Structure requirement and identification of a cryptic cleavage site in the mitochondrial processing of a plant F_1-ATPase β-subunit presequence

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Abstract We sought to determine the structural features involved in the processing of the mitochondrial F_1-ATPase β-subunit (F_1β) presequence (54 residues) from *Nicotiana plumbaginifolia*. The cleavage efficiency of F_1β presequence mutants linked to the green fluorescent protein (GFP) was evaluated in vivo in tobacco by in situ microscopy and Western blotting. The residue at position −1 (Tyr) was required to be an aromatic residue and the residue at position +2 (Thr) was found to be important for F_1β processing, while, unexpectedly, changing the distal (Arg-15) and proximal (Arg-5) arginine residues did not strongly reduce processing. In addition, results also supported the requirement of a helical structure around the cleavage position. Sequencing of the mature form of a precursor containing the first 30 residues of the F_1β presequence linked to GFP revealed the presence of a cryptic cleavage site between residues 26 and 27, which showed the features of a classical mitochondrial processing site, suggesting dual processing of the F_1β presequence. In vitro processing confirmed these data and showed that processing was sensitive to *o*-phenanthroline, thus catalyzed by mitochondrial processing peptidase.

Key words: Mitochondrial import; Processing; Cryptic cleavage; Plant; Mitochondrial processing peptidase

1. Introduction

Most of the mitochondrial proteins are synthesized in the cytosol as precursor proteins with an N-terminal extension called the presequence which is involved in mitochondrial import. The precursor is then cleaved off by a specific mitochondrial processing peptidase (MPP) releasing the mature protein from its N-terminal extension. MPP is a metalloendopeptidase constituted of two subunits (α and β) which are localized in the matrix in yeast and rat species and identified as the core I and core II proteins of the respiratory cytochrome bc_1 complex in plants (reviewed in [1]).

MPP recognizes a large variety of precursor proteins. Sequence comparison of the mitochondrial presequences revealed three amino acid patterns (R-2, R-3 and R-10) defined by the position of the last arginine residue before the cleavage point and a few conserved residues around it: RX↓XS, RX(Y/L)↓(S/A) and RX↓(F/I/L)(S/A)XXXXXX↓X, respectively [2,3]. The R-2 and R-3 motifs are related to a single processing by MPP, while the R-10 motif, not identified in plants so far [3], corresponds to a dual cleavage of the presequence by MPP and mitochondrial intermediate peptidase.

Nuclear magnetic resonance studies revealed that synthetic peptides to be cleaved by MPP have the potential to form a helix-linker-helix structure in which the linker acts as a helix breaker [4,5]. The role of the linker region was also demonstrated by the fact that glycine and proline residues inside it were important for the presequence cleavage [6] and that removing the linker prevented processing [7]. Moreover, a proximal arginine residue located at position −2 or −3 from the cleavage site and a distal basic residue located around position −10 are necessary for effective processing [6,8–10]. This means that the presequences belonging to the R-2 and R-3 categories, referred to before, often possess an arginine residue 6–16 residues before the cleavage site [11]. In addition, a hydrophobic or an aromatic residue at position +1 and a serine or threonine residue at position +2 and/or +3 are required [12,13].

Most of the studies on presequence processing have been made in vitro and hardly any were made on plant mitochondrial presequences which have been demonstrated to be longer and to have a higher serine content [13]. Processing assays with purified spinach mitochondria and mutagenized alternative oxidase presequences showed that modifying the Arg-2 had a drastic effect on processing [14]. Competition studies showed that a helical element in the C-terminal domain of a plant presequence was important for MPP recognition [15]. Therefore, to determine essential domains involved in the processing of a plant presequence, we carried out an in vivo mutagenesis study of the cleavage efficiency of the mitochondrial F_1-ATPase β-subunit (F_1β) presequence from *Nicotiana plumbaginifolia*. This demonstrated the essential role played by the tyrosine residue located at position −1 relative to the cleavage site, supported the requirement of a helical structure around the cleavage and revealed an additional cleavage in the middle of the F_1β presequence.

