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Mutations responsible for high light sensitivity in an atrazine-resistant mutant of *Synechocystis* 6714

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Abbreviations: DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PSII: photosystem II; RCII: reaction center II

Abstract

The primary target of photoinhibition is the photosystem II reaction center. The process involves a reversible damage, followed by an irreversible inhibition of photosystem II activity. During cell exposition to high light intensity, the D_1 protein is specially degraded. An atrazine-resistant mutant of *Synechocystis* 6714, AzV, reaches the irreversible step of photoinhibition faster than wild-type cells. Two point mutations present in the *psb*A gene of AzV (coding for D_1) lead to the modification of Phe 211 to Ser and Ala 251 to Val in D_1. Transformation of wild-type cells with the AzV *psb*A gene shows that these two mutations are sufficient to induce a faster photodamage of PSII. Other DCMU- and/or atrazine-resistant mutants do not differ from the wild type when photoinhibited. We conclude that the Q_B pocket is involved in PSII photodamage and we propose that the mutation of Ala 251 might be related to a lower rate of proteolysis of the D_1 protein than in the wild type.

Introduction

Photoinhibition is the decrease of photosynthetic activity induced by high light intensity (reviewed by [22, 6, 7]). The site of photodamage has been localized at the reaction center of photosystem II (PSII). The PSII reaction center contains a heterodimer of D_1 and D_2 which carries the primary and secondary donors (P_{680} and Z), the initial electron acceptor (a pheophytin molecule) and the quinonic acceptors (Q_A and Q_B) [37, 28, 3, 26]. Q_A is permanently bound to D_2. D_1 binds reversibly the secondary quinone Q_B and PSII-directed herbicides in a niche formed by the hydrophylic loop (on the stroma side) which links the fourth and fifth membrane spanning helixes [36].

The molecular mechanism of photoinhibition is still controversial. The sites of Q_A, pheophytin *a* and Q_B have alternatively been proposed to be the primary target of photoinhibition [5, 9, 21, 30]. Some publications suggest that photoinhibition leads to degradation of D_1 [21, 34, 24]. If exposure to high light intensity is not too long, photosystem II activity can be restored. The repair process involves *de novo* synthesis of thylakoid proteins among which the most prominent is D_1

[29, 32, 15, 24, 20]. However, other laboratories have reported that no loss of D_1 was observed during photoinhibition of spinach chloroplasts or *Oxalis oregona* leafs [4, 6].

In our previous publication [20] we have compared the behavior of wild-type and herbicide-resistant mutants of a cyanobacterium *Synechocystis* 6714 at high light intensity. In the wild type and the mutants the H_2O-to-Q_B electron transfer was lost faster than the H_2O-to-Q_A electron transfer. We therefore proposed that the Q_B niche might be the primary target of photoinhibition. One of the herbicide-resistant mutants, AzV, had an increased light sensitivity leading to a faster loss of the ability to recover PSII activity. AzV reached more rapidly an irreversible step of PSII degradation associated with a decrease of energy transfer from the phycobilisomes to PSII and with an inhibition of the synthesis or integration of thylakoid proteins [20]. The well-known rapid turnover of the D_1 protein is thought to allow an optimal functioning of PSII. D_1 is removed and replaced as fast as it is damaged. During photoinhibition the damage is so fast that the repair process can no longer keep up with it. It is possible that in AzV, the respective rates of damage and proteolysis are modified.

In the present paper, we show that the special behavior of AzV at high light intensity is indeed due to a change in the D_1 structure. We cloned and sequenced the *psb*A gene from three herbicide-resistant mutants: AzV, AzI and DCMU-II_B. All these mutants have a modified Q_B site but only the AzV is more sensitive to high light intensity. We propose that the mutations found in AzV cause a modification in the rate of D_1 proteolysis, generating the increased sensitivity of AzV to high light intensity.

Materials and methods

Growth conditions

Wild-type and mutant cells of *Synechocystis* 6714 and 6803 were grown under the conditions previously described [2, 20].

