are high and triazines are used (for reviews, see [7, 8]).

(2) They can be used to identify amino acids in the target which participate in herbicide binding, because the X-ray structure of photosystem II at high resolution is not yet available. The only other suitable method for the identification of herbicide-binding amino acids in the D-1 protein is photoaffinity labeling with radioactive, photolabile herbicide analogues and subsequent sequencing of the radioactive proteolytic fragments [3]. In this review article I will concentrate on item (2) though I will also briefly address item (1). I do not intend to list all the herbicide-resistant weeds that have emerged so far. The reason for that is, although weeds are resistant against certain types of herbicides, they can still be controlled by other types. Nevertheless, resistant weeds have begun to become an economic threat.

A great deal of our knowledge about the structure and function of photosystem II and the mode of action of herbicides therein is based on the X-ray structures of the reaction centers of purple photosynthetic bacteria. The high homology between the reaction center of purple bacteria and photosystem II was soon recognized as the first X-ray structure became available [9–11]. The structure of the purple bacterial photosynthetic reaction center has served as a model for all current ideas about the architecture of photosystem II. Thus, in this review the reaction centers of purple bacteria will be considered first.

The photosynthetic reaction center of purple bacteria

The photosynthetic reaction center of purple bacteria consists of three different subunits, the L-, M- and H-subunits. The L- and M-subunits cross the membrane in five transmembrane α-helical spans each, whereas the H-subunit has only one transmembrane span. The L- and M-subunits carry the functional components, which are four bacteriochlorophylls, two bacteriopheophytins, two quinones, one carotenoid and an iron atom. The quinones form the primary and secondary quinone electron acceptors Q_A and Q_B, respectively. Q_A is a menaquinone, a naphthoquinone in *Rhodopseudomonas viridis* and a ubiquinone in *Rhodobacter sphaeroides*. Q_B is a ubiquinone in both organisms. Photosynthetic electron transport occurs from an excited bacteriochlorophyll dimer via another bacteriochlorophyll on the active arm to bacteriopheophytin, Q_A, and over the iron atom to Q_B. During this process, Q_A is always single-reduced, whereas Q_B can be double-reduced. When Q_B^{2-} gets protonated, ubichinol leaves its binding site to be replaced by a new ubiquinone molecule from the endogeneous pool. The Q_A-site is located at the M-subunit, whereas Q_B is located at the L-subunit. The Q_B binding site at the L-subunit is localized between transmembrane helices D and E and a parallel helix DE oriented towards the cytoplasmatic side of the membrane [12–14].

Of the keto oxygens of the ubiquinone in the Q_B-site, one is hydrogen-bonded to His L_{190} and the other, in a

bifurcated way, to the side-chain oxygen of Ser L_{223} and to the peptide amino group of Gly L_{225}. In addition, Phe L_{216} forms an important part of the quinone binding site, and the aromatic moiety of the phenyl ring stacks directly onto the ubiquinone. This interaction suggests a contribution to the binding affinity of ubiquinone [15, 16]. Upon reduction, the ubisemiquinone moves 4.5 Å towards the cytoplasm with an accompanying 180° propeller twist around the isoprene tail [16].

Two pathways, denoted P1 and P2, constitute the proton pathways for hydrogenation of Q_B^{2-}. Pathway P1 proceeds about 23 Å from the Q_B-site via Glu L_{212} through the H-subunit to the cytoplasm. The second pathway P2 leads from Ser L_{223} to Asp L_{213} via the interface between the H- and M-subunits, parallel to the membrane surface at approximately the depth of the iron atom [16].

Certain inhibitors can compete with the binding of the native ubiquinone at the Q_B-site. They have a higher binding affinity than ubiquinone itself. Consequently, electron transport only proceeds to the state Q_A^- and is inhibited at the Q_B-site. These inhibitors also act at photosystem II, but their activity in the photosynthetic reaction centers of purple bacteria is much less pronounced. By soaking the crystals of the reaction center of *Rhodopseudomonas viridis* in solutions of the inhibitor, X-ray data for *o*-phenanthroline, terbutryn and stigmatellin in the binding niche have been obtained.

o-Phenanthroline binds close to the non-heme iron. The two nitrogen atoms of *o*-phenanthroline form a shared hydrogen bridge with the imidazole nitrogen of His L_{190}. In addition, *o*-phenanthroline is in close contact with Ile L_{229} and Leu L_{193}. In contrast, the binding site of the *s*-triazine herbicide terbutryn is further away from the non-heme iron atom. A hydrogen bond can be formed between the peptide nitrogen of Ile L_{224} and N-3 of the triazine ring system. Furthermore, a second hydrogen bridge between the ethylamino nitrogen of terbutryn and the hydroxyl group of Ser L_{223} can be observed. Further contacts are formed with Val L_{220}, Ile L_{229} and Phe $L_{216.}$ Glu L_{212} is also involved in terbutryn binding [17]. For stigmatellin, all the hydrogen bonds in ubiquinone binding are also involved in stigmatellin binding. Additional hydrogen bonds are accepted by the proximal methoxy group of stigmatellin and donated by its hydroxyl group, respectively, making the binding of stigmatellin more stable than that of ubiquinone [18].

A total of 25 single and 3 double herbicide-resistant mutants in the bacterial reaction center have been generated either by randomly induced or site-directed mutagenesis (table 1). It should be noted that only photosynthetically active mutants have been isolated. All the mutations lie within the L-subunit, with two exceptions: in the mutant Phe $L_{216} \rightarrow$ Ser an additional mutation in the M-subunit, Val $M_{263} \rightarrow$ Phe occurred. Furthermore, the mutation in the M-subunit Glu $M_{234} \rightarrow$ Lys confers resistance towards terbutryn, *o*-phenantroline and NH-thiazoles, without provoking any amino acid change in the L-subunit.

The region in the reaction centers of purple photosynthetic bacteria where resistance has been observed ranges from Gly L_{192} to Val L_{229} or Ile L_{229} and comprises a total of 38 amino acids. A picture of the herbicide binding niche of the photosynthetic bacterium *R. viridis* is shown in figure 1A and B. Figure 2 compares the sequences of the L-subunits of five different photosynthetic bacteria together with part of the sequence of the D1 protein from spinach. Amino acids where mutations have been observed are printed in italics.

The replacement of Gly L_{192} to Asp leads to resistance against triazines and *o*-phenanthroline (table 1; [19]). A change of the acidic Glu L_{212} to the basic Lys disrupts a hydrogen bond to a nitrogen of the triazine moiety, which leads to a loss of binding affinity (table 1; [20]). The participation of Phe L_{216} in ubiquinone binding has already been stressed above. Phe L_{216} has been mutated in a series of photosynthetic organisms to Leu, Pro, Thr, Val and Ser (table 1). All these mutations showed resistance towards triazine herbicides, whereas the sensitivity towards diuron was not affected (table 1; [21]). The most dramatic effects appear when Phe L_{216} is exchanged against Ser. This results in a tremendous increase in terbutryn and *o*-phenanthroline resistance (table 1; [22]). Phe L_{216} forms a major part of the terbutryn binding site by interaction with both π-electron systems [30], and its replacement by Ser dramatically decreases terbutryn binding and, to a lesser extent, also that of *o*-phenanthroline.

Tyr L_{222} has not been recognized yet as part of the herbicide binding site of the reaction center of purple photosynthetic bacteria. However, a change of Tyr L_{222} to either Phe or Gly dramatically changes the R/S-ratios (table 1). The Phe mutant is highly resistent against triazine herbicides such as ametryn, atrazine, desmetryn and terbutryn and, in addition, also to *o*-phenanthroline [22, 23]. In the Gly mutant only terbutryn and *o*-phenanthroline have been tested; they also exhibit high resistance against these chemicals [24]. Another feature of the Phe mutant is noteworthy of special attention. If Q_A or Q_B are single-reduced, semiquinones are formed which are paramagnetic species. They can be observed by electron paramagnetic resonance (EPR). In the Phe mutant, the $Q_B^- Fe^{2+}$ EPR signal is completely different from the signal of the wild type and any other photosynthetic bacteria and mutants. It has a peak at a g-value of 1.93 and in this respect is very similar to photosystem II. Consequently, the Phe mutant is sensi-

Table 1. Herbicide-resistant mutants of photosynthetic purple bacteria.