2. Materials and methods

2.1. Plasmids and DNA constructs

Plant transient (F_1β-green fluorescent protein (GFP) and GFP) or stable (st-F_1β-GFP, st-βΔ30-GFP and st-GFP) expression vectors were as described in [16]. Site-directed mutagenesis of the presequence was performed by triple PCR, using a 5′ primer located around the *Bgl*II site, a 3′ primer at the beginning of the *gfp* coding sequence and

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Abbreviations: F_1β, F_1-ATP synthase β-subunit; MPP, mitochondrial processing peptidase; GFP, green fluorescent protein

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pairs of internal primers containing the modified nucleotides. Amplified fragments were digested by *Kpn*I and *Bgl*II, cloned in $F_1\beta$-GFP by replacement of the wild-type presequence and sequenced.

2.2. Epifluorescence microscopy analysis

Epifluorescence microscopy was performed with a Leica DMR microscope featured with the Leica DC200 camera and a GFP filter set.

2.3. Protein analysis of the protoplasts

Transient expression was carried out in electroporated protoplasts [16]. Proteins from homogenates of 1.2×10^6 electroporated protoplasts were resolved on a 10–15% polyacrylamide–SDS gel and transferred onto a nitrocellulose membrane, which was then successively incubated with antibodies against GFP [16] and [^{125}I]protein A. Signals were detected and quantified by a phosphorimager (Bio-Rad GS-525 Molecular Imager® System).

2.4. Mitochondria purification

Mitochondria from tobacco protoplasts or plants were purified as described in [16,17]. Mitochondrial membrane proteins were obtained by sonicating purified mitochondria four times for 10 s and centrifuging the sample for 20 min at $100\,000\times g$.

2.5. In vitro processing

$F_1\beta$-GFP and $F_1\beta$-GFP mutants were processed according to [18]. The reaction mixture contained 4 μl ^{35}S-labeled precursor (synthesized in a reticulocyte lysate), 40 μg mitochondrial membrane proteins, 10 mM HEPES (pH 7.7), 2.5 mM $MnCl_2$, 1.25 mM phenylmethylsulfonyl fluoride, 1% Triton X-100 in a final volume of 20 μl. In some cases, mitochondrial membranes were preincubated for 10 min at 25°C with 20 mM 1,10-*o*-phenanthroline (10 min at 25°C). Processing was performed at 25°C for 40 min. Samples were analyzed by SDS–PAGE and the signals were treated by a phosphorimager.

2.6. Phenol extraction of proteins

Plant material (1 cm^2 leaf disk) was frozen in liquid nitrogen, ground and mixed with 300 μl phenol [19]. The aqueous phase was treated a second time with phenol and the proteins were precipitated by adding five volumes of cold precipitation buffer (0.1 M NH_4-acetate in methanol). Proteins were finally recovered by centrifugation ($7500\times g$ for 10 min).

3. Results

3.1. Transient expression of $F_1\beta$ processing mutants in tobacco protoplasts

Secondary structure prediction and alignment with 10 plant $F_1\beta$ presequences showed that, in addition to the typically well conserved N-terminal region of the presequence encompassing the amphiphilic α-helix, the amino acid sequence located between residues 47 and 60 and surrounding the cleavage site was well conserved and predicted to adopt an α-helix (Fig. 1).

Mutations were obtained for residues Arg40, Pro43, Leu47, Arg50, Tyr54, Ala55 and Thr56 which are well conserved and

Fig. 1. Secondary structure prediction and sequence conservation of the *N. plumbaginifolia* $F_1\beta$ presequence. The sequence of the first 66 residues of the *N. plumbaginifolia* $F_1\beta$ presequence is displayed. The arrow indicates the cleavage site and stars the residues that are conserved in at least nine among the $F_1\beta$ plant presequences available in the database. Below is shown the α-helix structure predicted according to Garnier et al. [22].