Construction of AzV and AzI library

AzV and AzI DNA were digested to completion with *Bam* HI and fractionated by gel electrophoresis in a 0.6% agarose gel. The 15 to 19 kb fraction was recovered by electroelution and ligated to $\lambda EMBL_3$ DNA (Stratagene) previously digested with *Eco* RI-*Bam* HI, following the procedure of Kaiser and Murray [19]. Ligation mixes were packaged (Gigapack kit, Stratagene) and then plated on *Escherichia coli* P_2 392. Libraries were screened by plaque hybridization as described by Maniatis *et al.* [25] using as a probe a nick-translated *psb*AII clone from *Synechocystis* 6803, kindly provided by Dr L. McIntosh [17] or the *psb*AI clone from *Synechocystis* 6714 (Ajlani *et al.*, submitted to Plant Molecular Biology).

DNA subcloning

The 15 kb fragment containing the *psb*A gene was subsequently digested by *Eco* RI-*Hin*d III. A 2 kb fragment which hybridized with the ^{32}P-*psb*A probe was recovered from a 1% agarose gel. This fragment was introduced into a Bluescript plasmid (Stratagene cloning systems).

Sequence analysis

Digestion of the recombinant plasmids with *Kpn* I produced four fragments. Three of them, of 0.7 kb, 0.4 kb and 0.2 kb, hybridized to the *psb*A clone. They were subcloned into Bluescript plasmid in order to perform dideoxy chain termination sequence reactions on double stranded DNA templates according to Toneguzzo *et al.* [35], using a Sequenase kit (US Biochemicals). Information on the initial sequence of the 0.7 kb plasmid (which was obtained using T7 and SK primers) was used to synthesize an oligonucleotide sequencing primer (20-mer) with a Milligen 7500 DNA synthesizer. The complete nucleotide sequence on both strands was obtained using this oligonucleotide as primer.

Transformation of Synechocystis *6803*

0.5 ml of wild-type *Synechocystis* 6803 at a concentration of 2×10^8 cells/ml was plated in top agar plates containing minimum medium. After solidification of the agar 10 μl of 2 kb fragments containing the *psb*A gene of the different mutants were spotted directly onto the surface of the plate. After 16 hours incubation in light, herbicides were added by the underlaying technique, giving a final concentration of 10^{-5} M DCMU or 10^{-4} M atrazine.

Photoinhibition experiments

Photoinhibition of cell suspensions (30 μg chlorophyll/ml) was carried out at 27 °C. The light intensity was about 2000 W/m^2. For recovery, the cells were centrifuged, resuspended in fresh growth medium at 30 μg chlorophyll/ml and incubated up to 4 h in standard conditions (shaking, 34 °C and light intensity of 20 W/m^2). During this period the culture growth is negligible at the cell concentration utilized.

Fluorescence induction

The kinetics of photoinhibition and recovery were followed by variable fluorescence measurements as previously described [38]. The fluorescence was excited with a tungsten lamp through 5–59 and 4–96 Corning filters. The fluorescence was detected in the red region through a 2–64 Corning filter and a Wratten 90 filter. The recording was done through a multichannel analyzer. The cell suspension contained about 1 μg chlorophyll/ml. The measurements were done in the presence of 10^{-5} M DCMU for AzV, AzI and wild type and 10^{-4} M atrazine for DCMU-II_B.

Degradation of D_1 protein

Wild-type and AzV cells (30 μg chlorophyll/ml) were incubated for 1 hour in the presence of $^{35}SO_4^{2-}$ (600 μCi/μmol, 4 μCi/ml) at 34 °C at a light intensity of 20 W/m^2. At the end of the incubation period non-radioactive sulfate was added. Thylakoids were isolated as previously described [20] and resuspended in a buffer containing 15 mM HEPES (pH 6.8), 30 mM $CaCl_2$ and 25% glycerol. Membrane suspensions (300 μg chlorophyll/ml) were incubated for 90 min at high light intensity (500 W/m^2). Samples were taken at different times. The thylakoid proteins were resolved in a SDS-polyacrylamide gel (12–17.5%) in the presence of 4 M urea using the method of Laemmli [23]. The gel was stained with Coomassie blue, dried and autoradiographed.

