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Mutation in cysteine bridge domain of the γ-subunit affects light regulation of the ATP synthase in *Arabidopsis*

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Introduction

The proton-translocating F_0F_1-ATPsynthase is a ubiquitous, multisubunit enzyme present in the plasma membrane of eubacteria, the inner mitochondrial membrane, and the photosynthetic membranes of higher plants, algae and cyanobacteria. While the activities of all ATP synthases are intricately regulated, redox regulation is a unique feature of the chloroplast ATP synthase from algae and higher plants. The CF_1 γ–subunit contains an intrapeptide disulfide bond between Cys-199 and Cys-205, which is located in an extra domain of the chloroplast subunit that is not present in mitochondrial or bacterial γ–subunit sequence (Miki et al., 1988). This cystine disulfide is the target of thioredoxin in the physiological reduction pathway (Shahak, 1982; Vallejos et al., 1983; Ketcham et al., 1984). The reduction of the disulfide bond is thought to cause a conformational change within the enzyme complex thereby decreasing the magnitude of the proton motive force required to maintain a catalytic activity (Mills et al., 1984). Site-directed mutagenesis experiments with *Chlamydomonas reinhardtii* showed that a serine substitution of the cysteine in the regulatory bridge domain results in the lost of redox modulation of ATP synthase activity (Ross et al., 1995). In order to further investigate the significance of the regulatory domain and the physiological significance of redox modulation, we are using genetic approaches to gain further insight into the critical process. We have designed four site-directed mutants in *Arabidopsis* of the γ-subunit gene, designated as C199S, C205S, C199S/C205S and Del (full deletion of regulatory domain).

Materials and methods

λC31 (Inohara et al., 1991) containing the atpC1 gene insert was subcloned into the multi-cloning Xba I site of PBI-121. The atpC1 gene encoding the ATP synthase γ-subunit was positioned downstream of the 35S promoter. Using QuikChange Site-Directed Mutagenesis kit (Stratagene) and the oligonucleotides, 5'-ATCTCTGACATTAATGGAACCTGTGTG-3', 5'-ATCTGTGACATTAATGGAACCTCCG-TG-3' or 5'-AT CTCTGACATTAATGGA ACCTCTGTG-3', the cysteine residues at the position 199, 205 or both of the atpC1 gene were substituted with serine residues by using PBI-121 containing the atpC1 gene insert. Those three different mutations were designated as C199S, C205S and C199S/C205S (Fig. 1) and validated by restriction digestion analyses since we intentionally created novel restriction enzyme digestion patterns for each construct. The forth mutation designated as Del was generated by deleting 9 amino acid residues in the regulatory domain including the two cysteine residues using the oligonucleotide 5'-CCTTTATCACCTAAAGGAGTGGATGCT

GCGGAAGAT-3'. These mutations were used to transform wild type Arabidopsis (Columbia ecotype) plants by vacuum infiltration (Clough et al., 1998). Mature seeds were harvested and screened on LB agar plates containing 50 μg/ml kanamycin. After 10 d, green seedlings were transferred to commercial potting soil.

The expression levels of atpC1 transgenes and the endogenous gene in T_0 plants were determined by RT-PCR followed by restriction analysis. Leaves from 3 week old plants were harvested and the total RNA isolated using TRIZOL Reagent (GIBCOBRL). All reverse transcription PCR reactions were performed by using MMLV-RT (Promega) to synthesize first-strand DNA that covered the regulatory domain followed by amplification of the cDNA template with Taq DNA polymerase. Primers used to amplify the mRNA were gamma 1 (5'-TCTGTTTCACCACTCCAAGCGTCT-3') and gamma 2A (5'-TCAAAGAGGGTTCAAAA CAAATCAAAC-3'). Tsp45 I was used to digest the RT-PCR products of C199S, C199S/C205S and Del, and Bsa JI was used to digest C205S product. The restriction products were separated on 1% agarose gel.

ΔA_{518} relaxation kinetics in the wild type and transgenic plants were measured at room temperature. Four week old plants were dark-adapted for 24 h to ensure that the γ-subunit regulatory cysteine disulfide bonds were fully oxidized. Then detached leaves were taken to measure the ΔA_{518} relaxation kinetics as described by Gabrys et al. (1994). The electrochromic changes were induced by a single 2 μs red flash. The reduction of γ-subunit was induced by 3 s of pre-illumination with continuous 65 μmol quanta m^{-2} s^{-1} red light. Thereafter the leaf was dark-readapted for 4 min to allow dissipation of the $\Delta\mu_{H^+}$ formed during the pre-illumination.