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
Single mutants					
$Gly_{192} \rightarrow Asp$	*Rubrivivax gelatinonosus*		Atrazine 6 *o*-Phenanthroline 3 Terbutryn 300		[19]
$Glu_{212} \rightarrow Lys$	*R. viridis*	MAV2	Atrazine		[20]
$Phe_{216} \rightarrow Leu$	*R. capsulatus*		Ametryn 3.1 Atrazine 4.2 Diuron 1 Prometon 1.8 Prometryn 1.4		[21]
$Phe_{216} \rightarrow Pro$	*R. capsulatus*		Ametryn 1.9 Atrazine 1.9 Diuron 1 Prometon 1.8		[21]
$Phe_{216} \rightarrow Thr$	*R. capsulatus*		Atrazine 3.5 Diuron 1		[21]
$Phe_{216} \rightarrow Val$	*R. capsulatus*		Atrazine 1.8 Diuron 1		[21]
$Phe_{216} \rightarrow Ser$	*R. viridis*	T6	*o*-Phenanthroline 333 Terbutryn >2800		[22]
$Phe_{216} \rightarrow Ser$	*R. viridis*	MAV3	Atrazine		[20]
$Tyr_{222} \rightarrow Phe$	*R. viridis*	T4	Ametryn 330 Atrazine >600 Desmetryn 50 *o*-Phenanthroline 10 Terbutryn 660 (>5000)	Diuron <0.001 Ioxynil <0.01	[22, 23]
$Tyr_{222} \rightarrow Gly$	*R. sphaeroides*	YG222	*o*-Phenanthroline 26 Terbutryn >3000		[24]
$Ser_{223} \rightarrow Pro$	*R. sphaeroides*	SP223	*o*-Phenanthroline 6.25 Terbutryn >3000		[24]
$Thr_{226} \rightarrow Ala$	*R. capsulatus*		Ametryn 4 Atrazin 3.1 Diuron 1 Prometon 1.8 Prometryn 1.4 Terbutryn 1.7	*o*-Phenanthroline 0.03 Terbutryn 0.6 (?)	[21, 25]
$Thr_{226} \rightarrow Met$	*R. capsulatus*		Ametryn 2.5 Atrazine 3.1 Diuron 1 Prometryn 1.1 Terbutryn 1.7		[21]
$Gly_{228} \rightarrow Arg$	*R. capsulatus*		Ametryn 4 Atrazine 5.4 Diuron 1 Prometon 1.2 Prometryn 1.1 Terbutryn 1.3		[21]
$Gly_{228} \rightarrow Val$	*R. capsulatus*		Ametryn 5.3 Atrazine 4.2 Diuron 1 Prometon 1.3 Prometryn 1.4 Terbutryn 1.5		[21]
$Ile_{229} \rightarrow Ala$	*R. capsulatus*		Atrazine 1.8 Diuron 1 Prometon 1.2		[21, 26]
$Ile_{229} \rightarrow Cys$	*R. capsulatus*		Atrazine 2 Diuron 1		[21, 26]
$Ile_{229} \rightarrow Leu$	*R. capsulatus*		Ametryn 2.5 Atrazine 3.5 Diuron 1		[21, 26]
$Ile_{229} \rightarrow Met$	*R. capsulatus*		Ametryn 5.3 Atrazine 5.4 Diuron 1 Prometon 1.2 Prometryn 2 Terbutryn 1.9		[21, 26]
$Ile_{229} \rightarrow Met$	*R. sphaeroides*		Atrazine >7		[27]
$Ile_{229} \rightarrow Met$	*R. sphaeroides*	IM229	*o*-Phenanthroline 11 Terbutryn 120		[24]

Table 1. (Continued).

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
$Ile_{229} \rightarrow Ser$	*R. capsulatus*		Atrazine 1.8		[21, 26]
$Ile_{229} \rightarrow Ser$	*R. capsulatus*		*o*-Phenanthroline 1.3	Terbutryn 0.4	[25]
$Ile_{229} \rightarrow Thr$	*R. capsulatus*		Ametryn 1.9 Atrazine 3.7 Prometon 1.2 Prometryn 1.4		[21, 26]
$GluM_{234} \rightarrow Lys$	*R. rubrum*	M6	*o*-Phenanthroline >25 Terbutryn >125 NH-Thiazoles (see text)		[28]
Double mutants					
$Phe_{216} \rightarrow Ser$ $ValM_{263} \rightarrow Phe$	*R. viridis*	T3	*o*-Phenanthroline 67 Terbutryn 166		[22]
$Arg_{217} \rightarrow His$ $Val_{220} \rightarrow Leu$	*R. viridis*	MAV4, MAV5	Atrazine		[20]
$Arg_{217} \rightarrow His$ $Ser_{223} \rightarrow Ala$	*R. viridis*		*o*-Phenanthroline 5 Terbutryn 222		[29]

tive towards diuron, a powerful photosystem II inhibitor which is inactive or only moderately active in most reaction center preparations from photosynthetic bacteria. Diuron in the Phe mutant is four orders of magnitude more active than in the wild type. Interestingly, supersensitivity also occurs against the phenolic herbicide ioxynil, this time about two orders of magnitude (table 1; [23]). Interestingly, Tyr L_{222} is about 7 Å away from the ubiquinone binding site and would not be a likely candidate for selection of site-directed mutagenesis. The hydroxyl group of Tyr L_{222} is hydrogen-bonded to Asp M_{43}. This interaction is important to the structure of the Q_B binding site, and hence the mutation of Tyr L_{222} to Phe leads to a structural rearrangement. This structural change leads to the impairment of terbutryn and *o*-phenanthroline binding but facilitates the interaction with diuron [22].

As already stressed, the hydroxyl group of Ser L_{223} forms a hydrogen bond to the ethylamino group of terbutryn. If Ser L_{223} is replaced by Pro, this hydroxy group is lost, and consequently resistance towards terbutryn is observed. In addition, resistance towards *o*-phenanthroline is also achieved (table 1; [24]). It should be noted that Ser L_{223} is conserved in the D1 protein of photosystem II (Ser_{264}), and its mutation leads to severe herbicide resistance there (see below).

Thr L_{226} by site-directed mutagenesis has been changed by Bylina's group [21, 25] to Ala and Met. Resistance against triazine herbicides is observed in both mutants (table 1), whereas the R/S-value of diuron remains 1. There exist conflicting data on terbutryn, which is resistant in one publication (R/S = 1.7; [21]) but appears to be supersensitive in another (R/S = 0.6; [25]). Noteworthy in the Ala mutant is its supersensitivity towards *o*-phenanthroline. Increased resistance against triazines and no change for diuron have also been observed for the mutation Gly L_{228} against both Arg and Val (table 1; [21]).

In the reaction centers of photosynthetic bacteria, Ile L_{229} is of special interest because it participates in the binding of both terbutryn and *o*-phenanthroline. Therefore, if this amino acid is exchanged, resistance and/or supersensitivity has to be expected. Ile L_{229} was mutated in *R. capsulatus* and *R. sphaeroides* to Ala, Cys, Leu, Met, Ser and Thr (table 1). Resistance against triazine herbicides was noted in all mutant strains with the exception of the *R. capsulatus* strain $Ile\ L_{229} \rightarrow Ser$, where terbutryn supersensitivity occurred. In all mutant strains so far tested, diuron sensitivity was unchanged compared with the wild type. In the *R. sphaeroides* $Ile\ L_{229} \rightarrow Met$ and the *R. capsulatus* $Ile\ L_{229} \rightarrow Ser$ strains resistance against *o*-phenanthroline was also observed.

The only case so far where an amino acid exchange in the M-subunit has caused herbicide resistance was in the *R. rubrum* mutant $Glu\ M_{234} \rightarrow Lys$ (table 1; [28]). This mutant was rendered resistant against 2-(1-phenyl)ethylamino-3-propionylamino-4-cyanothiazol, which belongs to a new class of thiazol inhibitors which are active in both purple bacterial photosynthetic reaction centers and photosystem II [31]. The Glu M_{234} mutant is resistant against other thiazols, but also against terbutryn and *o*-phenanthroline (table 1). It exhibits $Q_A^- Fe^{2+}$ and $Q_B^- Fe^{2+}$ EPR signals, which are completely different from wild-type EPR signals, indicating that the Q_A and Q_B environments are significantly changed as compared with the wild type. Glu M_{234} is located close to Glu M_{232}, which has been recognized as a bidentate ligand to the Fe atom. Change of the acidic Glu M_{234} to the basic Lys brings about a perturbation of the Q_A and Q_B environments, which results in a loss of herbicide binding affinity [28].

