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# Redox properties of *Arabidopsis* cytochrome $c_6$ are independent of the loop extension specific to higher plants

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**Abstract**

Cytochrome $c_6$ (cyt$c_6$) from *Arabidopsis* differs from the cyanobacterial and algal homologues in several redox properties. It is possible that these differences might be due to the presence of a 12 amino acid residue loop extension common to higher plant cyt$c_6$ proteins. However, homology modelling suggests this is not the case. We report experiments to test if differences in biochemical properties could be due to this extension. Analysis of mutant forms of *Arabidopsis* cyt$c_6$ in which the entire extension was lacking, or a pair of cysteine residues in the extension had been exchanged for serine, revealed no significant effect of these changes on either the redox potential of the haem group or the reactivity towards Photosystem I (PSI). We conclude that the differences in properties are due to more subtle unidentified differences in structure, and that the sequence extension in the higher plant proteins has a function yet to be identified.


*Keywords:* Cytochrome $c_6$; *Arabidopsis*; Protein expression; Spectroscopy; Redox midpoint potential; Laser flash spectroscopy

## 1. Introduction

Cytochrome $c_6$ (cyt$c_6$) is well-known to transport electrons between the cytochrome *bf* complex and Photosystem I (PSI) in the thylakoid lumen of many cyanobacteria and algae [1]. In many organisms, it substitutes for plastocyanin under copper-deficient conditions. It was widely believed that higher plants lacked cyt$c_6$ until genomic evidence and EST sequencing revealed in a wide range of higher plants the existence of a protein with homology to cyanobacterial and algal cyt$c_6$ [2]. The structure and function of cyt$c_6$ and plastocyanin from cyanobacteria and green algae have been thoroughly characterised [3,4]. Although cyt$c_6$ and plastocyanin belong to structurally different classes of protein, the distribution of charged residues and surface properties of the interaction regions are similar [5]. However, *Arabidopsis* cyt$c_6$ and plastocyanin have different distributions of charged residues in the areas believed to interact with the electron transport components, suggesting that cyt$c_6$ is not a simple substitute for plastocyanin. Experiments in vitro and homology modelling comparing cyt$c_6$ and plastocyanin from *Arabidopsis* revealed that cyt$c_6$ is not a suitable donor to PSI [6], contrary to the proposal of Gupta et al. [7] who reported that *Arabidopsis* cyt$c_6$ could substitute for plastocyanin. The former study [6] also found the redox potential of this new cytochrome to be much lower than that of all other known cyt$c_6$ proteins. In addition, a genetic analysis of *Arabidopsis* mutants showed that cyt$c_6$ cannot substitute for plastocyanin [8].

Both *Arabidopsis* cyt$c_6$ and cyanobacterial/green algal cyt$c_6$ contain the haem-binding motif CxxCH, characteristic of *c*-type cytochromes, and also an iron-ligating methionine. In addition, modelling has suggested that the plant and cyanobacterial/algal proteins have very similar secondary structures. However, higher plant cyt$c_6$ contains an additional stretch of 12 amino acids (–EKCTPRGQCTFG– in *Arabi-*

*Abbreviations:* cyt$c_6$, cytochrome $c_6$; $E_m$, redox midpoint potential; Pc, plastocyanin; p*I*, isoelectric point; PSI, Photosystem I

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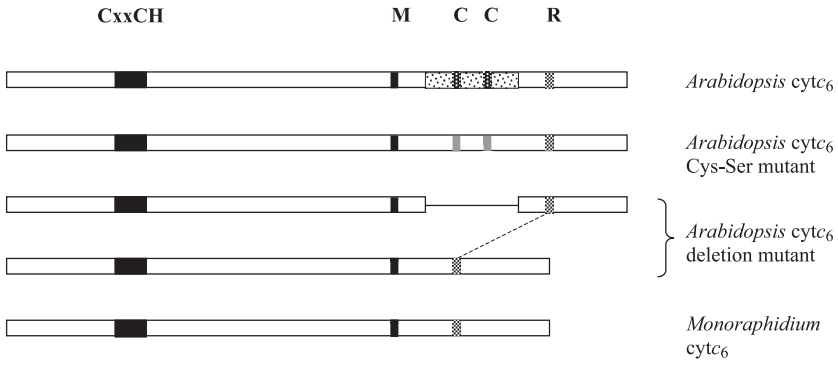


Fig. 1. Schematic overview of the *Arabidopsis* cyt$c_6$ and mutant protein forms used in this study and the main characteristic sequence motifs/features. Depicted are the haem binding motif (CxxCH, M), the 12 amino acid extension (dotted), the cysteine residues (C), the serine residues (grey) and the arginine residue (R).