Results and discussion

Figure 1 shows the four mutant constructs that were generated by PCR and transformed to the wild type *Arabidopsis* plants.

Arabidopsis C1 TLLPLSPKGE I **CD I NGTC**VDAAEDEFF

C199S TLLPLSPKGE I **S D I NGTC**VDAAEDEFF

C205S TLLPLSPKGE I **CD I NGT S**VDAAEDEFF

C199S/C205S TLLPLSPKGE I **S D I NGT S**VDAAEDEFF

Del TLLPLSPKG - - - - - - - VDAAEDEFF

Fig. 1. Partial amino acid alignments of γ-subunits of four *Arabidopsis* mutants, designated as C199S, C205S, C199S/C205S and Del were generated and used to transform to wild type plants. Amino acid positions deleted are shown as broken lines.

In order to quantify the expression of the transgenes we introduced new restriction patterns into the mutated gene constructs. Total RNA of T_0 transgenic plants was isolated and the expression levels of transgenes and endogenous genes were identified by RT-PCR following by restriction digestion. For the wild type plant, the1010bp product has two Tsp45 I sites and digestion results in 262bp, 380bp and 369bp fragments, whereas for C199S and C199S/C205S, their products lose a Tsp45 I site and result in 262bp and 749bp fragments. Similar results happen to Del transgenic plants except that the PCR products are 983bp fragments instead of 1010bp and the restriction products are 722bp instead of 749bp. In transgenic plants, 749bp bands are much brighter than 369bp and 380bp bands indicating that

the transgenes are more strongly expressed than original genes (Fig. 2). In C205S, we introduced a Bsa JI site that results in two intense bands, 378bp and 632bp. But the 1010bp bands are also intense due to the incomplete digestion. The results showed the transgenes were over expressed behind a constitutive 35S promoter, and the expression level is much greater than that of endogenous γ-subunit gene.

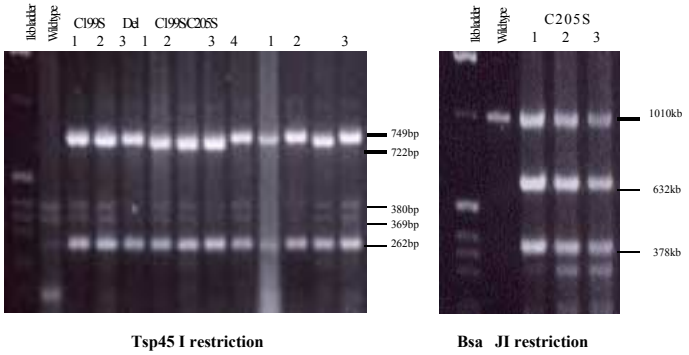

Figure 2. RT-PCR and restriction analysis of transgenic plants. The mRNA was isolated from transgenic plants and the fragments containing regulatory site were amplified by RT-PCR. Thereafter, the products were digested with the indicated restriction enzyme.

Table 1. ΔA_{518} relaxation time constants for wild-type and site-directed *Arabidopsis*. The plants were first dark adapted for 48 h (D_{ad}), followed by 3 s pre-illumination at the intensity of 65 μmol m^{-2} s^{-1}. The plants were then re-dark-adapted for 4 min.

Mutant type T_1	Plant number	Relaxation Time Constant (ms)
Wild type		**174 ± 44**
C199S	**1**	**85 ± 31**
	2	**88 ± 21**
	3	**76 ± 34**
C205S	**1**	**91 ± 34**
	2	**114 ± 66**
	3	**138 ± 36**
C199S/C205S	**1**	**82 ± 40**
	2	**77 ± 20**
	3	**84 ± 28**
Del	**1**	**84 ± 40**
	2	**102 ± 54**
	3	**106 ± 48**

Due to gated proton efflux through the ATP synthase, kinetic ΔA_{518} measurements can be employed to monitor the activation and reduction state of the enzyme (e.g., Gabrys et al., 1994). The ΔA_{518} relaxation kinetics of the dark-adapted transgenic mutant plants are summarized in Table 1. The results showed that the ΔA_{518} relaxation kinetics of the

transgenic mutant plants were faster than that of wild type indicating that the behavior of an unregulated enzyme caused by transgenes. This illustrates that at least a significant portion of the ATP synthase in the transformed plants were assembled using the engineered γ-subunits.

Our ongoing work is focusing on bromobimane (mBBr) labeling of cysteine residues to quantify the extent of substitution of the thiol group in γ–subunit of the transformed plants and investigating the physiological consequences of the loss of redox modulation.

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