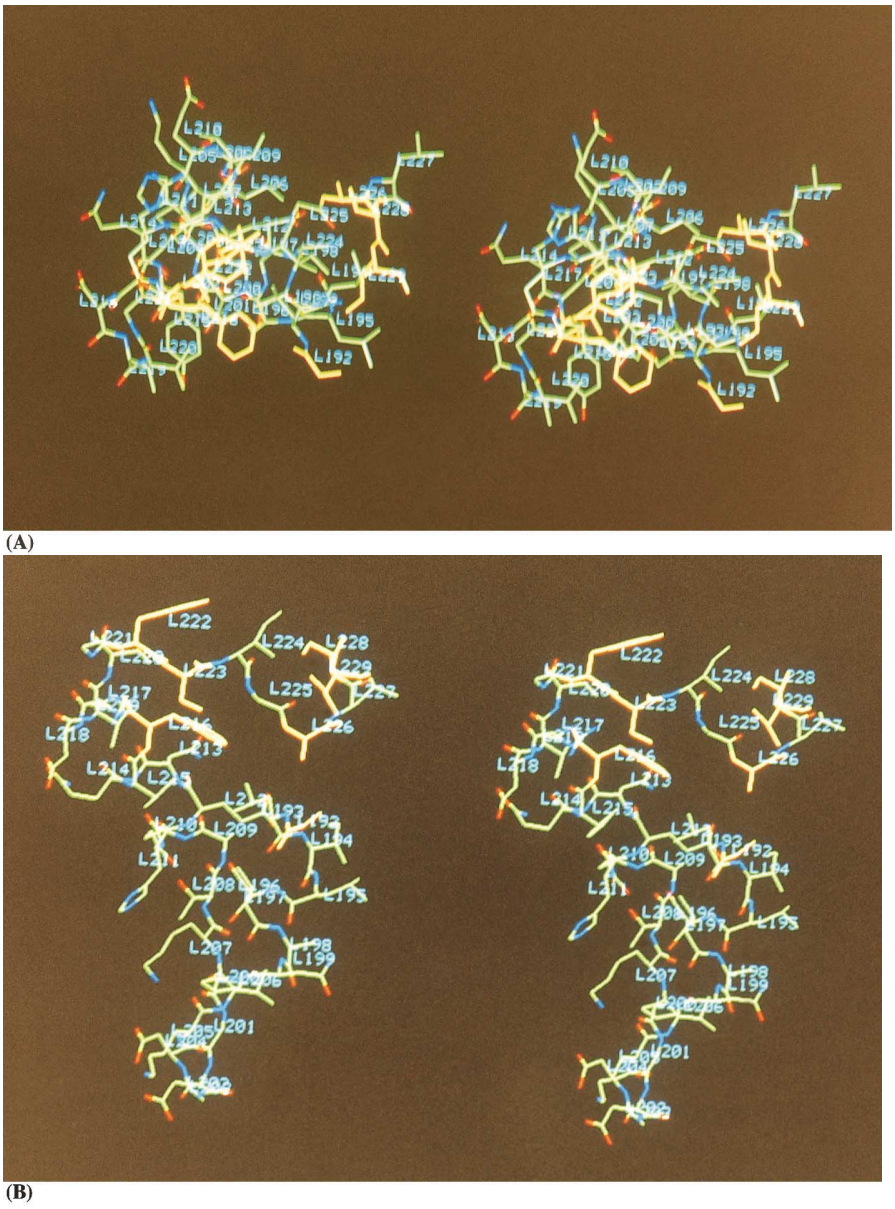

Figure 1A and B. Stereo view of the herbicide binding niche of the L-subunit of the photosynthetic reaction center from *R. viridis* from different view points [12]. The herbicide binding region reaches from Gly L_{192} to Ile L_{229}. Amino acids where mutations have been found are coloured in yellow. The model was constructed using Insight II, V. 95.0 (Biosym/MSI).

So far, three double mutants of *R. viridis* are known. The first one has one mutation in the L-subunit (Phe $L_{216} \rightarrow$ Ser) and another mutation in the M-subunit (Val $M_{263} \rightarrow$ Phe) (table 1). The Phe $L_{216} \rightarrow$ Ser mutation is already known as a single mutation, and has been shown to be resistant against terbutryn and *o*-phenanthroline. The same is true for the double mutant, though the resistance is less pronounced. The reason for the mutation Val $M_{263} \rightarrow$ Phe is unknown. Val M_{263} is located in the connecting loop between transmembrane helices IV and V and is part of the Q_A binding site. The next double mutant has mutations at positions Arg $L_{217} \rightarrow$ His and Val $L_{220} \rightarrow$ Leu and is resistant against atrazine. These mutations have not been observed yet in single mutants. The final double mutant has the same Arg $L_{217} \rightarrow$ His replacement, but in addition Ser $L_{223} \rightarrow$

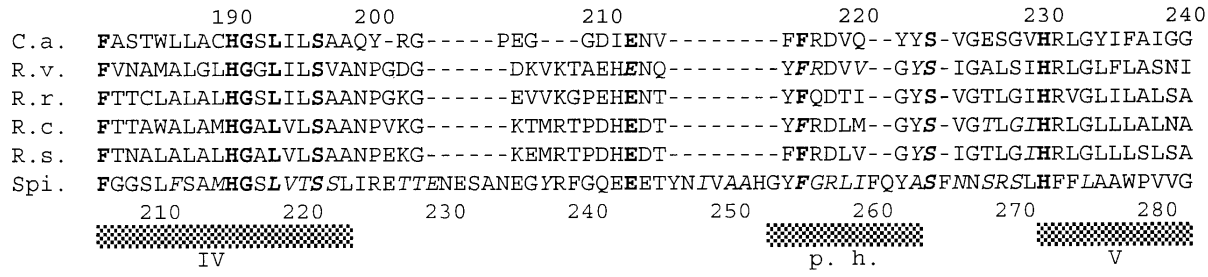

Figure 2. Sequence alignment of parts of L subunits from *Chloroflexus aurantiacus* (C. a.), *Rhodopseudomonas viridis* (R. v.), *Rhodospirillum rubrum* (R. r.), *Rhodobacter capsulatus* (R. c.) and *Rhodobacter sphaeroides* (R. s.) and the photosystem II D1 protein from spinach (Spi.). Amino acid residues conserved through all sequences are indicated in boldface. Amino acid residues where mutations have been found are indicated in italics (according to [3]; new mutations have been included).

Ala. Again, the Ser L_{223} mutation is already known as a single mutation (here Ser $L_{223} \rightarrow$ Pro) (table 1). Since Ser L_{223} is involved in terbutryn binding, it is not surprising that both the single and the double mutant are resistant against triazine herbicides (table 1).

In conclusion, in addition to the amino acids involved in either terbutryn (Phe L_{216}, Val L_{220}, Ser L_{223}, Ile L_{229} and Ile L_{244}) or *o*-phenanthroline binding (His L_{190}, Leu L_{193} and Ile L_{229}) as realized from the X-ray data, the following five amino acids are involved in herbicide binding in the reaction centers of photosynthetic bacteria: Gly_{192}, Glu L_{212}, Tyr L_{222}, Thr L_{226} and Gly L_{228}.

The reaction center of photosystem II

Our knowledge of the photosystem II reaction is much less advanced when compared with the photosynthetic reaction center of purple bacteria. Though two-dimensional [32] and three-dimensional crystals [33] are available, the resolution is yet too low to see details in the amino acids, though helices and pigments are well resolved. Thus, the current model of photosystem II is based on the X-ray structure of the reaction center of purple photosynthetic bacteria.

The homology between the photosynthetic reaction center of purple bacteria and the acceptor side of photosystem II was soon recognized [34]. Trebst has presented a folding model of the photosystem II reaction center proteins D1 and D2 [9–11]. D1 is homologous to the bacterial L-subunit, whereas D2 is homologous to the M-subunit. D1 and D2 are coded by the chloroplast genome, by genes *psb*A and *psb*D, respectively. D1 and D2 on an SDS-polyacrylamide gel electrophoresis (PAGE) gel show approximate molecular weights of about 32 kDa; their names are attributed to their 'diffuse' staining on the gel by Coomassie Blue. D1 and D2 bind the pigments and the primary and secondary quinone acceptors, Q_A and Q_B, respectively, which are both plastoquinones. There are six chlorophyll and two pheophytin molecules per photosystem II reaction center. The primary quinone acceptor Q_A is located at D2, whereas the secondary quinone acceptor Q_B is associated with D1. As in the reaction center of purple bacteria, both quinones are connected via a non-heme iron atom. The nature of the primary electron donor P_{680} is still a matter of debate; it may be either a chlorophyll monomer or dimer. The center-to-center distance between P_{680} and Q_A is about 27 Å [35]. The electron is transported via pheophytin to Q_A and ultimately to Q_B. The head group of plastoquinone bound to the Q_B-site in photosystem II requires a structural reorientation for its reduction to the semiquinone [36]. The double-reduced Q_B^{2-} gets protonated and leaves the binding pocket. It should be noted that the donor side of photosystem II bears no resemblance at all to the reaction center of purple photosynthetic bacteria. In addition, the intrinsic proteins cytochrome b_{559} and the light-harvesting proteins CP43 and CP47 are unique and indispensable constituents of a functionally intact photosystem II (for reviews on photosystem II, see [37–41]).

When its identity as a photosystem II reaction center protein was not known yet, the D1 protein was also called the 'herbicide binding protein' because it is radioactively labeled by either radioactive azido-atrazine [42–48] or azido-triazinone [49]. However, it was early recognized that quinones and herbicides bind in a different way within the Q_B binding site [50, 51].

Until 1997, sequences of the D1 protein were known from a total of 41 species [52]. Figure 2 depicts a sequence comparison between the herbicide binding region of the L-subunit of four different photosynthetic bacteria (amino acids 181–240) and the D1 protein of photosystem II from spinach (amino acids 206–282).

This region includes parts of the helices IV and V and the parallel helix, as indicated. As compared with the bacterial L-subunit, the D1 protein contains 17 additional amino acids. This immediately creates the problem of how to incorporate these additional amino acids into a model, a problem which will be discussed later. The shorter loop between helices IV and V in the bacterial L-subunit as compared with the larger loop in the D1 protein may explain the lack of inhibition by certain inhibitors in the bacterial system [53]. Trebst has presented a folding model of this 'extra loop' which brings Tyr_{237} and Arg_{238} of D1 in contact with Arg_{234} of D2. By this contact an inhibitor present in the Q_B-site of D1 also influences the Q_A-binding protein D2 [54].
There is homology at 9 positions out of 77 in the sequence alignment of the fragments in figure 2. In particular, His_{215} (D1) and His_{272} (D1), which serve as ligands to the iron atom, are conserved. Furthermore, His_{215} (D1) is hydrogen-bonded to Ser_{264} (D1), which is also conserved. Another conserved ligand to Q_B is Glu_{243} (D1) [30]. Ser_{264} and its counterpart in the L-subunit, Ser L_{223}, are important for herbicide resistance, as will be discussed below. Also conserved is the amino acid Phe_{255} (D1). In the equivalent position in the L-subunit, the aromatic moiety of Phe L_{216} interacts with terbutryn. Finally, conservation of Gly_{191} (D1) allows for close contact of helices IV of D1 and V of D2, which would be prevented by the presence of a side chain [30].