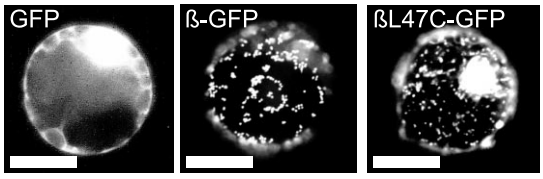

Fig. 2. In situ analysis of protoplasts expressing GFP constructs. Fluorescence microscopic images of tobacco protoplasts expressing GFP alone (GFP), GFP linked to the first 66 residues of the $F_1\beta$ presequence or to the L47C mutant. The bars correspond to 25 μm.

suspected to play an important role in the cleavage according to the proposed model [2].

The modified $F_1\beta$ presequence (54 residues of the presequence and 12 residues of the mature protein) was linked to the reporter *gfp* and expressed transiently in electroporated tobacco protoplasts. Mitochondrial import of GFP was checked by fluorescence microscopy and compared with two controls: GFP linked to the unmodified $F_1\beta$ presequence and GFP alone (Fig. 2). Reduction of mitochondrial import was considered when more than 20% of the transformed cells showed GFP in the cytosol and nucleoplasm, in addition to mitochondria. Fluorescence microscopy showed that all the $F_1\beta$ presequence mutants behaved like the wild-type presequence ($F_1\beta$-GFP, Fig. 2) except for the L47C mutant for which import was partial (Fig. 2).

Cleavage of the mutants was followed by immunodetection of the GFP mature and precursor forms in a protoplast homogenate. The signals were quantified by image analysis and cleavage efficiency was calculated as the ratio between the mature form and the total GFP detected (mature and precur-

Table 1
Cleavage efficiency of the mutated presequences[a]

Construct	Cleavage ratio (%)
$F_1\beta$-GFP	100
βR40F	83*
βP43S	96
βP43N	117
βL47C	95 (+C-ter)
βR50N	88
βR50D	80*
βR50F	106
βR50L	81*
βY54N	43* (+N-ter)
βY54H	41*
βY54L	78*
βY54V	58*
βY54D	53* (+N-ter)
βY54F	107
βA55C	100 (+N-ter)
βA55V	90
βA55N	98
βA55R	93 (+C-ter)
βA55G	101
βT56C	79*
βT56D	45*
βT56Y	86
βT56N	80*

[a]Cleavage ratio was calculated as the ratio between the mature and the total GFP detected (mature and precursor forms) reported as a percentage of the value obtained with the control $F_1\beta$-GFP. N-ter and C-ter indicate that cleavage occurred upstream or downstream of the normal cleavage site, respectively. Asterisk indicates significant decrease in cleavage efficiency (Student's *t*-test, $\alpha=0.01$) compared to the control $F_1\beta$-GFP.