*dopsis*) that is highly conserved amongst all known higher plant sequences. This stretch forms an extension to a loop found in all cyt$c_6$. Within the stretch are two cysteine residues that are absolutely conserved in the same position in all plant sequences [2]. Furthermore, the extension shifts the position relative to the iron-ligating methionine residue of a conserved arginine residue that has been shown to be involved in the interaction and electron transfer between cyt$c_6$ and PSI in *Anabaena* [9]. Therefore, it is possible that this loop extension, which is found in all higher plant cyt$c_6$: is the main reason why the electron transfer properties of cyt$c_6$ from *Arabidopsis* and from algae (or cyanobacteria) differ so markedly. However, homology modelling suggests that the extra loop extension is unlikely to influence the redox properties of the protein significantly, as it would be unlikely to interact with the haem, so as to change the surface properties significantly. To test this, mutants of *Arabidopsis* cyt$c_6$ were generated in which either the entire extension of 12 amino acids was deleted or the two cysteine residues in the extension were changed to serine residues (Fig. 1). Mutant and wild-type proteins were compared with regard to midpoint potentials, p*I* and the kinetics of reduction of *Arabidopsis* PSI.

## 2. Materials and methods

### 2.1. Protein expression

Recombinant cyt$c_6$ was obtained by expression of a synthetic gene containing the coding region of mature *Arabidopsis* cyt$c_6$ fused to that for the signal peptide from *Anabaena* cyt$c_6$ [10]. The synthetic gene was designed by back-translation with the codon usage of *Escherichia coli* and the amino acid sequences of *Arabidopsis* cyt$c_6$ and *Anabaena* signal peptide as template [6,10]. The sequence of the *Arabidopsis* cyt$c_6$ synthetic gene was: ATGAAAAAAATTTTCTCTCTGGTGCTGCTGGGTATCGCGCTGTTCACGTTCGCGTTCTCGTCCCCGGCGCTGGCGCAGACGCTGGATATTCAGCGTGGTGCGACCCTGTTTAACCGGGCGTGTATTGGTTGTCATGATACGGGCGGCAATATTATCCAGCCGGGTGCGACCCTGTTTACCAAAGATCTGGAACGCAACGGCGTGGATACCGAAGAAGAAATTTACCGCGTGACCTATTTCGGCAAAGGCCGCATGCCGGGCTTTGGCGAAAAATGCACCCCGCGTGGTCAGTGCACCTTTGGCCCGCGTCTGCAGGATGAAGAAATCAAACTGCTGGCGGAATTTGTGAAATTTCAGGCGGATCAGGGCTGGCCGACCGTGTCAACCGATTGATGA. The same principle was used to design a synthetic gene for the *Arabidopsis* plastocyanin (main isoform): ATGAAAAAAATTTTCAGTCTTGTGCTTCTTGGTATTGCCCTTTTTACCTTTGCGTTCAGTAGCCCGGCCCTTGCGATTGAAGTGCTTCTTGGTGGTGGCGACGGCAGCCTTGCCTTCATTCCGAACGATTTTAGTATCGCGAAAGGTGAAAAAATTGTGTTTAAAAACAATGCGGGTTACCCGCACAATGTGGTGTTCGATGAAGACGAAATCCCGAGTGGCGTGGATGTGGCGAAAATCAGTATGGACGAACAGGATCTTCTTAATGGTGCGGGTGAAACCTATGAAGTGGCGCTTACCGAACCGGGCACCTACAGTTTTTATTGCGCGCCGCATCAAGGTGCGGGCATGGTGGGTAAAGTGACCGTGAACTGATGA. In addition, an RBS at the 5′-end and a *Sal*I restriction site at the 3′-end were created for each gene. The designed genes were made in a single PCR step using two conventional primers and four oligonucleotides of ca. 110 bases as templates, with overlaps of 10–20 base pairs. The PCR was performed in a Mastercycler 5330 (Eppendorf) thermocycler. The amplified sequences were cloned in the pGEM-T vector (Promega), transformed in *E. coli* XL1Blue and the insert cloned into the expression vector pBluescriptII SK(+) (Stratagene). Constructs generated using PCR were sequenced to check that no mutations had been introduced. The synthetic genes were expressed in *E. coli* GM119. *Arabidopsis* cyt$c_6$ and plastocyanin were expressed and cyt$c_6$ purified as described previously for *Synechocystis* cytochrome $c_M$ [10]. Plastocyanin purification was also carried out as previously described [10] with two modifications: (i) plastocyanin was eluted from the DEAE-cellulose column with a linear gradient of 5–500 mM NaCl, and (ii) after resolution of the chromatofocusing step, plastocyanin remained bound to the column and was eluted with a 0–300 mM NaCl gradient. The final protein yield amounted to