Single mutations

A list of herbicide-resistant mutants which have a mutation in the D1 protein is given in table 2. In addition, in figure 2 all known mutated amino acids are indicated in italics. All mutations are in a region ranging from Phe_{211} to Leu_{275}. Thus, the herbicide binding site consists of at least 65 amino acids. It includes part of transmembrane helix IV, the stromal parallel helix and part of transmembrane helix V.
The most important mutation and the only one which occurs in higher plants in vivo is the mutation of Ser_{264} to Gly. When the *psb*A gene of *Amaranthus hybridus*, the first triazine-resistant plant, reported in 1970 [5], was sequenced in 1983, three nucleotide differences were found when compared with the wild type. From these three differences, two are silent, that is they do not result in amino acid changes, whereas the third leads to a change from AGT to GGT, which replaces Ser by Gly. It should be noted that this change was located at position 228 in the original paper because the authors erroneously assumed that the transcription of the D1 protein commenced at Met_{37} [6]. It has been established without doubt, however, that the transcription begins at a Met which is 37 positions before that assumed by Hirschberg and McIntosh [109]. In the following years, the $Ser_{264} \rightarrow$ Gly mutation has been found in a variety of other triazine-resistant weeds, such as *Amaranthus retroflexus*, *Amaranthus bouchonii*, *Brassica campestris*, *Solanum nigrum*, *Senecio vulgaris*, *Chenopodium album*, *Poa annua* and *Phalaris paradoxa* (table 2), to cite only a few. To date, triazine resistance due to the $Ser_{264} \rightarrow$ Gly change has been confirmed in over 20 countries in biotypes of more than 58 species [1]. For more details, the interested reader should refer to the book *Herbicide Resistance in Plants* [110].
The loss of the hydroxymethyl group in Ser_{264} upon changing to glycine leads to dramatic effects in triazine binding. The hydrogen bond between the alkylamino group of triazines and the hydroxymethyl group of the original serine is lost, leading to the much-decreased binding affinity of triazines. In *A. hybridus* this is established for ametryn, atrazine and atraton (table 2). It should be noted that if an interval in the R/S-values is given in table 2, the numbers come from different groups. They have used different test systems, and that may account for the differences in R/S-values. *A. hybridus* is also resistant against phenmedipham, whereas the binding of urea herbicides, with the exception of lenacil, is not affected Slight supersensitivity can be noted for phenolic herbicides and bentazon (table 2). The same pattern of R/S-values is observed for *A. retroflexus*; in addition, triazinone resistance has been reported here (table 2). Astonishingly, diuron resistance is found in *A. bouchonii*. Similar to the *Amaranthus* species, triazine resistance with little change in diuron sensitivity is also observed in *B. campestris*, *C. album*, *P. annua*, *Senecio vulgaris* and *Solanum nigrum*. It should be noted that *C. album* is highly resistant against fenuron and that *P. annua* is also resistant against various ureas (table 2). The amino acid change from $Ser_{264} \rightarrow$ Gly has also been engineered into the cyanobacterium *Synechococcus* PCC7942 by site-directed mutagenesis. As in higher plants, the mutant is highly resistant against triazines and metribuzin, whereas ureas are little affected. Again, the phenolic herbicide ioxynil exhibits supersensitivity.
The exchange of Ser_{264} in the D1 protein is not restricted to Gly. Other amino acid changes include Ala, Asn, Pro and Thr (table 2). The $Ser_{264} \rightarrow$ Asn mutation was found in *Nicotiana plumbaginifolia* and renders this mutant insensitive towards terbutryn. The $Ser_{264} \rightarrow$ Thr mutation occurred in cell cultures of *N. tabacum* and *Solanum tuberosum* as well and confers resistance towards triazines, metribuzin, some ureas and supersensitivity towards dinoseb (table 2). The same behaviour is found in the $Ser_{264} \rightarrow$ Thr *Euglena* mutant. Replacing $Ser_{264} \rightarrow$ Pro has basically the same effect, but diuron sensitivity is not affected.

Table 2. Mutations in the photosystem II reaction center D1 protein conferring herbicide resistance and/or supersensitivity.

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
Single mutants					
$Phe_{211} \rightarrow Ser$	*Synechococcus* sp. PCC7002		Atrazine 7 Diuron 2		[55]
	Synechocystis PCC6714	AzI	Atrazine 10 Metribuzin 8		[56, 57]
$Val_{219} \rightarrow Ile$	*Chlamydomonas*	Dr2	Atrazine 2 Benzthiazuron 16 BNT 2 Bromanil 1.2–2 Cyanoacrylate 2 Dinoseb 7.5 Diuron 15–32 Ioxynil 50 Metabenzthiazuron 62 Metamitron 1.2 Metribuzin 200 Triazinones (see text)	Ketonitrile 0.6 Lenacil 0.8 Triazinones (see text)	[53, 58–62]
	Synechococcus sp. PCC7002		Atrazine 2 Diuron 10		[55]
$Tyr_{237} \rightarrow Phe$	*Synechocystis* sp. PCC6803		Atrazine 2 Diuron 5 Ioxynil 5	BNT 0.2	[63]
$Lys^{*}_{238} \rightarrow Val$	*Synechocystis* sp. PCC6803		Diuron 1.3 Ioxynil 2.3	Atrazine 0.6 BNT 0.4	[63]
$Ile_{248} \rightarrow Thr$	*Synechocystis* PCC6714	M30	Diuron 2 Metribuzin 28		[64]
$Ala_{250} \rightarrow Arg$	*Chlamydomonas*		Metamitron 2.5 Phenmedipham 6.3 Terbutryn 1.3	Atrazin 0.25 Bromoxynil 0.3 Ioxynil 0.5 Metribuzin 0.5	†
$Ala_{250} \rightarrow Asn$	*Chlamydomonas*		Ioxynil 4 Metamitron 8 Phenmedipham 4 Terbutryn 1.3	Atrazine 0.3 Bromoxynil 0.2 Metribuzin 0.8	†
$Ala_{250} \rightarrow Asp$	*Chlamydomonas*		Metamitron 5 Metribuzin 1.3 Phenmedipham 5	Atrazine 0.25 Bromoxynil 0.16 Ioxynil 0.2 Terbutryn 0.4	†
$Ala_{250} \rightarrow His$	*Chlamydomonas*		Atrazine 2.8 Ioxynil 1 Metamitron 8 Metribuzin 1.3 Phenmedipham 10 Terbutryn 1.8	Bromoxynil 0.16	†
$Ala_{250} \rightarrow Ile$	*Chlamydomonas*		Ioxynil 1 Metamitron 1 Phenmedipham 2.5	Atrazine 0.5 Bromoxynil 0.3 Metribuzin 0.25 Terbutryn 0.5	†
$Ala_{250} \rightarrow Tyr$	*Chlamydomonas*		Metamitron 2 Phenmedipham 20 Terbutryn 1.3	Atrazine 0.4 Bromoxynil 0.4 Ioxynil 0.8 Metribuzin 0.6	†
$Ala_{251} \rightarrow Cys$	*Chlamydomonas*		Bromoxynil 6.3 Ioxynil 10 Metamitron 6.3 Metribuzin 2.5 Terbutryn 1.0	Atrazin 0.8 Phenmedipham 0.8	†
$Ala_{251} \rightarrow Gly$	*Chlamydomonas*		Atrazin 1 Bromoxynil 2.5 Ioxynil 4 Metamitron 10 Phenmedipham 25 Terbutryn 1	Metribuzin 0.6	†
$Ala_{251} \rightarrow Ile$	*Chlamydomonas*		Atrazine 2 Bromacil 11 Metribuzin 55	Diuron 0.6	[65]
$Ala_{251} \rightarrow Leu$	*Chlamydomonas*		Atrazine 6 Bromacil 26		[65]

Table 2. (Continued).

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
			Diuron 4 Metribuzin 108		
$Ala_{251} \rightarrow Val$	*Chlamydomonas*	MZ2	Atrazine 25 Benzthiazuron 16 BNT 8 Cyanoacrylate 16 Dinoseb 3 Diuron 5–8 DNSJ 1.6 Ioxynil 25–40 Lenacil 160 Metamitron 124 Metabenzthiazuron 126 Metribuzin 1000 Triazinones (see text)	Ketonitrile 0.5 Triazinones (see text)	[53, 61, 62, 64, 66, 67]
	Synechocystis PCC6714	M35	Atrazine 23 Diuron 3 Ioxynil 10 Metribuzin 200		
$Phe_{255} \rightarrow Tyr$	*Chlamydomonas*	Ar207$^+$	Atrazine 15 Cyanoacrylate 39 Dinoseb 3 Ioxynil 2.5 Triazinones (see text)	Benzthiazuron 0.3 Bromacil 0.9 BNT 0.6 Diuron 0.6–0.8 Ketonitrile 0.6 Metabenzthiazuron 0.3 Metamitron 0.3 Metribuzin 0.6 Triazinones (see text)	[53, 58–61]
	Synechococcus PCC7942	Tyr5	Atrazine 25 Diuron 1.5 Ioxynil 4	Metribuzin 0.8	[68]
$Gly_{256} \rightarrow Asp$	*Chlamydomonas*	BR24	Atrazine 15 Bromacil 10 Diuron 3		[60, 69]
$Arg_{257} \rightarrow Val$	*Synechocystis* sp. PCC6803		Atrazine 30 Diuron 38	BNT 0.3 Ioxynil 0.8	[63]
$Ala_{263} \rightarrow Pro$	*Synechocystis* sp. PCC6803		Atrazine 2000 Diuron 60 Ioxynil 1 Metribuzin 1600 Terbutryn 160		[70]
$Ser_{264} \rightarrow Ala$	*Chlamydomonas*	MZ1, MZ3, MZ5	Atrazine 125–500 Benzthiazuron 49–80 Bromacil 106 Cyanoacrylate 30 Diuron 200 Ioxynil 1.3 Metamitron 30 Methabenzthiazuron 25 Metribuzin 5000–10000 Phenisopham 40 Triazinones (see text)	BNT 0.3 i-Dinoseb 0.7 DNSJ 0.3 Hydroxyquinoline 0.5 Ioxynil 0.5 Ketonitrile 0.6 Triazinones (see text)	[53, 61, 62, 66, 71–74]
$Ser_{265(264)} \rightarrow Ala$	*Euglena*	ZR	Atrazine 2.3 Chloroxuron 480 Diuron 270 Neburon 2.3 o-Phenanthrolin 5 Siduron 2.5		[75–77]
$Ser_{264} \rightarrow Ala$	*Anacystis nidulans* R2		Atrazine 10 Diuron 100 HQNO 6		[78]
$Ser_{264} \rightarrow Ala$	*Synechocystis* PCC6714	DCMU II_A	Atrazine 70 Diuron 500 Metribuzin >3000		[56, 67]

Table 2. (Continued).