Results

We compared the behavior at high light intensity of three herbicide-resistant mutants, AzV, AzI and DCMU-II_B. DCMU-II_B is a double mutant isolated in the presence of 10^{-5} M DCMU. AzI has been screened with 10^{-4} M atrazine and AzV was derived from AzI in the presence of 2.5×10^{-4} M atrazine [2]. Their resistance to herbicides is described in Table I. AzV and AzI are atrazine- and metribuzin-resistant. The resistance to both herbicides is largely increased in AzV cells. DCMU-II_B is resistant to DCMU and metribuzin and sensitive to atrazine [1]. We have previously shown that AzV cells have an increased sensitivity to high light intensity while DCMU-II_B cells behave as wild-type cells regarding photoinhibition [20]. In order to determine

Table 1. I_{50} concentrations of different herbicides in herbicide-resistant mutants of *Synechocystis* 6714[a].

	I_{50} atrazine (M)	I_{50} DCMU (M)	I_{50} metribuzin (M)
Wild type	3×10^{-6}	2×10^{-7}	10^{-6}
AzI	2×10^{-5}	2×10^{-7}	8×10^{-6}
AzV	2×10^{-4}	5×10^{-7}	4×10^{-4}
DCMU-II B	3×10^{-6}	10^{-4}	2.5×10^{-4}

[a] I_{50}: concentration of the herbicide needed to block half of the maximal variable fluorescence.

whether the mutation responsible for the sensitivity to high illumination of AzV is also present in the AzI genome, we studied the behavior of AzI cells under photoinhibitory conditions. The cells were exposed to high-light illumination for 60 and 90 min, and then transferred to low light to allow them to recover their PSII activity. This recovery was determined by measuring the variable fluorescence, which is correlated with recovery of photosynthetic electron flow via Q_B. Figure 1 shows that AzI cells behave like wild-type cells. They recovered their variable fluorescence with a time-course similar to that of wild-type cells. In contrast, as previously shown [20], AzV cells were unable to recover PSII activity when photoinhibited for 90 min and regained only partially the initial variable fluorescence when they were photoinhibited for 60 min. These results demonstrate that the increased sensitivity to high light intensity is specific for AzV. It was not observed in AzI from which AzV was derived and neither in DCMU-II_B. Since it has been proposed that D_1 might be involved in the photoinhibition process [21, 24, 20], we decided to clone the *psb*A gene of all three mutants and to perform transformation experiments.

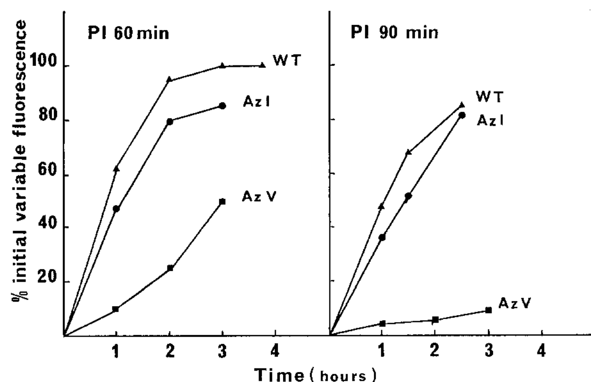

Fig. 1. Recovery of variable fluorescence ($F_V = F_M - F_0/F_0$) in cells of wild type (▲–▲), AzI (●–●) and AzV (■–■) mutants of *Synechocystis* 6714 photoinhibited for 60 or 90 min. Cell suspensions (1 μg chlorophyll/ml) were excited at 440 nm in the presence of 10^{-5} M of DCMU. 100% of F_V is equal to 1–1.5. F_0 is constant during the time of recovery. PI: photoinhibition.

