TWO DIFFERENT PLANT-TYPE FERREDOXINS IN EACH OF TWO PETUNIA SPECIES

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1. Introduction

The presence of two different molecular species of ferredoxin has been reported by several authors. The phenomenon has been observed most frequently in bacteria [1–6] but also in blue–green algae [7,8] and some higher plants [9,10].

In the present paper the occurrence of two different plant-type ferredoxins in each of two Petunia species, *Petunia inflata* Fries., and *Petunia axillaris* Lam., is described and discussed. An account is given of their isolation and purification and of a number of physical and chemical characteristics.

The results are taken to indicate a duplication of the ferredoxin gene which occurred before the evolutionary divergence within the Petunia line.

2. Materials and methods

2.1. *Plant material*

P. inflata and *P. axillaris* (inbred lines) were grown in greenhouses. Leaves of two months old were used for the isolation of ferredoxins.

2.2. *Isolation of ferredoxin*

The complete ferredoxin fraction isolated from both Petunia species as described [11] was adsorbed onto a DEAE-cellulose column (70 × 1.5 cm) and eluted very slowly with Tris–HCl, pH 7.5, containing 0.22 M Cl^{-1}. As the elution continued the initially brown band split up in two clearly separated bands which were both isolated.

2.3. *Analysis*

Polyacrylamide gel electrophoresis of the isolated proteins and their tryptic digests was performed as described [11].

S-Carboxymethylation of ferredoxin, digestion with trypsin, and preparation of fingerprints on thin-layer plates were carried out as in [12].

The Ouchterlony double diffusion analysis was performed as in [13], using antibodies raised against *Nicotiana tabacum* ferredoxin [14].

Amino acid analyses were carried out on a Beckman Multichrome-M amino acid analyser following the procedure in [15], using 100 μg protein samples.

Dodecylsulphate electrophoresis was performed according to [16,17].

Optical spectra were recorded on a Perkin Elmer Double Beam 124 spectrophotometer.

$NADP^+$ reduction was determined using the procedure in [18]. The illuminated cuvette contained 100 μg chlorophyll, 0.048 mM dichlorophenol–indophenol, 9 mM ascorbate, 0.6 μmol $NADP^+$ in 30 mM K-phosphate buffer, pH 8.0, containing 400 mM saccharose and 10 mM KCl.

Fe contents were determined as in [19].

EPR-spectroscopy was carried out on a Varian-E3 EPR spectrophotometer.

All chemicals used were of analytical grade.

3. Results

Upon chromatography of ferredoxin on a DEAE-23 column the initially brown–reddish band split up in a

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fast moving major component and a minor component. In both species the major/minor ratio appeared to be about 5:1 (by wt).

When subjected separately to polyacrylamide gel electrophoresis all isolated proteins moved as single brick-red bands close to the marker dye. After staining destaining only one band was seen in all cases, indicating that all proteins were homogeneous.

Results of electrophoresis of tryptic digests on polyacrylamide gels are shown in fig.1. There are unmistakable differences in the peptide patterns of the major and minor components of both Petunia species. Both major components, however, are very similar and the same holds for the two minor ones. Under the conditions described they yield three and two detectable peptide bands, respectively.

Figure 2 shows the results of tryptic peptide mapping of 30 μg samples derived from carboxymethylated proteins. Thin-layer mapping reveals 7 spots in the case of the major and 6 spots for the minor

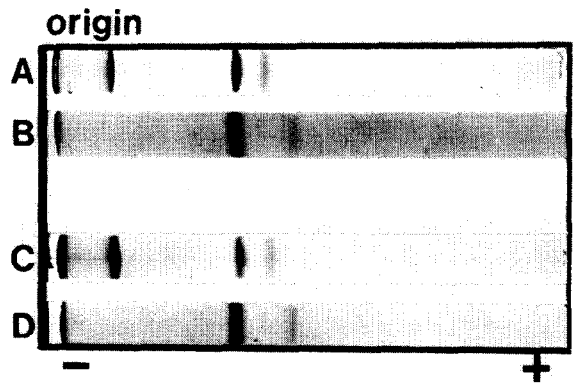

Fig.1. Resolution of tryptic peptides of apoferredoxin obtained from *P. axillaris*, major component (A), minor component (B), and *P. inflata*, major component (C), minor component (D). About 30 μg digest as applied to 15% polyacrylamide gels and electrophoresed at pH 8.6. Only the negatively-charged peptide bands show up on the gel patterns.