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
$Ser_{264} \rightarrow Ala$	*Synechococcus* PCC7942	Di1	Bromacil 33 Metamitron 3.3 Metribuzin 5000 6 Triazines 3–60 7 Ureas 10–1000	Ioxynil 0.4	[68, 79, 80]
$Ser_{264} \rightarrow Ala$	*Synechocystis* sp. PCC6803		Atrazine 30 Diuron 207 Ioxynil 1.2	BNT 0.1	[63]
$Ser_{264} \rightarrow Gly$	*Amaranthus hybridus*		Ametryn 500 Atrazine 966–1033 Atraton 1000 Bromacil 20 Chloroxuron 3 Diuron 1.5 Fenuron 1.1–1.4 Lenacil 1880–2040 Phenmedipham 1266–2040	Bentazon 0.2 Bromnitrothymol 0.6 Diuron 0.1–0.13 DNOC 0.8	[6, 81, 82]
$Ser_{264} \rightarrow Gly$	*Amaranthus retroflexus*		Ametryn 460 Atraton 1000 Atrazine 250–1100 Bromacil 20–>2000 Chloroxuron 794 Diuron 1.0–4 Fenuron 1.1–1.4 Lenacil 50–590 Linuron >3100 Metribuzin 260–>1500 Metamitron 40 Neburon 501 Phenmedipham 6.3; 1033–1100	Bentazon 0.6 Bromnitrothymol 0.22–2 *i*-Dinoseb 0.5 DNOC 0.14–0.5 Fluometuron 0.25 Ioxynil 0.64–1.6 Picric acid 0.3	[82–84]
$Ser_{264} \rightarrow Gly$	*Amaranthus bouchonii*		Atrazine 857 Diuron 809 Fenuron 13.5 Lenacil 4.4 Phenmedipham 3.8		[82]
$Ser_{264} \rightarrow Gly$	*Brassica campestris*		Ametryn 600 Atraton 800 Atrazine 600 Bromacil 40 Diuron 2.5		[81]
$Ser_{264} \rightarrow Gly$	*Chenopodium album*		Ametryn 400 Atraton 100 Atrazine 100–>3000 Bromacil 30 Chloroxuron 8 Diuron 1.3–2 Fenuron 1692–2115 Lenacil 13.5–14.1 Metribuzin 500 Phenmedipham 5.0–5.9	Bentazon 0.5	[81, 82, 85, 86]
$Ser_{264} \rightarrow Gly$	*Phalaris paradoxa*		Triazines 2–11 Ureas 62–1000		[80, 87]
$Ser_{264} \rightarrow Gly$	*Poa annua*		Atrazine		[88]
$Ser_{264} \rightarrow Gly$	*Senecio vulgaris*		Bromacil 100 Diuron 1.7 Triazines 250–1000		[81, 89–91]
$Ser_{264} \rightarrow Gly$	*Solanum nigrum*		Atrazine >1000		[92]
$Ser_{264} \rightarrow Gly$	*Synechococcus* PCC7942	G264	6 Triazines 4–50 7 Ureas (45–1000) Metribuzin 1500	Ioxynil 0.4	[68, 80]
$Ser_{264} \rightarrow Asn$	*Nicotiana plumbaginifolia*		Terbutryn		[93]
$Ser_{264} \rightarrow Pro$	*Synechocystis* sp. PCC6803		Atrazine 10000 Diuron 1 Ioxynil 5 Metribuzin 25 Terbutryn 300		[70]

Table 2. (Continued).

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
$Ser_{264} \rightarrow Thr$	*Nicotiana tabacum* (cell culture)		Bromacil 64 Metribuzin 290 11 Phenylureas 6.3–200 11 Triazines 47–430	Dinoseb 0.13	[94–96]
$Ser_{264} \rightarrow Thr$	*Euglena*	MSI	Atrazine 63 Benzthiazuron 20 Cyanoacrylate 50 Diuron 20 Metribuzin 63	BNT 0.3 Ioxynil 0.2 Ketonitril 0.8	[97]
$Ser_{264} \rightarrow Thr$	*Solanum tuberosum* (cell culture)		Atrazine 65 Atrazine 1		[98]
$Asn_{266} \rightarrow Asp$	*Synechocystis* PCC6714	IoxIIa	Ioxynil 2.5	Diuron 0.7	[99]
$Asn_{266} \rightarrow Thr$	*Synechocystis* PCC6714	IoxI	Atrazine 1 Bromoxynil 15 Ioxynil 9	Diuron 0.7	[100]
$Ser_{268} \rightarrow Pro$	*Glycine max* (cell culture)	STR7	Atrazine 50 Diuron 3		[101]
$Arg_{269} \rightarrow Gly$	*Chlamydomonas*		Terbutryn 8		[102]
$Leu_{275} \rightarrow Phe$	*Chlamydomonas*	Br202	Atrazine 1 Bromacil 4.5 Diuron 5		[60, 69]
		MZ4	Atrazine 1 Benzthiazuron 4 BNT 1.5 i-Dinoseb 3 Diuron 5 Ketonitrile 1.3 Lenacil 3 Metabenzthiazuron 1.2 Metamitron 63 Metribuzin 20–26 Phenmedipham 1.5	Cyanoacrylate 0.5 Ioxynil 0.2	[53, 62, 103]
Double mutants					
$Phe_{211} \rightarrow Ala$ $Met_{214} \rightarrow Thr$	*Chlamydomonas*		Atrazine 3 Cyanoacrylate 6 Ioxynil 3 Metamitron 2 Metribuzin 1 Phenmedipham 25	Diuron 0.8	†
$Phe_{211} \rightarrow Gly$ $Met_{214} \rightarrow Gln$	*Chlamydomonas*		Atrazine 6.3 Cyanoacrylate 5 Diuron 2 Metribuzin 1 Phenmedipham 4	Ioxynil 0.8 Metamitron 0.4	†
$Phe_{211} \rightarrow Gly$ $Met_{214} \rightarrow Ser$	*Chlamydomonas*		Atrazine 4 Cyanoacrylate 6 Diuron 5 Ioxynil 1.6 Metamitron 8 Metribuzin 1.6 Phenmedipham 79		
$Phe_{211} \rightarrow Ile$ $Met_{214} \rightarrow Gly$	*Chlamydomonas*		Atrazine 2 Cyanoacrylate 25 Diuron 5 Ioxynil 1.3 Metamitron 1 Phenmedipham 79	Metribuzin 0.5	†
$Phe_{211} \rightarrow Ser$ $Ala_{251} \rightarrow Val$	*Synechocystis* PCC6714	AzV	Atrazine 100 Diuron 3 Ioxynil 20 Metribuzin 4000		[56, 64]
$Phe_{211} \rightarrow Thr$ $Met_{214} \rightarrow Gly$	*Chlamydomonas*		Atrazin 4 Cyanoacrylat 13 Diuron 10 Ioxynil 1.3 Metamitron 2 Metribuzin 1 Phenmedipham 63		†

Table 2. (Continued).

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
$Leu_{219(218)} \rightarrow Phe$ $Ser_{265(264)} \rightarrow Ala$	*Euglena*	ZR250^{+}‡ ZR250^{-}‡	Diuron 4226 Atrazine 63 Diuron 1922		[104]
		ZR480^{+}‡ ZR480^{-}‡	Diuron 4045 Atrazine 71 Diuron 1735		
$Val_{219} \rightarrow Ile$ $Ala_{251} \rightarrow Thr$	*Chenopodium rubrum* (cell culture)	L4, L6, L7	Atrazine 4–14 BNT 0.6–11 Bromacil 2–25 Dinoseb 4–8 Diuron 3–13 Metribuzin 6–794 Phenmedipham 2–3 Propanil 2–71		[105]
$Val_{219} \rightarrow Ile$ $Ala_{251} \rightarrow Val$	*Chenopodium rubrum* (cell culture)	L1, L8	Atrazine 8–9 BNT 25 Bromacil 15–20 Dinoseb 6–8 Diuron 2–3 Metribuzin 1259/5012 Phenmedipham 1.5 Propanil 14–15		[105]
$Ser_{221} \rightarrow Leu$ $Ser_{222} \rightarrow Ala$	*Synechocystis* sp. PCC6803		Atrazine 14 BNT 2 Diuron 560 Ioxynil 6.2		[63]
$Thr_{227} \rightarrow Ala$ $Thr_{228} \rightarrow Ala$	*Synechocystis* sp. PCC6803		Atrazine 3 BNT 2.4 Diuron 14 Ioxynil 3		[63]
$Ala_{250} \rightarrow Ser$ $Phe_{255} \rightarrow Ile$	*Chlamydomonas*		Metamitron 1.6 Phenmedipham 10	Atrazine 0.5 Bromoxynil 0.2 Ioxynil 0.2 Metribuzin 0.5 Terbutryn 0.9	†
$Phe_{255} \rightarrow Leu$ $Ser_{264} \rightarrow Ala$	*Synechococcus* PCC7942	Di22	Atrazine 2.6 Bromacil 37 Diuron 2647 Ethidimuron 16 Ioxynil 5 Metamitron 40 Metribuzin 175 Terbutryn 1.5 Thebuthiron 6.2		[68, 79] [56]
	Synechocystis PCC6714	$DCMUII_b$	Atrazine 1 Diuron 600		
$Phe_{255} \rightarrow Tyr$ $Ser_{264} \rightarrow Ala$	*Synechococcus* PCC7942	D5	Bromacil 17 S-Cyanoacrylate 250 R-Cyanoacrylate 13 Metamitron 2 Metribuzin 2000 Phenmedipham 2000 6 Triazines 88–660 7 Ureas 2.5–250	Bromoxynil 0.8 Dinoseb 0.08 Ioxynil 0.5	[106]
$Phe_{255} \rightarrow Tyr$ $Ser_{264} \rightarrow Ala$	*Synechococcus* PCC7942	TG	Triazines Atrazine 40	 BNT 0.16	[107]
$Ser_{264} \rightarrow Ala$ $Asn_{266} \rightarrow Thr$	*Chlamydomonas*		Benzthiazuron 50 Cyanoacrylate 130 Diuron 80 Metamitron 50 Metribuzin 1580 Phenmedipham 1000	Ioxynil 0.8 Ketonitrile 0.4	[72]

