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The products of the mitochondrial *orf25* and *orfB* genes are F_O components in the plant F_1F_O ATP synthase

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Abstract The F_O portion of the mitochondrial ATP synthase contains a range of different subunits in bacteria, yeast and mammals. A search of the Arabidopsis genome identified sequence orthologs for only some of these subunits. Blue native polyacrylamide gel electrophoresis separation of Arabidopsis mitochondrial respiratory chain complexes revealed intact F_1F_O, and separated F_1 and F_O components. The subunits of each complex were analysed by mass spectrometry and matched to Arabidopsis genes. In the F_1F_O complex a series of nine known subunits were identified along with two additional proteins matching the predicted products of the mitochondrial encoded *orfB* and *orf25* genes. The F_1 complex contained the five well-characterised F_1 subunits, while four subunits in the F_O complex were identified: subunit 9, d subunit, and the *orfB* and *orf25* products. Previously, orfB has been suggested as the plant equivalent of subunit 8 based on structural and sequence similarity. We propose that orf25 is the plant b subunit based on structural similarity and its presence in the F_O complex. Chimerics of orf25, orfB, subunit 9 and subunit 6 have been associated with cytoplasmic male sterility in a variety of plant species, our additional findings now place all these proteins in the same protein complex.

Key words: Plant mitochondria; Proteomics; Cytoplasmic male sterility; F_O

1. Introduction

The membrane bound F_1F_O-type H^+ ATP synthase of mitochondria catalyses the terminal step in oxidative respiration converting the generation of the electrochemical gradient into ATP for cellular biosynthesis. The general structure and the core subunits of the enzyme are highly conserved in both prokaryotic and eukaryotic organisms. The structure consists of two distinct parts, the hydrophilic F_1 which contains the nucleotide-binding site and the F_O which channels protons through the membrane. The F_1 enzyme consists of five subunits designated α, β, γ, δ, and ε in a 3:3:1:1:1 stoichiometry in both prokaryotes and eukaryotes [1,2]. The F_O consists of three subunits in the *Escherichia coli* enzyme, a, b and c in a 1:2:12 stoichiometry. In mammals and yeast the F_O contains a central core of three subunits analogous to the *E. coli* proteins in a 1:1:9–12 stoichiometry and a series of associated proteins involved in F_1–F_O interactions and as components of the second or 'stator' stalk [3]. This second stalk prevents free rotation of the αβ dimers under the influence of the γ rotor, thus ensuring coupling between proton translocation and ATP generation.

The number of associated subunits varies between mammals and yeast. Both species share homologues of subunits IF1, A6L (or subunit 8), oligomycin sensitivity conferring protein (OSCP), d, f, F_6 (or h), e and g [3,4]. Yeast also contains additional subunits, I and k and the F1 inhibitor stabilising factor (STF) containing a 15 kDa and a 9 kDa protein encoded by *STF1* and *STF2* respectively [2]. The exact functions of all the peripheral subunits of the eukaryotic complex are unclear. Subunits e, g and k are known in yeast to be required for dimerisation of the complex [3]. In *E. coli* two identical b subunits form the stator stalk while in the eukaryotic mitochondrial ATP synthases the b subunit (or subunit 4) along with the associated subunits d, OSCP and F_6 appear to primarily fulfil this function [5].

The plant mitochondrial ATP synthase also displays the classical F_1 five subunit structure. Searches of the Arabidopsis nuclear and organellar genomes identify orthologs for all five of the F_1 subunits (α, NP_085571; β, At5g08670, At5g08680, At5g08690; γ, At2g33040; δ, At5g47030; ε, At1g51650). The β-subunit alone is encoded in a small multigene family of three members on chromosome 5 and these contain over 98% sequence identity at an amino acid level. The subunits OSCP (sometimes referred to as δ′ in plants) and IF1 have been identified in the plant F_1F_O ATP synthase complex [6–9]. The genes encoding subunit 6 and subunit 9 of the F_O are present in various plant mitochondrial genomes, and the subunit 9 protein has been identified in the potato complex [7]. In Arabidopsis, orthologs for subunit 6 and 9 components of the F_O core (NP_085569 and NP_085561), OSCP (At5g13450) and d-subunit (At3g52300) peripheral components are also present in the genome. Several species appear to contain a plant specific subunit termed F_Ad. This component was originally proposed as a b subunit based on cross-reactivity with an antibody raised against the mammalian b or PVP-subunit [6,8], however sequence obtained from the soybean gene had little similarity to b subunits and its predicted hydrophilic nature did not reflect the structural role of subunit b in *E. coli*, mammals or yeast [10]. Plant sequences for F_Ad are now available from a wide variety of plants, including the