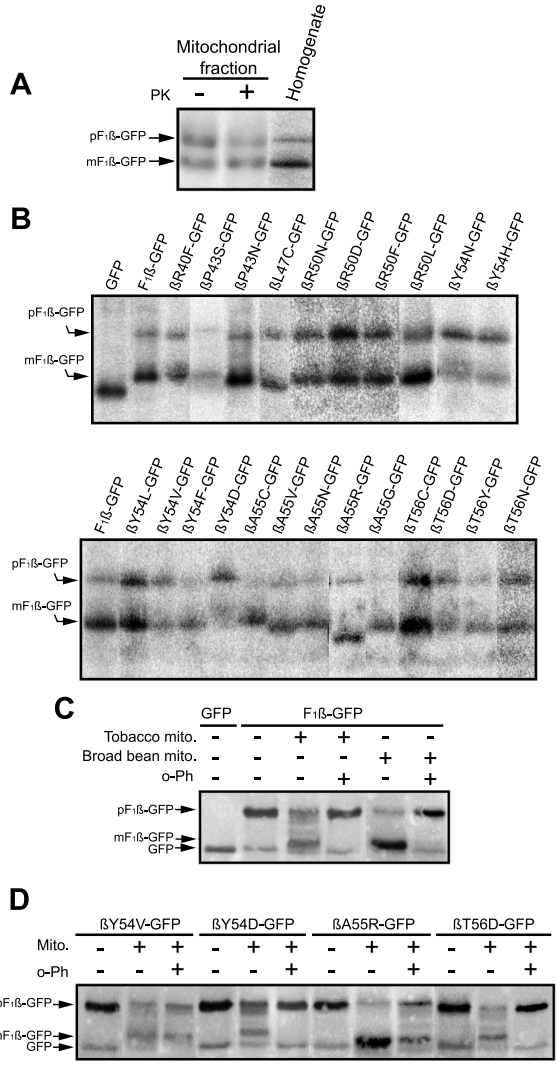

Fig. 3. Western blotting analysis of the $F_1\beta$ presequence mutants in a transient expression system and in an in vitro processing assay. A: The precursor form of $F_1\beta$-GFP in protoplast homogenates is located inside mitochondria. A crude mitochondrial extract isolated from $F_1\beta$-GFP-transformed protoplasts (1.2×10^6 cells) was treated or not with proteinase K (PK) (50 μg/ml) for 15 min on ice, washed and its protein content was resolved by SDS–PAGE and GFP Western blotting. A homogenate from $F_1\beta$-GFP-transformed protoplast is shown as a control. B: Homogenates of protoplasts expressing transiently mutant $F_1\beta$-GFP constructs were electrophoresed and subjected to Western blotting. Positions of mature ($mF_1\beta$-GFP) and precursor ($pF_1\beta$-GFP) forms of $F_1\beta$-GFP are indicated. C: In vitro cleavage of $F_1\beta$-GFP. ^{35}S-labeled precursors were incubated with 40 μg of tobacco or 20 μg of broad bean mitochondrial membrane proteins for 40 min at 25°C in the presence or not of 20 mM *o*-phenanthroline (o-Ph). D: In vitro cleavage of the βY54V, βY54D, βY55R and βT56D mutants linked to GFP. Processing was performed as in C with tobacco mitochondrial membrane proteins. For C and D, positions of GFP and the precursor and mature forms of $F_1\beta$-GFP are indicated.

sor forms) reported in percentages of the value obtained with the control $F_1\beta$-GFP. Values came from three independent experiments and the significance of the cleavage reduction was estimated by Student's *t*-test ($\alpha = 0.01$) (Fig. 3B, Table 1).

About 20% of the control $F_1\beta$-GFP was found as a non-cleaved precursor. To rule out the possibility that this band was bound outside the mitochondria and thus not imported, a crude mitochondrial extract was subjected to proteinase K treatment. GFP immunodetection of Western-blotted mitochondrial protein revealed that the precursor form was detected in both treated and non-treated proteinase K mitochondrial fractions (Fig. 3A).

Modification of the distal Arg40 into Phe and the proximal Arg50 into Asp and Leu resulted in a slight reduction of the cleavage efficiency. The R50N and R50F mutations did not affect the cleavage significantly. Furthermore, neither did modification of Pro43, located between the two arginine residues, show any reduction in cleavage activity (Fig. 3B, Table 1). These arginine and proline residues have therefore a weak role in $F_1\beta$ processing.

Mutation of the conserved Leu47 into Cys resulted in a smaller mature form, indicating a downstream shift of the cleavage position (Fig. 3B).

Modification of Thr56 (position +2 compared to the cleavage site) led to a slight reduction of cleavage with the T56C and T56N mutants. The T56D mutant induced a sharp decrease in the cleavage reaction while the T56Y mutant had a non-significant decrease. Presence of a hydroxylated or an aromatic residues at +2 is thus preferred to other residues (Fig. 3B, Table 1).