13 mg of cyt$c_6$ and 25 mg of plastocyanin from 15-l *E. coli* cultures. The extinction coefficient of the *Arabidopsis* cyt$c_6$ at 554.5 nm (26 $mM^{-1}$ $cm^{-1}$) was determined using the pyridine haemochrome method [11]. In order to check that the protein was correctly processed, the N-terminus was sequenced.

The Cys–Ser mutant was constructed by a single PCR step with the QuickChange kit (Stratagene). The deletion mutant was generated with a protocol comprising three PCR steps, using two conventional primers that anneal to the ends of the gene sequence, and two primers that anneal to the same sequence on opposite strands, designed with half of its sequence complementary to each flanking region of the deletion. The two first PCRs were performed in a Mastercycler 5330 (Eppendorf) thermocycler with the expression vector including the fusion gene, in order to amplify each region flanking the deletion. The two products of both PCRs were fused in a third PCR step using the two conventional primers. The constructs were sequenced to check that the mutations had been introduced. Expression and purification were achieved according to the procedures as described above. Yields for the deletion mutant and the Cys–Ser mutant protein were 4 and 2 mg from 10-l *E. coli* culture, respectively.

### 2.2. Preparation of other proteins

*Monoraphidium braunii* cyt$c_6$ was isolated from cell cultures as described previously [12]. Thylakoid membranes from *Arabidopsis thaliana* (var. Columbia) were obtained as described previously [13]. PSI particles were prepared from thylakoid membranes by solubilisation with β-dodecyl maltoside and discontinuous sucrose gradient centrifugation [14] with two modifications: first, membranes were solubilised with 1% maltoside for 20 min, and second, 15%, 20%, 25% and 30% sucrose was used in the gradients. The chlorophyll/P700 ratio of the final PSI sample was 280:1.

### 2.3. Analytical methods

Redox titrations were performed in a dual wavelength spectrophotometer as described previously [15]. Low temperature spectra were recorded in an Applied Photophysics single beam spectrophotometer. Laser flash-induced kinetics of PSI reduction were monitored by following the absorbance changes at 820 nm [16]. The reaction mechanism was analysed according to the kinetic models previously proposed [17].

## 3. Results and discussion

### 3.1. Expression of cyt$c_6$ and mutant proteins

Plant cyt$c_6$ was expressed in *E. coli* using a synthetic fusion gene coding for mature *Arabidopsis* cyt$c_6$ preceded by the signal peptide from *Anabaena* cyt$c_6$, which has recently been shown to direct export of haem proteins to the periplasmic space of *E. coli* cells [10]. The UV/visible absorption spectrum of the purified *A. thaliana* cyt$c_6$ in the reduced form showed typical spectroscopic features of *c*-type cytochromes and was similar to that of *M. braunii* cyt$c_6$ except that the absorption bands of the former were shifted slightly towards the red (Fig. 2A). The α-peak, in particular, is at 554.5 nm. This is in contrast to the value of 553 nm previously reported for *Arabidopsis* cyt$c_6$ expressed in *Synechocystis* sp. PCC 6803 [7], which could be ascribed to the presence of small amounts of *Synechocystis* cyt$c_6$. Expression and purification of the two mutant proteins were performed as described for the wild-type cyt$c_6$. The UV/visible spectra for the extension-deletion