Cloning of psb*A genes from AzV, AzI and DCMU-II_B of* Synechocystis *6714 and transformation experiments*

In higher plants, the *psb*A gene is a chloroplast gene [10, 39], present as a single copy. On the other hand, the genome of cyanobacteria contains a family of *psb*A genes [8, 27, 12]. The *Synechocystis* 6714 genome contains three copies of *psb*A as does the genome of *Synechococcus* 7942 and *Synechocystis* 6803 (Ajlani *et al.*, submitted to Plant Molecular Biology; [13, 17]). Several phages containing a 15 kb *Bam* HI DNA fragment with one of the three copies of *psb*A were identified by screening the $\lambda EMBL_3$ libraries with radioactive *psb*A specific probes (see Materials and methods). All three 15 kb fragments isolated from AzV, AzI and DCMU-II_B had an identical restriction map (data not shown). They were subsequently digested with the restriction endonucleases *Eco* RI and *Hin*d III, obtaining 2 kb DNA fragments which hybridized with the radioactive *psb*A probe.

In order to test if the cloned copy of *psb*A carries the mutation conferring herbicide resistance we used the 2 kb DNA fragments containing the *psb*A gene to transform wild-type cells and study their phenotypes. *Synechocystis* 6803 was utilized as the recipient strain, since *Synechocystis* 6714 is poorly transformable. This heterologous transformation was reported for the first time by Grigorieva and Shestakov [16]. The *Synechocystis* 6803 transformed cells were selected in agar plates containing either 3×10^{-5} or 10^{-4} M atrazine to screen for AzI and AzV phenotypes or 10^{-5} M DCMU to screen for the DCMU-II_B phenotype. No AzI transformed cells have been obtained until now. Several clones of AzV and DCMU-II_B transformed cells were isolated and transferred to liquid medium. The transformed cells were called AzV_{6803} and DCMU-II_{B6803} in order to differentiate them from the original mutants in *Synechocystis* 6714. All the clones of AzV_{6803} tested presented the same herbicide resistance as the original mutant AzV_{6714} (Table 2; compare Table 1). AzV_{6803} and AzV_{6714} are 70-fold more resistant to atrazine than the

Table 2. I_{50} concentrations of different herbicides in transformed cells of *Synechocystis* 6803 [a].

	I_{50} atrazine (M)	I_{50} DCMU (M)	I_{50} metribuzin (M)
Wild-type$_{6803}$	3×10^{-6}	2.5×10^{-7}	5×10^{-7}
AzV$_{6803}$	2×10^{-4}	5×10^{-7}	4×10^{-4}
DCMU-II B$_{6803}$	3×10^{-6}	10^{-4}	3×10^{-4}

[a] I_{50}: concentration of the herbicide needed to block half of the maximal variable fluorescence.

respective wild types of *Synechocystis* 6803 and 6714. DCMU-II$_{B6803}$ also presents the same phenotype as the original mutant. These results confirm that the cloned copy of *psb*A carries the mutation conferring the original herbicide-resistant phenotype.

Characteristics of transformed cells regarding photoinhibition and recovery

Since *Synechocystis* 6803 was the recipient strain used for transformation, photoinhibitory experiments were performed with the wild-type 6803 and the transformants AzV$_{6803}$ and DCMU-II$_{B6803}$. Cells photoinhibited for 60 or 90 min were resuspended in fresh medium and incubated at low light intensity for up to four hours. The time courses of recovery for WT$_{6803}$, AzV$_{6803}$ and DCMU-II$_{B6803}$ are shown in Fig. 2. For the wild-type 6803 photoinhibition was still reversible after 90 min of cell exposure to high light intensity. On the other hand, AzV$_{6803}$ cells were unable to recover any activity when photoinhibited for 90 min and only partially when photoinhibited for 60 min. Thus in AzV$_{6803}$ the irreversible step was reached faster than in wild-type cells, as was the case in AzV$_{6714}$. In DCMU-II$_{B6803}$ the initial variable fluorescence was recovered with the same time course as for the wild type. The similarity of behavior between wild-type cells and DCMU-II$_{B6803}$ rules out the possibility that the introduction of exogenous *psb*A in *Synechocystis* 6803 modified the behavior of the cells under high light. These results demonstrate that the mu-