Mutation of Ala55 resulted in an upstream and downstream shift of the cleavage site in the case of A55C and A55R, respectively. The A55V, A55N and A55G mutations had no effect on cleavage (Fig. 3B, Table 1).

All the mutations affecting Tyr54 altered cleavage efficiency, except for Y54F. Strong reduction of cleavage activity was observed with Y54H, Y54V, Y54N and Y54D. Y54L mutation reduced cleavage to a lesser extent. In addition, N-terminal shift of the cleavage position was observed with Y54N or Y54D. Residue 54, or −1 to the cleavage site, is therefore essential and required to be aromatic.

To check that the processing observed in vivo was linked to the MPP, an in vitro processing assay was performed with tobacco and broad bean mitochondrial membrane fractions in the presence or not of *o*-phenanthroline, a specific inhibitor of MPP [20]. The precursor synthesized in vitro was accompanied by a minor band corresponding to a translation start at the *gfp* initiation codon (Fig. 3C). After incubation with tobacco mitochondrial membrane proteins, a mature protein appeared, which was also the case with proteins extracted from broad bean. We also tested two mutants, Y54V and T56D, which had shown a reduced cleavage in vivo. In vitro, their cleavage was almost as efficient as for the wild-type construct, indicating that in vitro processing does not reflect the in vivo kinetics (Fig. 3D). Two mutants that had shown in vivo cleavage displaced at an up (Y54D) or down position (A55R) displayed the same shift in vitro. In all cases, cleavage was sensitive to *o*-phenanthroline, thus reflecting the action of MPP (Fig. 3D). In addition to the expected mature form, two minor intermediate bands appeared, suggesting progressive processing.

3.2. Cryptic cleavage of the $F_1\beta$ presequence

Expression of the first 47 or even 30 residues of the $F_1\beta$ presequence linked to GFP in transgenic tobacco showed that they were still able to import GFP into mitochondria [16]. However, although devoid of the normal cleavage site (between residues 54 and 55), βΔ47-GFP and βΔ30-GFP were