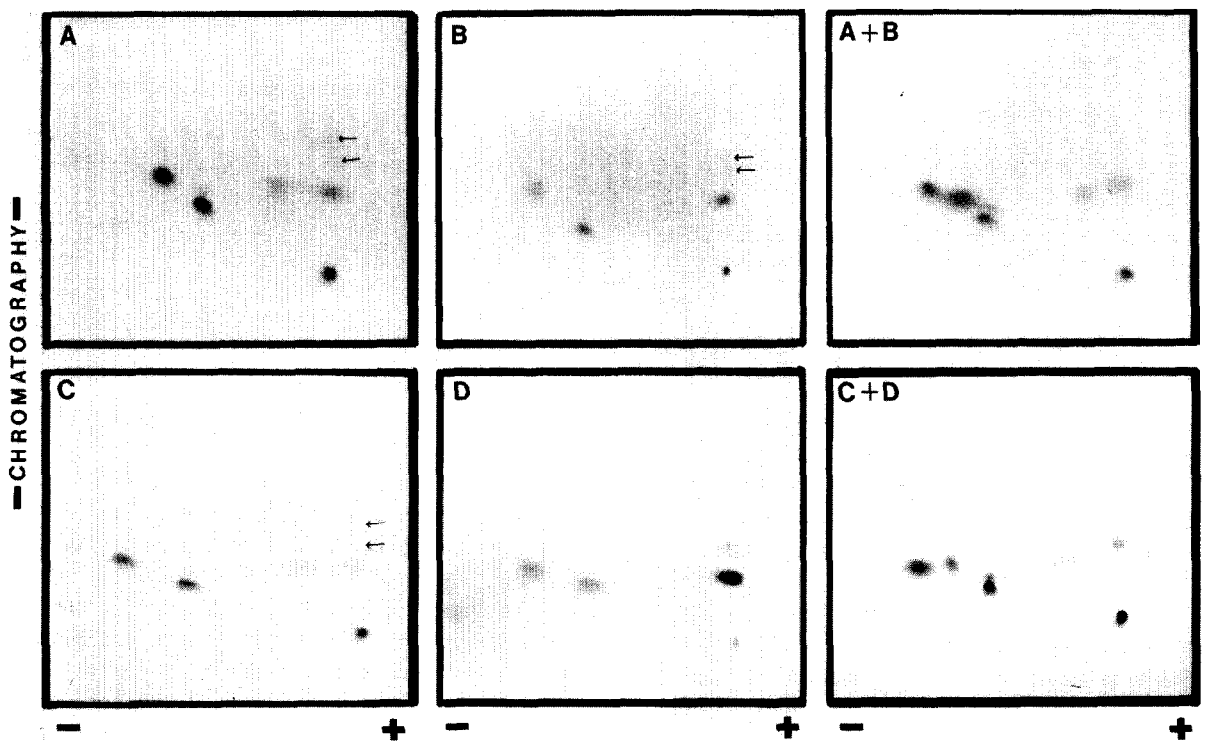

Fig.2. Peptide maps produced from tryptic digests of *S*-carboxy-methylated ferredoxins. (A) major component; (B) minor component; (A+B) mixture of both components derived from *P. axillaris*. (C) major component; (D) minor component; (C+D) mixture of both components derived from *P. inflata*.

Table 1
Amino acid compositions of major and minor components of ferredoxins from *P. inflata* and *P. axillaris*

	Residues in			
	P. axillaris		*P. inflata*	
	Major	Minor	Major	Minor
Asx	14	14	14	14
Thr	6	7	6	7
Ser	6	8	6	8
Glx	10	11	10	11
Gly	7	7	7	7
Ala	7	7	7	7
Pro	5	5	5	5
Val	10	9	10	9
Met	0	1	1	1
Ile	3	3	2	3
Leu	8	7	8	7
Tyr	4	3	4	3
Phe	2	3	2	3
Lys	5	4	5	4
His	3	2	3	2
Arg	1	1	1	1
Cys[a]	5	5	5	5
Trp[b]	1	0	1	0

[a]Cysteine was determined as cysteic acid after performic acid oxidation

[b]Tryptophan was determined spectrophotometrically in apoferredoxin

The data are averages of analyses after 24 h acid hydrolysis, of three different preparations of each ferredoxin being analyzed in triplicate

components. This is in agreement with the differences in lysine residues (see table 1).

The four isolated fractions showed an approximately equal degree of immunological cross-reaction (fig.3). The precipitation lines formed by each antigen are confluent, indicating immunological identity.