Table 2. (Continued).

Mutation	Organism	Code name	Resistance R/S	Supersensitivity R/S	Ref.
Triple mutants					
$Phe_{211} \rightarrow Val$ $Val_{218} \rightarrow Phe$ $Val_{219} \rightarrow Ile$	*Bumilleriopsis filiformis*		Diuron		[108]
$Val_{219} \rightarrow Ile$ $Thr_{220} \rightarrow Ala$ $Ser_{270} \rightarrow Tyr$	*Chenopodium rubrum* (cell culture)	L5	Atrazine 8 Bromacil 2 Diuron 2 Metribuzin 25 Phenmedipham 2 Propanil 13	BNT 0.6 Dinoseb 0.7 BNT 0.6	[105]
$Val_{219} \rightarrow Ile$ $Glu_{229} \rightarrow Gly$ $Ser_{270} \rightarrow Phe$	*Chenopodium rubrum* (cell culture)	L3	Atrazine 14 Bromaxil 5 Dinoseb 1.1 Diuron 3 Metribuzin 25 Phenmedipham 3 Propanil 2	BNT 0.6	[105]
$Val_{219} \rightarrow Ile$ $Ala_{251} \rightarrow Val$ $Asn_{266} \rightarrow Thr$	*Chenopodium rubrum* (cell culture)	L2	Atrazine 8 BNT 5 Bromacil 120 Dinoseb 10 Diuron 3 Metribuzin 251 Phenmedipham 9 Propanil 2		[105]
$Ile_{259} \rightarrow Ser$ $Ser_{264} \rightarrow Ala$ $Asn_{266} \rightarrow Thr$	*Chlamydomonas*		Atrazine 20 Benzthiazuron 20 Cyanoacrylate 30 Diuron 13 Metamitron 50 Metribuzin 1550 Phenmedipham 1260	BNT 0.1 Ioxynil 0.13 Keteonitrile 0.4	[72]
Deletion mutants					
ΔGlu_{229}–$Gln(Ala)_{233}$	*Synechocystis* sp. PCC6803		Atrazine 1.6 BNT 1 Diuron 2.6 Ioxynil 1.5		[63]
ΔAsn_{234}–Gly_{236}	*Synechocystis* sp. PCC6803		Atrazine 5 BNT 1.4 Diuron 8.6 Ioxynil 6		[63]
ΔTyr_{237}-Phe_{239}	*Synechocystis* sp. PCC6803		Atrazine 6 Diuron 23 Ioxynil 4	BNT 0.4	[63]
ΔGly_{240}-Gln_{241}	*Synechocystis* sp. PCC6803		Atrazine 21 Diuron 26 Ioxynil 1.4	BNT 0.5	[63]
Deletion/insertion mutant					
ΔAla_{250}–Ala_{251} $+Asn_{247} \rightarrow Tyr$	*Synechocystis* sp. PCC6803		Atrazine 80 BNT 2 Diuron 280 Ioxynil 5		[63]

* Arg in other organisms.
† Johanningmeier U. et al., unpublished results.
‡ Denotes growth in the presence (+) or absence (−) of diuron at micromolar concentration.

Of special interest is a comparison of the $Ser_{264} \rightarrow Gly$ versus the $Ser_{264} \rightarrow Ala$ mutant. The latter mutation has been found in *Chlamydomonas* and *Euglena* and was-generated by site-directed mutagenesis in three different cyanobacteria. These mutants retain their resistance towards triazines (much less than in the Gly mutation) and to a much higher extent towards triazinones (metribuzin). We tested a variety of 30 different triazinones which showed either resistance or supersensitivity in the *Chlamydomonas* mutant. The details are discussed

in [61]. Furthermore, diuron resistance is far more pronounced compared with the Gly mutant. Obviously, the additional methyl group in alanine prevents an efficient binding of diuron for steric reasons. Supersensitivity is again observed for phenolic herbicides such as ioxynil, BNT (bromonitrothymol), i-dinoseb and DNSJ (2-iodo-4-nitro-*i*-butylphenol).

After dealing with the most important D1 mutation, which causes dramatic variations in resistance, I will only briefly discuss the other mutations. Phe_{211} is located at the beginning of the herbicide binding region, and its mutation to Ser causes resistance against atrazine, diuron and metribuzin (table 2). Notable in the $Val_{219} \rightarrow Ile$ mutant is diuron resistance; a report on resistance and supersensitivity against 30 triazinones is reported in [61]. The $Ile_{248} \rightarrow Thr$ mutant is highly resistant against metribuzin. By site-directed mutagenesis with 'wobbling' base pairs we have generated six mutants at position 250 (Arg, Asn, Asp, His, Ile and Tyr). All mutants have resistance against metamitron and phenmedipham, but are supersensitive against atrazine, metribuzin, ioxynil and bromoxynil (table 2). The $Ala_{251} \rightarrow Val$ mutant in *Chlamydomonas* was originally generated by chemical mutagenesis by Pucheu et al. [66] and sequenced by Johanningmeier et al. [67]. It stands out because of its high metribuzin resistance. By site-directed mutagenesis four other herbicide-resistant mutants (Cys, Gly, Ile and Leu) have been obtained (table 2). A long-known, atrazine-resistant mutant is the result of the transformation $Phe_{255} \rightarrow Tyr$ in *Chlamydomonas*. It should be noted that a series of triazinones have been tested in both the $Ala_{251} \rightarrow Val$ and the $Phe_{255} \rightarrow Tyr$ *Chlamydomonas* mutants. In general, the Ala_{251} mutant was resistant against triazinones, whereas the Phe_{255} mutant was supersensitive [61]. Mutants $Gly_{256} \rightarrow Asp$ and $Arg_{257} \rightarrow Val$ are both resistant against atrazine and diuron; the latter is supersensitive against phenolic herbicides. The mutation $Ala_{263} \rightarrow Pro$ leads to a high degree of resistance against atrazine, diuron, metribuzin and terbutryn (table 2). Replacement of Asn_{266} by either Thr or Pro leads to ioxynil tolerance and negative cross-resistance to diuron. Mutation of $Arg_{269} \rightarrow Gly$, which probably participates in bicarbonate binding, causes terbutryn resistance. Finally, the last amino acid which defines the herbicide binding niche of the D1 protein is Leu_{275}. It is close to the carboxy terminus of the D1 protein. Its switch to Phe causes resistance to bromacil, diuron and metribuzin, to cite only a few.

Double mutations

Table 2 also contains a list of 16 double mutants with altered herbicide-binding characteristics. These alterations are more difficult to interpret, because two amino acids are involved. From the 16 double mutants, five bear mutations which are already known from single mutants. Nine have mutations where one is already known from single mutants and one is a new mutation. Only two mutants are entirely new. Again, the mutants will only be discussed briefly.

In the double mutant $Phe_{211} \rightarrow Ser$ and $Als_{251} \rightarrow Val$, atrazine resistance is much higher ($R/S = 100$) than in the atrazine-resistant single mutants $Phe_{211} \rightarrow Ser$ ($R/S = 7$) and $Ala_{251} \rightarrow Val$ ($R/S = 25$). This mutant is also highly resistant against metribuzin ($R/S = 4000$). This indicates that both amino acids participate in both triazine and triazinone binding. The mutant $Val_{219} \rightarrow Ile$ and $Ala_{251} \rightarrow Val$ from the *C. rubrum* cell culture has a high metribuzin resistance. This high ratio, however, has to be expected from the $Ala_{251} \rightarrow Val$ mutation alone. The same is true for the mutant $Phe_{255} \rightarrow Tyr$ and $Ser_{264} \rightarrow Ala$, where the single mutant $Ser_{264} \rightarrow Ala$ exhibits high metribuzin tolerance. This mutant also has a high resistance against phenmedipham. This feature, however, cannot be evaluated, because phenmedipham has not been tested in either single mutant. The $Ser_{264} \rightarrow Ala$ and $Asn_{266} \rightarrow Thr$ *Chlamydomonas* mutant is resistant against atrazine, benzthiazuron, diuron, metribuzin and phenmedipham, as can be expected from the $Ser_{264} \rightarrow Ala$ mutation alone. However, it is slightly supersensitive against ioxynil, which is unexpected because both single mutants are resistant.