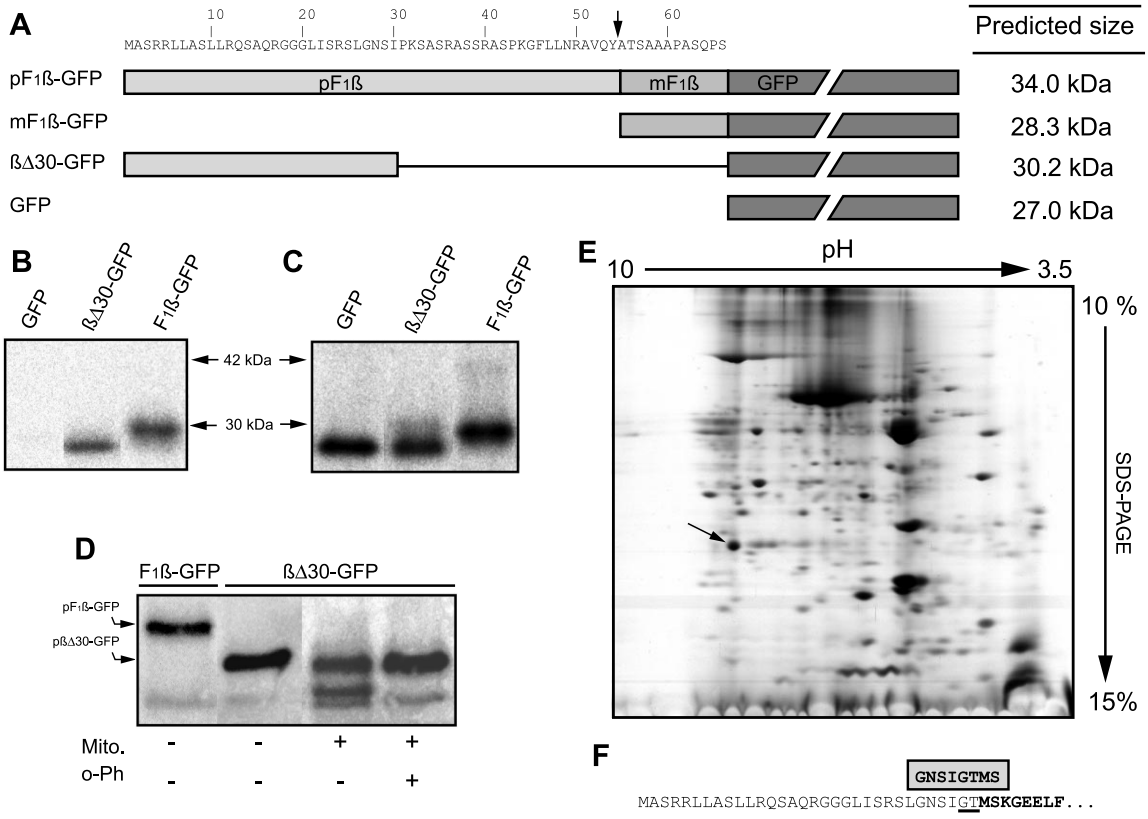

Fig. 4. Detection of the cryptic cleavage site of the βΔ30-GFP construct. A: Schematic presentation of GFP, the F_1β-GFP precursor (p) and mature (m) forms and the deletion retaining the first 30 residues of the F_1β presequence (βΔ30) linked to GFP. B, C: Western blotting of mitochondrial fractions and homogenates of stable transgenic tobacco expressing GFP, βΔ30-GFP and F_1β-GFP. Mitochondrial proteins (5 μg) (B) and phenol-extracted homogenate proteins (20 μg) (C) were resolved by SDS–PAGE and subjected to GFP Western blotting. Exposure in B and C was adjusted to obtain a similar intensity. (D) In vitro processing of βΔ30-GFP. Processing was performed as in Fig. 3C. Positions of the precursor of F_1β-GFP and βΔ30-GFP are indicated. E: 2-D gel electrophoresis of purified mitochondria. Mitochondria were purified from st-βΔ30-GFP-transformed plants and the soluble proteins were resolved on a 2-D gel [23] and stained by Coomassie blue. The arrow indicates the localization of the βΔ30-GFP mature protein determined by GFP Western blotting of a twin gel. F: Amino acid sequence of the N-terminal part of βΔ30-GFP mature form aligned with its precursor. The linker sequence between the presequence and GFP is underlined and the GFP sequence is in bold. The N-terminal sequence of the mature βΔ30-GFP protein, determined experimentally, is shaded in gray. This sequence was obtained by transferring the preparative gel onto a polyvinylidene difluoride membrane, cutting and sequencing the spot of interest with an Applied Biosystems pulsed liquid-phase sequencer (model 477A).

smaller than the mature F_1β-GFP ([16] and Fig. 4A,B for βΔ30-GFP), suggesting the presence of a cryptic cleavage site within the first 30 residues of the F_1β. GFP Western blotting of a mitochondrial fraction isolated from βΔ30-GFP showed a band close to the size of GFP (27 kDa). To rule out that this cleavage occurred in vitro during cell homogenization or sample preparation, homogenates of plants expressing β-GFP, βΔ30-GFP and GFP were prepared in the presence of phenol. The same pattern was observed, indicating that cleavage of βΔ30-GFP occurred in vivo (Fig. 4C). In vitro processing performed on a ^{35}S-radiolabeled βΔ30-GFP precursor showed that the cryptic maturation was sensitive to *o*-phenanthroline, thus linked to MPP (Fig. 4D).