Results of the amino acid analyses are given in table 1. The composition of the two minor components is identical. The major components differ by one residue (Met → Ile). More differences do exist between major and minor components. The major ones contain one more lysine and one more histidine than the minor components, which may account for the fact that the major components are eluted sooner from the DEAE column than the minor ones.

The sedimentation patterns of both ferredoxins

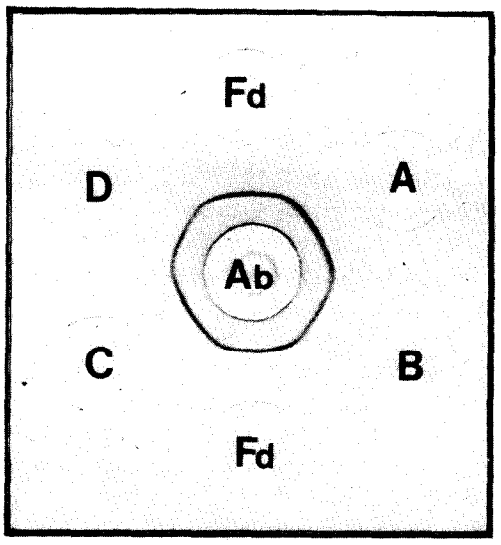

Fig.3. Double diffusion analysis of ferredoxin components obtained from both Petunia species and from *N. tabacum* ferredoxin (Fd) against anti-*N. tabacum* serum (Ab). (A) 4 μg *P. axillaris* major component; (B) 4 μg *P. axillaris* minor component; (C); 4 μg *P. inflata* major component: (D) 4 μg *P. inflata* minor component.

from *P. axillaris* and spinach ferredoxin as determined at 420 nm with an MSE analytical ultracentrifuge are identical, indicating that all three have the same molecular weight. Calculations of the molecular weights from SDS electrophoresis according to [17] yielded values of 10 500 for both components. The results of SDS electrophoresis according to [16] yielded molecular weights of 10 500 and 13 000 for the major and minor components, respectively, (cf. [10]).

Figure 4 shows optical spectra of the major and minor component of *P. inflata.* Absorption maxima are found at 276 nm, 330 nm, 420 nm and 468 nm for both proteins. The difference in A_{280} between both components corresponds to the presence of one Trp residue in the major component. The $A_{420/276}$ ratio of 0.48 and 0.68 for the major and minor component, respectively, is in agreement with spectra of all known plant-type ferredoxins [20]. The same results were obtained with both components of *P. axillaris* ferredoxin.

The four proteins were capable of catalyzing $NADP^+$ photoreduction and a rate of 0.05 μmol $NADP^+$/min/100 μg chlorophyll was obtained for all proteins.

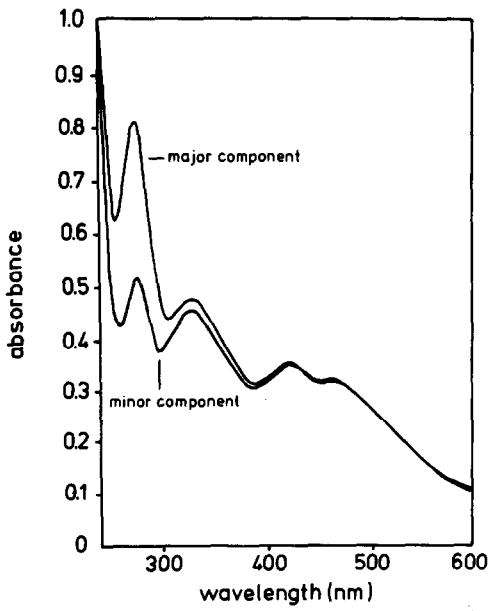

Fig.4. Absorption spectra of the major and minor component in the oxidized form obtained from *P. inflata* ferredoxin. The concentration was about 450 μg/ml for both components.

Fe contents of the major components appeared to be 1.92, 1.95, and 1.95 and 1.96 for the minor components. These values express the number of Fe-atoms/molecule protein.

EPR spectra of the major and minor components of *P. inflata* are given in fig.5. The spectra appear to be identical and the g value of 1.96 (g_x = 1.887, g_y = 1.947, g_z = 2.042) corresponds with the value found for 4Fe–4S chromophores as well as for 2Fe–2S chromophores [21]. The EPR spectra of the proteins obtained from *P. axillaris* gave the same value.