We have constructed *Chlamydomonas* mutants by wobbling the nucleotides at the positions coding for Phe_{211} and Met_{214}. This strategy was chosen because Phe_{211} was already known as a single mutant and Met_{214} was a target for the photoaffinity label azido-atrazine [111, 112]. So far, the following double mutants have been obtained: $Phe_{211} \rightarrow Ala$ and $Met_{214} \rightarrow Thr$, $Phe_{211} \rightarrow Gly$ and $Met_{214} \rightarrow Gln$, $Phe_{211}Gly$ and $Met_{214} \rightarrow Ser$, $Phe_{211} \rightarrow Ile$ and $Met_{214} \rightarrow Gly$ and $Phe_{211} \rightarrow Thr$ and $Met_{214} \rightarrow Gly$. As expected, all mutants were resistant against atrazine and showed enhanced resistance against the biscarbamate herbicide phenmedipham (table 2). Notable for both $Phe_{255} \rightarrow Leu$ and $Ser_{264} \rightarrow Ala$ mutations in *Synechococcus* and *Synechocystis* as well is high diuron resistance, which, however, can be implicated by the $Ser_{264} \rightarrow Ala$ mutation alone. The two double mutants which have mutations unknown so far as single mutations comprise two adjacent amino acids: $Ser_{211} \rightarrow Leu$ and $Ser_{222} \rightarrow Ala$ and $Thr_{227} \rightarrow Ala$ and $Thr_{228} \rightarrow Ala$. Both are resistant against atrazine, BNT, diuron and ioxynil.

It should be noted that Horowitz et al. [106] have given an equation by which the I_{50}-value of a mutant can be calculated, providing the I_{50}-value of three other mutants or the wild type, respectively, is known, and there is a lack of interaction between the amino acids. Let us consider the wild-type (WT) D1-$Phe_{255}Ser_{264}$, the single mutants Di1, D1-$Phe_{255}Ala_{264}$ and Tyr5 D1-

$Tyr_{255}Ser_{264}$, and the double mutant D5 D1-$Tyr_{255}Ala_{264}$. The equation for the I_{50}-values will then be:

$$I_{50}(D5)/I_{50}(Di1) = I_{50}(Tyr5)/I_{50}(WT)$$

The authors have genetically engineered the mutant Tyr5 and compared the calculated I_{50}-values with the measured ones. Twenty out of 22 predictions for herbicides were in agreement with the experimental results. Only two herbicides, bromoxynil and fluomethuron, clearly did not fit the experimental results.

Triple mutations

The five known triple mutants of the D1 protein contain either one, two or three mutations already known from the single mutants. Two of these mutants are worthy of special attention: the $Val_{219} \rightarrow Ile/Ala_{251} \rightarrow Val/Asn_{266} \rightarrow Thr$ mutant, because it is highly resistant against metribuzin, and the $Ile_{259} \rightarrow Ser/Ser_{264} \rightarrow Ala/Asn_{266} \rightarrow Thr$ mutant, which is highly resistant to metribuzin and phenmedipham. All five mutants show supersensitivity against phenolic herbicides in tests performed thus far.

Deletion and deletion/insertion mutations

Kless et al. [63] have constructed four deletion mutants in the loop of the D1 protein which connects transmembrane helix IV and the lumenal parallel helix IV/V. This region of the D1 protein is of special interest for the following reasons: (i) it is missing in the photosynthetic reaction center of purple bacteria, (ii) two amino acids, Tyr_{237} and Val_{249}, are tagged by the photoaffinity labels azido-monuron [113] or azido-ioxynil [114], respectively, and (iii) the connecting loop is the processing site when the D1 protein is cleaved during rapid turnover. All four deletion mutants are resistant against atrazine, diuron and ioxynil. The same is true for the deletion/insertion mutant $\Delta Ala_{250}Ala_{251} + Asn_{247} \rightarrow Tyr$.

Defining the binding niche for photosystem II herbicides

The data obtained from the above mutations can be used to define the binding area of the various herbicides and inhibitors within the Q_B binding site. Though all inhibitors compete with the native plastoquinone for binding in the Q_B site, it is entirely clear that no common binding pattern exists, and each herbicide has to be viewed differently.

At first glimpse the enormous difference between the 'classical' herbicides (triazines, triazinones, ureas) and the phenolic herbicides (bromoxynil, ioxynil, BNT, dinoseb) becomes evident. Whereas the 'classical' herbicides show a tremendous resistance, the phenolic herbicides either exhibit supersensitivity or have R/S-values that are only slightly different from the wild type. This behaviour has prompted Trebst [115] to define two major binding areas for these two types of herbicides. The 'classical' herbicides orient themselves preferentially towards Ser_{264} and can form a hydrogen bridge to the hydroxymethyl group of serine. Contrary, phenolic herbicides possess a hydroxyl group which cannot bind to the serin hydroxymethyl group. Consequently, phenolic herbicides are pushed away from Ser_{264} towards His_{215}. There also exists differential sensitivity between the phenolic herbicides, such as the resistance of ioxynil, dinoseb and bromonitrothymol in the $Val_{219} \rightarrow Ile$ and $Ala_{251} \rightarrow Val$ mutants. It also depends on the length of the alkyl side chain in nitro- and dinitrophenols [116].

All $Ser_{264} \rightarrow Gly$ mutants are highly resistant against triazines and, in addition, against metribuzin, a triazinone. Metamitron, which is itself a triazinone, is only moderately resistant, which indicates that the binding affinities differ even within one group of compounds. The thiomethyl group of metribuzin is in contact with Ala_{251} because its affinity is weakened by a larger amino acid like Val, Leu or Ile. The difference between metribuzin and metamitron is further evident in the $Val_{219} \rightarrow Ile$ mutant, where only metribuzin but no metamitron resistance is observed. This indicates that the binding site of metribuzin also includes transmembrane helix IV.

As far as tests indicate, atrazine and metribuzin resistance run parallel, with two exceptions: in the $Phe_{255} \rightarrow Tyr$ mutation resistance is acquired for atrazine but not for metribuzin; in the $Leu_{275} \rightarrow Phe$ mutation the opposite is the case.

Cyanoacrylates stand out when compared with the other inhibitors because they are resistant in the $Phe_{255} \rightarrow Tyr$ mutation, a property which is not shared by triazines, triazinones or phenolic inhibitors.

The R/S-values of diuron are only slightly modified in the $Ser_{264} \rightarrow Gly$ mutations. This changes, however, dramatically when Gly is exchanged for Ala. This mutation is highly diuron-resistant. Obviously, diuron cannot be accommodated any more due to steric hinderance.

The binding affinities of neither ketonitriles, tetrabromopyridinol nor quinolines are highly affected in the mutations. In this context, it is of interest that the quinolines have been grouped into the phenolic herbicides for their inhibitory pattern [117].

Molecular modeling of the herbicide binding niche

A further refinement of herbicide binding within the Q_B binding niche can be achieved by molecular modeling. In this way amino acids participating in herbicide binding which so far have not been mutated can be iden-

tified. All models are based on the three-dimensional structure of the photosynthetic reaction center of purple bacteria, because an X-ray structure of photosystem II at a sufficient resolution is not yet available.

So far, four different models have been developed. The first model was constructed by Croft's group [118, 119]. At that time, the atomic coordinates of the reaction center of *R. viridis* were not yet available, and the three-dimensional model was constructed by triangulation from the stereo pair diagrams as published by Michel et al. [17]. Croft's model includes Phe_{206}-Arg_{225} and Asn_{247}-Gly_{282}, that is parts of helix IV and V and the parallel helix. The connecting loop between helix IV and the parallel helix has been omitted because it is missing in the reaction center of purple bacteria. Proposals for the structure of this loop have been made by Kleier et al. [120] and by Trebst [54].

The second model, similar to the first, was constructed by scientists at Bayer AG in collaboration with Trebst [62]. It also used the coordinates of *R. viridis*. This model includes Phe_{211}-Ala_{276}, again under omission of the interconnecting loop. According to Tietjen et al., [62] the following amino acids are in contact with the Q_B binding niche: Phe_{211}, Met_{214}, His_{215}, Leu_{218}, Val_{219},