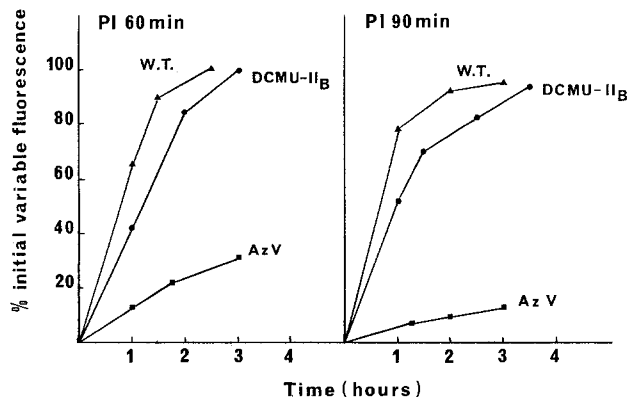

Fig. 2. Recovery of variable fluorescence of *Synechocystis* 6803 (wild type) (▲–▲) and of transformed cells, DCMU-IIB$_{6803}$ (●–●) and AzV$_{6803}$ (■–■) photoinhibited for 60 and 90 min. Experimental conditions as in Fig. 1.

tation(s) present in the AzV *psb*A gene is sufficient to confer the increased sensitivity to high light intensity and that no other gene is involved.

Sequencing of the psb*A gene from AzI and AzV*

In order to find the mutation responsible for photoinhibition sensitivity in AzV we sequenced the *psb*A genes from AzV and AzI and compared them to the *psb*A gene of the wild type. To localize the *psb*A gene, the cloned 2 kb DNA fragment was subcloned into a Bluescript plasmid and digested with *Kpn* I. The partial restriction maps of the 2 kb fragments from AzV, AzI and wild-type cells are identical (Fig. 3). Comparison of the nucleotide sequence of the *psb*A gene of *Synechocystis* 6714 with the sequence of the *psb*A gene from other cyanobacteria [12, 11] has permitted to localize the sequence coding for the Q_B pocket of *Synechocystis* 6714 in the 0.7 kb *Kpn* I fragment. Since all the mutations shown to confer DCMU or atrazine resistance were found between amino acids residues 211 and 275, we decided to subclone and sequence the 0.7 kb fragments. The analysis of the different 0.7 kb fragment sequences showed a single nucleotide change in AzI with respect to the wild type. This nucleotide change – TTC to TCC – results in the modification of Phe 211 to Ser in the herbicide-

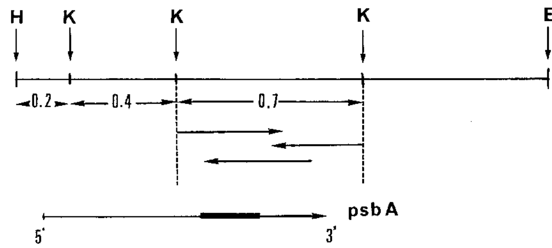

Fig. 3. Partial restriction map of the 2 kb *Eco* RI-*Hin*d III fragment containing the entire *psb*A gene. Strategy of sequencing the 0.7 *Kpn* I fragment is indicated by horizontal arrows under the 0.7 fragment. Location of the *psb*A gene is represented by an arrow at the bottom of the figure. The dark box shows the position of the Q_B niche coding region. Restriction endonucleases: E, *Eco* RI; H, *Hin*d III; K, *Kpn* I.

resistant protein (Fig. 4). In AzV a second nucleotide change – GCC to GTC – was observed in codon 251 leading to a replacement of Ala for a Val (Fig. 4). No other nucleotide differences were found between the 0.4 and 0.2 kb fragments of AZV and of the wild type (data not shown). The sequence of the *psb*A gene of DCMU-II_B showed that Ser_{264} was changed to Ala and Phe_{255} to Leu in D_1 (Ajlani *et al.*, submitted to Plant Molecular Biology).