To determine precisely the cryptic cleavage site of βΔ30-GFP, mitochondria of transgenic plants expressing βΔ30-GFP were purified and the soluble fraction resolved on a preparative 2-D gel (Fig. 4E). GFP was identified by Western blotting of a twin gel. The spot of interest was recovered and subjected to N-terminal sequencing. The obtained sequence (GNSIGTMS) indicated that cleavage occurred between residues 26 and 27 (Fig. 4F).

4. Discussion

In order to study the cleavage information contained within a plant mitochondrial presequence, site-directed mutagenesis was undertaken on the well-conserved residues surrounding the cleavage site of the F_1β presequence. Analysis of the different mutants demonstrated that the residue located at position −1 (Tyr54), relative to the cleavage site, was required to be aromatic. Modification of the residue at position +2 (Thr56) indicated that besides threonine, the presence of an aromatic residue was tolerated.

A helical structure encompassing the cleavage site is generally predicted and its involvement in the recognition by MPP has been demonstrated in vitro [15]. This observation is supported here since mutations such as L47C and A55C, which, according to structure prediction, completely disrupt the C-terminal α-helix of the presequence, shifted the cleavage position.

Surprisingly, modification of the proximal Arg50 and the distal Arg40, as well as the Pro43, did not significantly affect the cleavage, revealing the poor role of these residues in the

processing information of the $F_1\beta$ presequence. Therefore, although being cleaved by MPP in vitro ([15], Fig. 3), the $F_1\beta$ presequence does not belong to the R-2 or R-3 category and seems to harbor particular cleavage information in which arginine residues are not involved.

A particular effect observed with some $F_1\beta$ presequence mutants was the upstream or downstream shift of the cleavage site position. When cleavage occurred upstream of residue 54, it was actually impossible to distinguish between the creation of a new cleavage site and the existence of an upstream site exposed by the disruption of the normal one. The second hypothesis is in agreement with the discovery of a cryptic site between residues 26 and 27 of the $F_1\beta$ presequence. When cleavage happened more downstream than residue 54, one can speculate only that the cleavage was shifted. This is probably the case with the A55R mutation, since arginine residues have been demonstrated to play a preponderant role in the cleavage information of other presequences. Shift in the cleavage position has been recently reported in in vitro import experiments with mutants of the alternative oxidase presequence [14].

The expression in transgenic plants of C-terminally truncated $F_1\beta$ presequences, which did not possess the normal cleavage site (residues 54–55) [16], revealed that the mature truncated $F_1\beta$-GFP were shorter than expected, indicating that they were processed or degraded. This occurs in vivo since the same bands were seen by Western blotting of proteins obtained after phenol treatment of the plant material. Sequencing of the mature form of the $\beta\Delta30$-GFP chimeric protein revealed a cleavage site located between residues 26 and 27. Within this region were found a proximal and distal arginine residue at position -3 and -10 relative to the cleavage point as well as three helix breaker residues (glycine) between the two. This corresponds to the R-3 pattern of recognition by MPP, indicating that this cleavage is most probably due to a specific reaction between the precursor and the MPP. This hypothesis is supported by the fact that cleavage was sensitive to *o*-phenanthroline, a specific inhibitor of MPP.

Since the involvement of another peptidase can be ruled out by the fact that the $F_1\beta$ presequence has been shown to be cleaved in vitro by the plant MPP [15], we propose that the $F_1\beta$ presequence is cleaved in two steps by MPP. An alternative hypothesis is that, although $F_1\beta$ is cleaved by MPP in vitro, another peptidase might be actually involved in vivo.

A cleaved presequence has never been recovered from mitochondria once released from the precursor, indicating that mitochondria possess a rapid system of presequence degradation. In mitochondria, precursors are thought to be quite stable and the presequence is rapidly degraded by a matrix-located (probably carboxy) exopeptidase only once it is removed from the mature protein by the MPP endopeptidase [21]. However, up to now, no clear information concerning a possible carboxy-exopeptidase has been obtained in mitochondrial import studies, to our knowledge. Therefore, the presence of several cryptic sites within the presequence might increase the rate of presequence degradation.

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