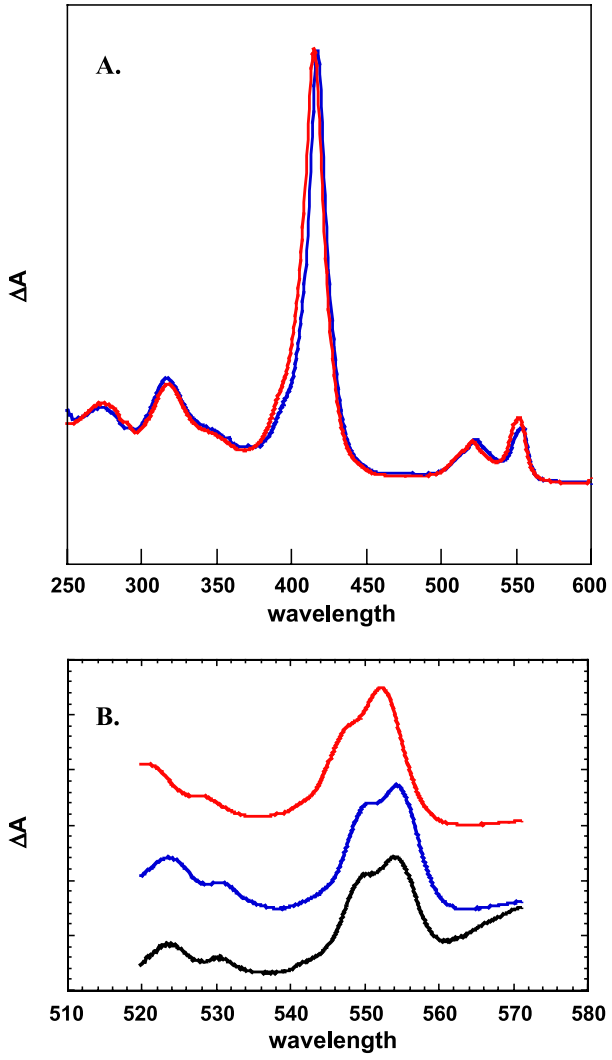


Fig. 2. Spectroscopic analysis. UV/visible absorption spectra of cyt$c_6$ of *Arabidopsis* (blue) and *Monoraphidium* (red) in the reduced state (A). Low temperature spectra of *Arabidopsis* cyt$c_6$ (blue), deletion mutant protein cyt$c_6$ (black) and *Monoraphidium* cyt$c_6$ (red) (B). The low temperature spectra were recorded as redox difference spectra against oxidised protein, respectively. (For color see online version).

mutant and the Cys–Ser mutant were indistinguishable from that of the wild-type protein (data not shown). We also recorded spectra at 77K in order to analyse α-band splitting. Cytochromes, especially those of type *c*, frequently show split α-bands, which is a reflection of the asymmetry of the π electron system of the haem ring. At low temperature, the splitting is more readily observed because of the sharpening of the bands, and there is also a small shift towards the blue. The spectra shown in Fig. 2, and the peak positions recorded in Table 1 indicate that at 77 K, the α-band of cyt$c_6$ was shifted 0.8–0.9 nm to the blue. The splitting of the α-band could be more clearly seen at 77 K and the peak positions indicated that the difference between the main peak and its satellite was close to 3 nm for both *Arabidopsis* and *Monoraphidium* cyt$c_6$. As expected, the loop-extension deletion mutant of *Arabidopsis* cyt$c_6$ showed the same low temperature spectrum as wild-type. These results indicate that the difference in haem environment which is responsible for the red shift of *Arabidopsis* compared to *Monoraphidium* introduces no additional asymmetry. This is in contrast to the spectral behaviour of cytochrome *f* of *Phormidium laminosum* compared to *Chlamydomonas*, where a tryptophan residue in contact with the haem edge in the *Phormidium* protein causes a red shift of the main peak but not the satellite band [18].

### 3.2. Redox midpoint potential

The midpoint redox potential ($E_m$) of *Arabidopsis* cyt$c_6$ was previously shown to be 140 mV, which is ca. 200 mV lower than that of *Arabidopsis* plastocyanin (365 mV) and *M. braunii* cyt$c_6$ (358 mV) [6]. A possible cause of the large difference in $E_m$ between the two types of cytochromes was the presence of the 12 amino acid residue loop in the *Arabidopsis* protein. However, the results of potentiometric titrations of both the extension-deletion mutant and the double Cys–Ser mutant (Table 2) ruled out this possibility. The $E_m$ values for both mutant proteins differed from that of wild-type by 10 mV or less, which would not be enough to influence the reactivity significantly.

Table 1
α-Band absorption maxima of *Arabidopsis* cyt$c_6$ and *Monoraphidium* cyt$c_6$ at different temperatures

| | Maximum 298 K (nm) | Maximum 77 K (nm) | Shift (nm) |
|---|---|---|---|
| *Arabidopsis* cyt$c_6$ | 554.5 | 553.7<br>550.7 | 0.8 |
| *Arabidopsis* cyt$c_6$ deletion mutant | 554.5 | 553.9<br>550.4 | 0.6 |
| *Monoraphidium* cyt$c_6$ | 552.5 | 551.6<br>548.6 | 0.9 |

The table shows the absorption maxima of the alpha bands and the shift. For the low temperature spectra, the satellite peaks are shown, too.