Discussion

In our previous publication [20] we have proposed that the Q_B niche was the primary target of photoinhibition and that photoinhibition resulted in a damage of D_1. An atrazine-resistant mutant, AzV, proved to be more sensitive to photoinhibition than the wild type. The electron transfer from H_2O to Q_A and the ability of the cells to recover photosystem II activity were lost faster in AzV than in the wild-type cells. In the present work, we present further proof that D_1, and more specifically the Q_B binding domain, is involved in the photoinhibition process. By transforming wild-type cells with a DNA fragment containing the *psb*A gene encoding for D_1 we show that the mutations of D_1 are sufficient to account not only for the atrazine resistance but also for the increased sensitivity of AzV to photoinhibition. AzV is a double mutant; D_1 presents two point mutations, Phe 211 to Ser and Ala 251 to Val. On the other hand, AzI, from which AzV was derived, has a single mutation (Phe 211 to Ser) and behaves as the wild type with regard to photoinhibition. A more definitive proof that the Ala 251 to Val change alone is sufficient to confer high light sensitivity could come from studies performed with a mutant presenting only the Ala 251-to-Val change. Directed DNA mutation experiments are currently being performed in order to obtain this type of mutant. A metribuzin-resistant mutant of *Chlamydomonas reinhardtii* having the mutation Ala 251 to Val has been reported [18] but its behavior under photoinhibitory conditions has not been described.

Herbicide resistance has been obtained in AzI, AzV and DCMU-II_B by mutations of amino acids located in the Q_B binding domain. The electron transfer between Q_A and Q_B has been found to be altered in all three mutants (Etienne *et al.*, submitted to Biochimica et Biophysica Acta) but AzV is the only mutant presenting a higher sensitivity to photoinhibition. We therefore propose that changes in electron transfer between Q_A and Q_B are not responsible for the higher sensitivity to high light intensity.

A specific region of the Q_B domain might be involved in the D_1 degradation occuring during photoinhibition. This region could be modified in AzV but not in the other mutants. Greenberg *et al.* [14] have demonstrated that the product of the *in vivo* breakdown of the D_1 protein in *Spirodela*

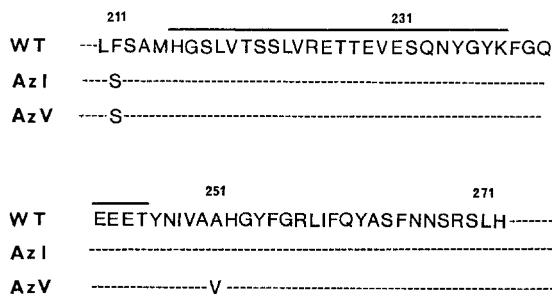

Fig. 4. Comparison of the amino acid sequence of the Q_B niche from *Synechocystis* 6714 wild type (WT) with those of AzI and AzV mutants. For the mutants only the differences are given. The possible position of the PEST signal is overlined.