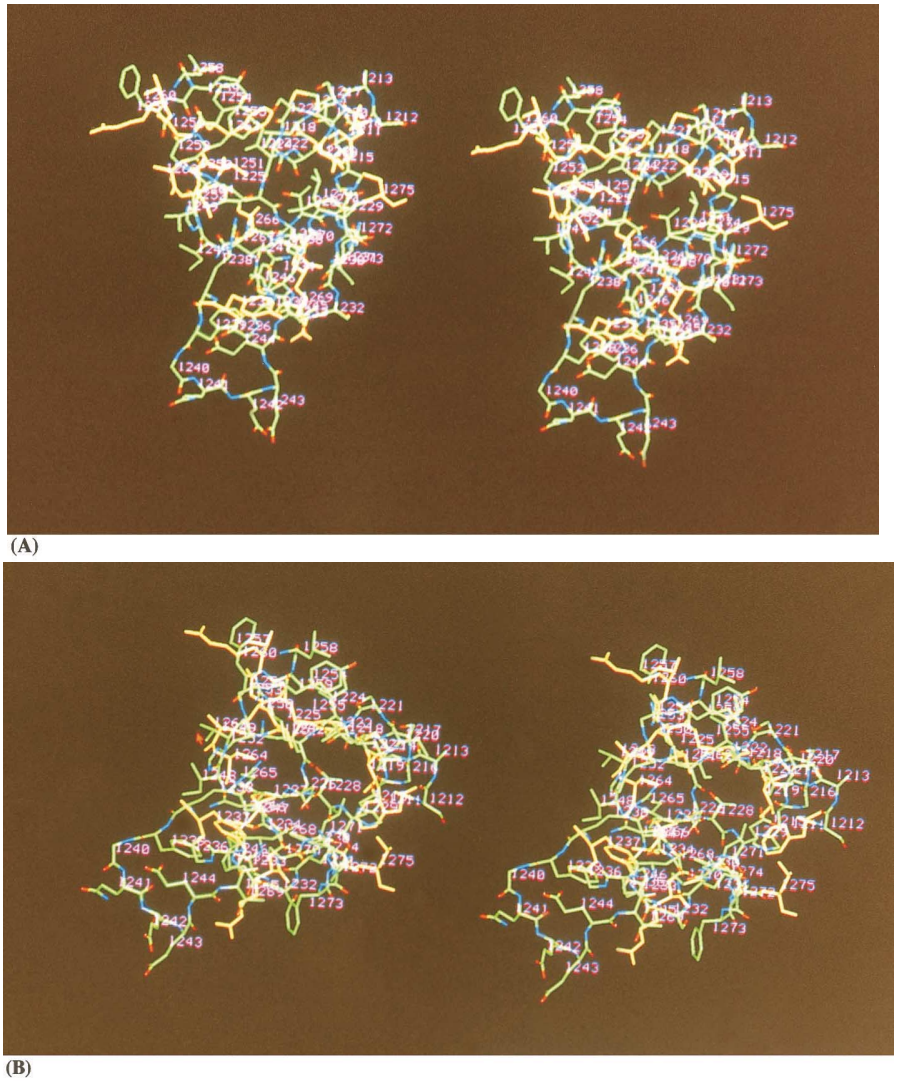

Figure 3A and B. Stereo view of the herbicide binding niche of the photosystem II D1 protein from *Chlamydomonas reinhardtii* from different viewpoints. The coordinates were obtained from Govindjee [123]. The herbicide binding niche reaches from Phe_{211} to Leu_{275}. Amino acids where mutations have been found are coloured in yellow. The model was constructed using Insight II. V. 95.0 (Biosym/MSI).

Tyr_{237}, Ile_{248}, Ala_{251}, His_{252}, Phe_{255}, Gly_{256}, Ala_{263}, Ser_{264}, Phe_{265}, Asp_{266}, Ser_{268} and Leu_{275}.

The next model, the photosystem II D1/D2 reaction center from pea (*Pisum sativum*), was devised by Ruffle et al. [121]. For the first time, the interconnecting loop was also constructed. It was built by matching fragments from solved structures and then added to the framework which was derived from the X-ray structures of both *R. viridis* and *R. sphaeroides*. The latest model, which also includes the interconnecting loop, was constructed by Xiong et al. [122, 123] (Fig. 3A, B).

The study of binding of selected herbicides increased what is known from modeling, as discussed below.

Atrazine. The ethylamino side chain of atrazine is hydrogen-bonded to the side chain hydroxyl of Ser_{264}. The aromatic ring nitrogen is hydrogen-bonded to the backbone amide nitrogen of Phe_{265}, and the ethylamino side chain is also near to Ala_{251}. The loss of activity in mutants Ser_{264} and Ala_{251} is explained [62]. A detailed study of the interaction of optically active triazine herbicides with the D1 protein of photosystem II was performed by Mackay and O'Malley [124].

Lenacil. The two carboxyl groups of lenacil (3-cyclohexyl-5,6-trimethyleneuracil) can form hydrogen bonds to Phe_{265} backbone nitrogen, to Ser_{264} hydroxyl and to His_{251} $\delta 1$ nitrogen, simultaneously. The cyclopentene ring is close to Ala_{251}, causing resistance in the mutation to the more space consuming Val [62].

Metribuzin. The free amino group of metribuzin is hydrogen-bonded to the side chain hydroxyl group of Ser_{264}. Its carbonyl group is hydrogen-bonded to the backbone amide nitrogen of Phe_{265}, and the methylthio substituent is in contact with the side chain of Ala_{251}. This explains the strong impairment of metribuzin binding in the mutants Ser_{264} and Ala_{251} [62].

Cyanoacrylates. A cyanoacrylate [2-cyano-3-methylthio-(1-phenyl)ethylaminoacrylic acid-2-ethyloxyethanolate] is hydrogen-bonded to Ser_{264} and to Phe_{265} backbone amide nitrogen. The amino substituent forms a hydrogen bond to Ala_{263} backbone amide oxygen. Furthermore, a hydrogen bond of the alkoxy substituent in the ester alcohol moiety to the Ser_{264} hydroxyl group is possible [62]. The interaction of cyanoacrylates and optical isomers with the D1 protein of photosystem II has been extensively discussed by Mackay and O'Malley [125, 126].

Ioxynil. Ioxynil is in contact with Ala_{251}; its binding is decreased upon mutation to Val. However, no interactions with either Phe_{255}, Ser_{264} or Leu_{275} take place, because the inhibitory properties of ioxynil are not affected in the corresponding mutants or because supersensitivity occurs.

The binding of other types of inhibitors is also described in the literature. The interested reader is directed to the references: NH- and SH-thiazoles [62], phenylureas [119] and other triazinones [37].

Other mechanisms of resistance against photosystem II herbicides

Tolerance against photosystem II herbicides is not due to a mutation in the D1 protein in a variety of herbicide-resistant weeds. Recently, a velvetleaf (*Abutilon theophrasti*) was found in Maryland, USA, which is resistant to atrazine due to an enhanced capacity to detoxify the herbicide via glutathione detoxification. It should be noted that maize is naturally resistant to atrazine because of this mechanism. The level of glutathione S-transferase was about fivefold greater in leaf and stem tissue of the resistant biotype as compared with the wild type [127]. *Lolium rigidum* is resistant against simazine and chlorotoluron, because these compounds can be rapidly detoxified. The detoxification proceeds via a dealkylation. A cytochrome P_{450} enzyme system is responsible for this because the resistance can be abandoned by inhibitors of cytochrome P_{450} [128, 129].

A very promising approach to achieve herbicide resistance is to introduce a foreign gene into a plant. The enzyme system coded by the gene chemically modifies the herbicide and renders it ineffective. This approach was successfully carried out with the gene for the bacterial enzyme nitrilase. Nitrilase from the natural soil bacterium *Klebsiella ozaenae* converts the photosystem II herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) to 3.5-dibromo-4-hydroxybenzoic acid, which is no longer an active herbicide. The gene *bxn* coding for the nitrilase was sequenced and the properties of the nitrilase investigated [130]. For expression in plants, the *bxn* gene was placed under the control of a light-regulated tissue-specific promoter, the small subunit of ribulose bisphosphate carboxylase. Transfer of this chimeric gene into tobacco plants by *Agrobacterium tumefaciens* resulted in a tobacco group which was insensitive to high levels of a commercial formulation of bromoxynil [131]. Other promotors were also successfully incorporated to yield transgenic tobacco plants which were resistant to up to 20 times the lethal doses of bromoxynil [132]. In 1995, the *bxn* gene was successfully integrated into cotton. BXN cotton is resistant against bromoxynil, and yield, fiber strength and fiber length are not reduced relative to commercial varieties. Furthermore, the *bxn* gene has been introduced into other species (for review, see [1]).

Physiological aspects of the photosystem II mutants

Most physiological studies have been performed with the $Ser_{264} \rightarrow Gly$ mutant because a broad variety of resistant plants are available. As already stressed, Ser_{264} not only participates in triazine and triazinone binding but also in the binding of the native plastoquinone at the Q_B-site. Consequently, by substitution of Ser by Gly, the rate of electron transfer between Q_A and Q_B decreased. Furthermore, the equilibrium constant $Q_A \rightarrow Q_B$ is lower, the quantum yield is decreased by 23% in the mutant and the number of active photosystem II reaction centers is decreased by 25%. The mutant is more susceptible towards photoinhibition and more heat-sensitive. There is also an increase in thylakoid grana stacking and an increase in fatty acid unsaturation in grana lamellae. Overall carbon assimilation is lower in the mutant [133–137]

These limitations in photosynthesis will affect a crop which consists of herbicide-resistant plants. The yield will be lower in general, and lower costs for herbicides may or may not outweigh the losses in the harvest. It is beyond the scope of this review to consider all the implications. The interested reader is directed to a series of monographs which deal extensively with this subject [110, 138–141].

Outlook

Herbicide-resistant crops are now a commercial reality, and their use will increase in the future. This will not necessarily be true for crops resistant to photosystem II herbicides; the use of triazine-resistant rape in Canada amounts to only a few percent of the total rape crop. The current interest focuses on herbicides which interfere with amino acid biosynthesis, such as glyphosate (inhibitor of the 5-enolpyruvylshikimi acid-3-phosphate (EPSP)-synthase in the shikimate pathway), glyfosinate (inhibitor of glutamine synthetase) or imidazolinones and sulfonylureas (inhibitors of acetolactate synthethase). By genetic manipulation, soybeans, cotton, corn, canola, sugar beet, oilseed rape, tobacco, tomato and potato, to cite only a few, have been obtained which are resistant against the herbicides inhibiting amino acid biosynthesis.

The pros and cons of herbicide-resistant crops have been vigorously discussed within the scientific and political communities and will continue to be debated (for review, see [1]).

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