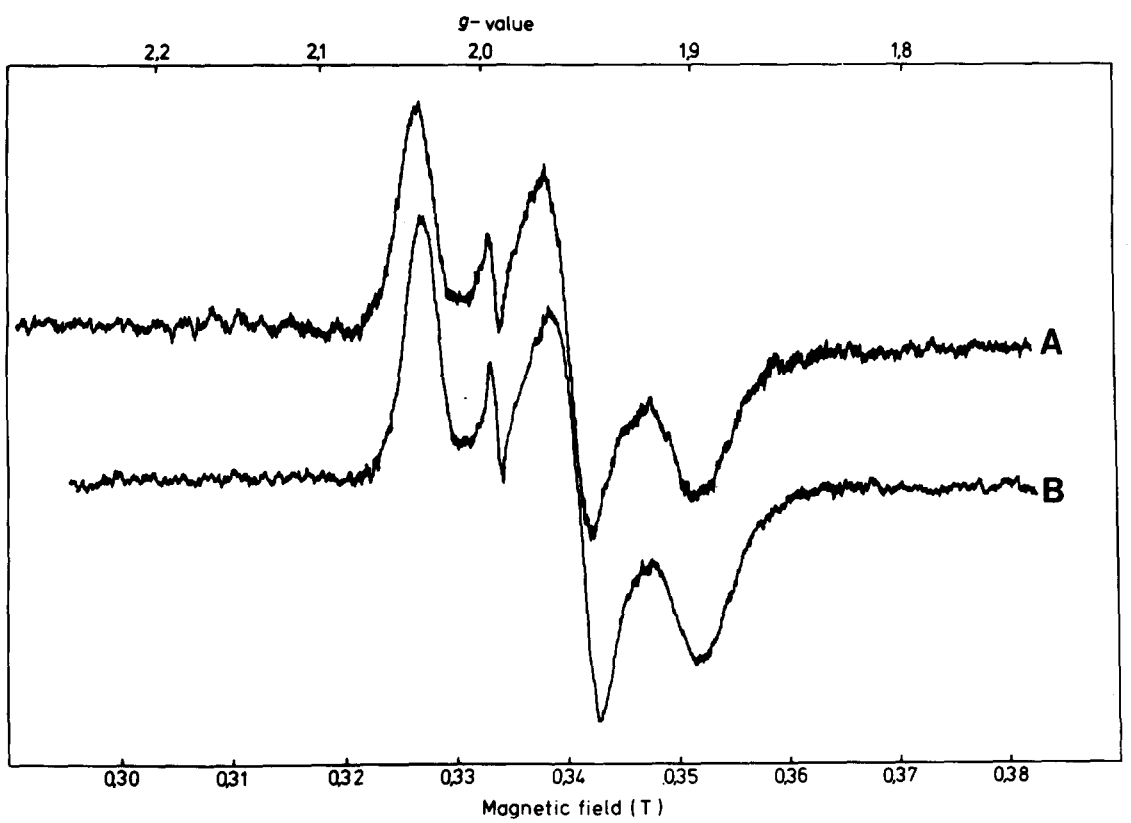

Fig.5. EPR spectra of major (A), and minor component (B), of *P. axillaris*, conditions of measurements were: frequency 9.34 GHz; modulation amplitude 1.25 mT; temp. 24°K, scan speed 25 mT/min.

4. Discussion

All four isolated proteins show a striking resemblance to each other as for biological activity, EPR and optical spectra and cross-reactivity to ferredoxin antibodies. The number of five cysteine residues and two iron atoms/molecule, indicate the existence of a 2Fe–2S chromophore. We may conclude that all proteins analyzed are plant-type ferredoxins.

That both Petunia species contain two distinct ferredoxins was apparent from the behaviour on DEAE-cellulose, the peptide analysis on acrylamide gels, fingerprinting on cellulose plates, and difference in absorption ratio. Moreover, the amino acid analyses revealed that in each of the two Petunia species the major and minor components differ considerably in their compositions, whereas both minor components are identical and both major components differ in only one residue. The authors would like to suggest the following explanation for this phenomenon. By a duplication of the ferredoxin gene in the common ancestor of both Petunia species, there arose two separate identical ferredoxin genes, which as a result of mutations gradually became different, resulting in the production of two unmistakably different ferredoxins. Assuming that the two Petunia species evolved relatively recently from their common ancestor explains why the amino acid compositions of both minor ferredoxins are still identical, whereas in the major components one Met residue is replaced by an Ile residue.

This is the first report of the isolation and detailed characterization of two plant-type ferredoxins in each of two species of a higher plant.

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