Table 2
Physicochemical properties of wild-type cyt$c_6$ and mutant forms thereof in comparison with green algal cyt$c_6$ and *Arabidopsis* plastocyanin

| Protein | Molecular mass | p*I* | $E_m$ (mV) | $k_2$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|---|
| *Arabidopsis* cyt$c_6$ | 12.3 | 5.1 | +140 | $1.27 \times 10^6$ |
| Deletion mutant | 10.8 | 4.6 | +150 | $1.13 \times 10^6$ |
| Cys–Ser mutant | 12.3 | 5.1 | +130 | $0.84 \times 10^6$ |
| *Monoraphidium* cyt$c_6$ | 9.9 | 3.6 | +358 | $8.8 \times 10^7$ |
| *Arabidopsis* Pc | 10.5 | 4.1 | +365 | $8.7 \times 10^7$ |

The molecular mass, p*I*, midpoint potential and $k_2$ (the second-order rate constant for the reaction with PSI) are displayed for *Arabidopsis* cyt$c_6$, mutant forms thereof, *Monoraphidium* cyt$c_6$ and *Arabidopsis* plastocyanin (Pc), $k_2$ was calculated from the observed rate constant ($k_{obs}$) of the reaction of the protein with *Arabidopsis* PSI after Hervás et al. [17]. $k_2$values for *Monoraphidium* cyt$c_6$ and *Arabidopsis* Pc were calculated from the slope of the initial linear dependence (at low concentrations) of the slow phase for PSI reduction (Ref. [6] and see Fig. 3).

### 3.3. Isoelectric point

The isoelectric points (p*I*) of cyt$c_6$ and plastocyanin show wide variations among different photosynthetic organisms, but are very similar to each other within any one strain [3]. As electrostatic forces are crucial in driving the interactions of plastocyanin and cyt$c_6$ with PSI, the p*I* values of the two proteins should be closely correlated to allow for their physiological exchangeability. Thus among cyanobacteria, the p*I* values of cyt$c_6$ and plastocyanin are 5.6 and 5.5, respectively, in *Synechocystis*, 5.1 and 5.2 in *Phormidium*, 8.0 and 8.4 in *Prochlorothrix*, 9.0 and 8.8 in *Anabaena* [3]. In eukaryotic organisms, the p*I* values of the two proteins are always acidic: 3.6 and 3.7 for cyt$c_6$ and plastocyanin, respectively, in *Monoraphidium*; and 4.2 for spinach plastocyanin. The p*I* value of *Arabidopsis* plastocyanin was determined to be 4.1 (Table 2), which is practically the same as that of spinach plastocyanin, but the p*I* value of *Arabidopsis* cyt$c_6$ is 5.1. This difference in the p*I* of the two *Arabidopsis* proteins already suggests that cyt$c_6$ cannot substitute for plastocyanin in the thylakoid lumen of *Arabidopsis*, which is consistent with the results of two recent studies [6,8]. The *Arabidopsis* cyt$c_6$ with the 12 amino acid extension deleted had a p*I* value of 4.6, and the mutant with the cysteines replaced by serine residues had a value of 5.1. The shift in the p*I* value of the former is best explained by the loss of two basic amino acid residues which are part of the deleted sequence. The p*I* value and the $E_m$ suggest that the two mutant forms of *Arabidopsis* cyt$c_6$ would be no more effective than the wild-type protein in substituting for plastocyanin in normal photosynthetic electron transfer. This was tested directly by measurement of the kinetics of reduction of *Arabidopsis* PSI by the mutants.