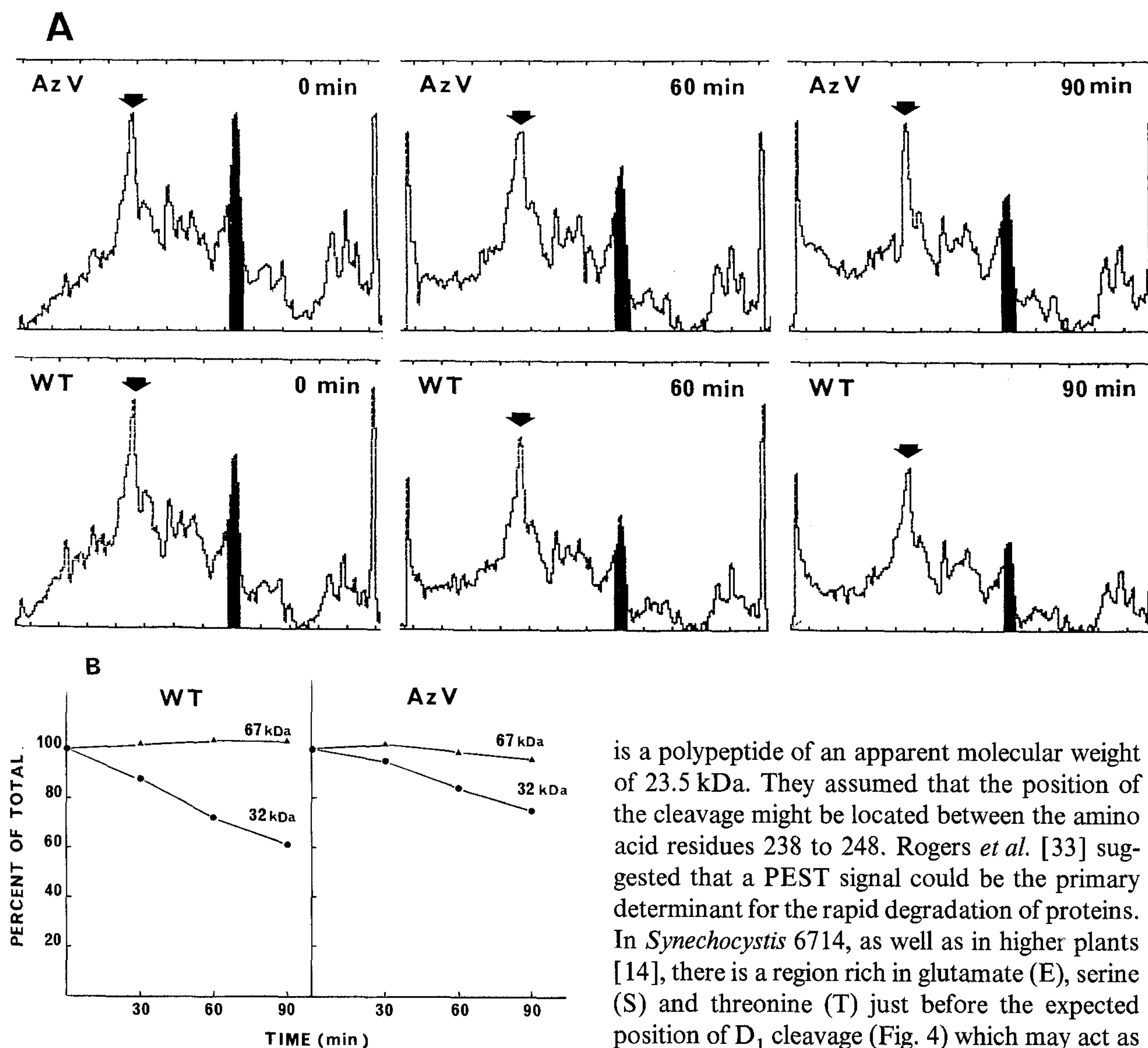

Fig. 5. Degradation of the D_1 protein during photoinhibition of thylakoids isolated from *Synechocystis* 6714 wild-type and AzV mutant cells. A. Densitometry scans of autoradiograms showing the chase of radioactively labelled proteins during photoinhibition. The black surfaces locate the peak at 32 kDa (D_1). The arrows indicate the band at 67 kDa. B. Percent of the initial radioactivity in the 32 kDa (●–●) and the 67 kDa (▲–▲) bands remaining in the samples photoinhibited for 30, 60 and 90 min. The radioactivity of the bands was calculated as a percentage of the total radioactivity. As seen in A, the 32 kDa band amounts to 8% of the total radioactivity present in the sample at time 0. The 67 kDa band corresponds to 12% and 11% of the total radioactivity measured at time 0 in the wild type and the AzV respectively.

is a polypeptide of an apparent molecular weight of 23.5 kDa. They assumed that the position of the cleavage might be located between the amino acid residues 238 to 248. Rogers *et al.* [33] suggested that a PEST signal could be the primary determinant for the rapid degradation of proteins. In *Synechocystis* 6714, as well as in higher plants [14], there is a region rich in glutamate (E), serine (S) and threonine (T) just before the expected position of D_1 cleavage (Fig. 4) which may act as a PEST signal. Since in AzV the mutation of Ala 251 into Val is near the region of cleavage one might postulate that this change could be related to the mechanism of proteolysis of D_1. *In vitro* experiments of photoinhibition with thylakoids of AzV and the wild type indicate that the rate of proteolysis of D_1 is slower in AzV than in the wild type (Fig. 5). Whether this difference is associated to the faster inactivation of water to Q_A electron transfer remains to be elucidated. However, it is clear that these two modifications are due to the mutations present in the D_1 protein of AzV.

Acknowledgements

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