### 3.4. Laser flash-induced kinetics of Arabidopsis PSI

The relatively low midpoint potential of *Arabidopsis* cyt$c_6$ renders it a very inefficient electron acceptor from cytochrome *f* ($E_m$ = 320 mV), but might be expected to

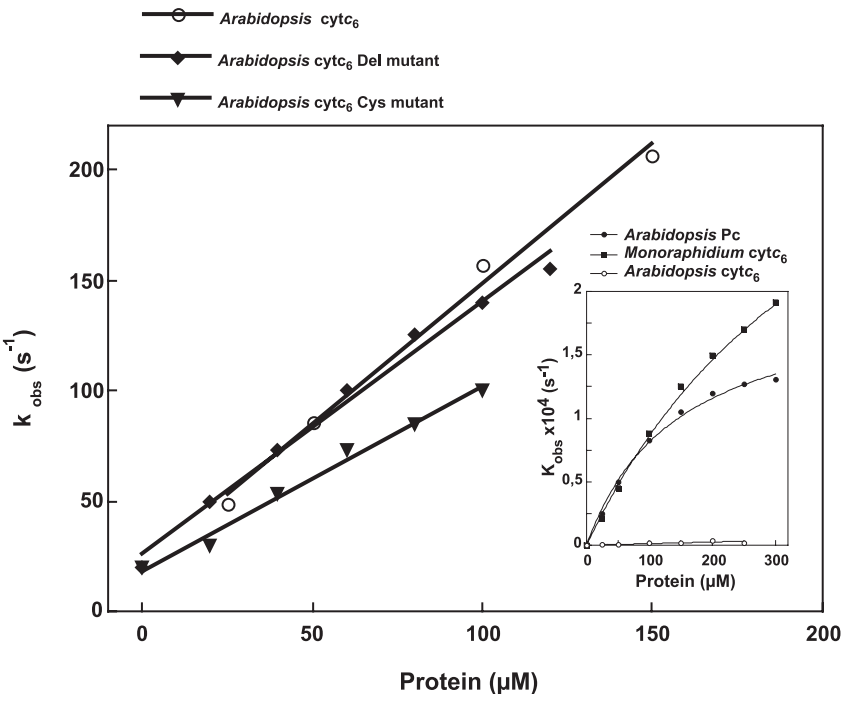


Fig. 3. Reduction of *Arabidopsis* PSI by cyt$c_6$ and mutant forms. Reduction of *Arabidopsis* PSI by *Arabidopsis* cyt$c_6$, the extension-deletion mutant and the double Cys–Ser mutant *Arabidopsis* plastocyanin (Pc), cyt$c_6$ and *Monoraphidium* cyt$c_6$. The figure shows the dependence of the observed rate constant ($k_{obs}$) upon donor concentration. The insert focuses on wild-type *Arabidopsis* plastocyanin (Pc), cyt$c_6$ and *Monoraphidium* cyt$c_6$ [6].

stimulate the reaction with PSI, in accordance with Marcus' theory [19]. In fact, *Arabidopsis* cyt$c_6$ was found to react with PSI 100 times more slowly than either *Arabidopsis* plastocyanin or *Monoraphidium* cyt$c_6$ [6]. This raised the question of whether the presence of the 12 aa residue extension in *Arabidopsis* cyt$c_6$ could be the cause of this slow rate of reaction. The results of a laser-flash kinetic study shown in Fig. 3 demonstrate that removal of the loop extension, or its modification in the Cys–Ser mutant, has little effect on the very slow rate of reaction of the wild-type protein. The overall rate constant showed no increase with increasing ionic strength for the deletion mutant or the Cys–Ser mutant protein (data not shown).

## 4. Conclusions

These results show that wild-type *Arabidopsis* cyt$c_6$ is inappropriate to substitute for plastocyanin, consistent with earlier proposals [6,8]. Furthermore, we have shown that, as predicted by homology modelling, the low redox potential, unsuitable p*I* and lack of reactivity with PSI of the *Arabidopsis* protein are not due to the extension of the loop or to the two cysteine residues within it. Therefore, other features must be responsible for the shift in the midpoint potential of *Arabidopsis* cyt$c_6$ and for its lack of reactivity with PSI. The loop serves another function, which is not yet known. *Arabidopsis* cyt$c_6$ could possibly play a role in transmitting measurement of redox poise, perhaps as part of a control pathway [2,20]. In cyanobacteria and algae cyt$c_6$ is located in the thylakoid lumen and Gupta et al. [7] proposed a lumenal location for *Arabidopsis* cyt$c_6$ too. However, proteomic approaches have not identified cyt$c_6$ in *Arabidopsis* [21,22], or any other higher plant [22]. In silico analysis of the targeting signals also failed to predict consistently the precise location of cyt$c_6$. Whereas ChloroP [23] suggested cyt$c_6$ to be located in chloroplasts, PSORT [24] suggested a location in the mitochondria. Confirming the location will be an important step to identifying the function of the protein.

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