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Abbreviations: CMS, cytoplasmic male sterility; F_O, the F_O subcomplex of the ATP synthase; F_1, the F_1 subcomplex of the ATP synthase; OSCP, oligomycin sensitivity conferring protein

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Arabidopsis gene At2g21870, and none appear likely to be b subunit orthologs. Gray et al. [11] have pointed out that a common protist gene and a plant mitochondrial orf, *orfB*, contain a number of features that indicate they are analogous to the eukaryotic subunit 8, but no experimental confirmation of this proposal has been presented. Thus to date, no plant homologues of the central F_O core b subunit (subunit 4) or of the peripheral subunits, encoded by ATP14 and ATP17-21 in yeast, have been identified. This suggests that the plant F_O may be composed of substantially different proteins or substantially fewer proteins than those observed in mammals and yeast.

Using blue native polyacrylamide gel electrophoresis (BN-PAGE) and mass spectrometry (MS) we have attempted to characterise the protein components of the F_1F_O ATP synthase complex and link these directly back to specific gene products in Arabidopsis. In this process we have identified two mitochondrial-encoded orfs in Arabidopsis that encode ATP synthase subunits not previously characterised as associated with this protein complex. These are likely to represent the plant equivalents of the F_O components, AL6 (or subunit 8) and b subunit (or subunit 4).

2. Materials and methods

2.1. Cell culture growth and mitochondrial isolation

A heterotrophic *Arabidopsis thaliana* cell culture, established from callus of ecotype Lansberg *erecta* stem explants, is maintained by weekly subculture in Murashige and Skoog basal media supplemented with 3% (w/v) sucrose, 0.5 mg/l naphthaleneacetic acid and 0.05 mg/l kinetin [12]. The cell cultures were maintained in 250 ml conical flasks in the dark at 22°C in an orbital shaker (150 rpm). At 6–7 days, each flask (120 ml of cell culture) contained 8–10 g fresh weight cells and growth was approximately in the middle of the log phase. Subculture of 20 ml of culture to 100 ml of fresh media initiated the cycle again. A total of 1.0–1.2 l of 7-day-old dark-grown cell suspension culture was filtered through gauze to remove media and then the cells disrupted in a Waring blender by three successive 15 s bursts. Disruption of 60 g of cells was performed in 200 ml of grinding medium (0.45 M mannitol, 50 mM sodium pyrophosphate, 0.5% (w/v) bovine serum albumin, 0.5% (w/v) PVP-40, 2 mM EGTA, 20 mM cysteine, pH 8.0). Filtered cell extract was separated by differential centrifugation and mitochondria purified from the resultant organelle pellets by two Percoll gradients according to Millar et al. [13].

2.2. BN-PAGE/SDS–PAGE

BN-PAGE was performed largely according to Schägger et al. [14] and Jänsch et al. [7]. Gels consisted of a separating gel (5–15% w/v acrylamide) and a stacking gel (4% w/v acrylamide) formed in a solution of 0.25 M ε-amino-*n*-caproic acid and 25 mM Bis–Tris–HCl (pH 7.0). Subsequent SDS–PAGE was performed in Tris–glycine buffers to resolve higher molecular mass subunits (100–12 kDa), and Tris–tricine buffers to resolve subunits with molecular masses <12 kDa.

2.3. Quadrupole time-of-flight MS (Q-TOF MS)

Q-TOF MS/MS was performed on an Applied Biosystems Q-STAR Pulsar (Q-TOF MS) using an electrospray source. Protein spots to be analysed were cut from the 2D PAGE gel, destained, dried at 50°C in a dry block heater and stored at −70°C. For the sequencing analysis, the proteins were digested with trypsin according to Sweetlove et al. [15] injected into the electrospray source in 50% (v/v) methanol/0.1% (v/v) formic acid, and selected multiply charged peptides, identified in TOF MS mode, fragmented by N_2 collision and analysed by MS/MS. Mass spectra and collision MS/MS data were analysed with Analyst QS and BioAnalyst software (Applied Biosystems, Sydney, Australia) and Mascot (Matrix Science, London, UK).

2.4. Gene identification and hydropathy plots

Sequence orthologs were identified by BLAST analysis of yeast genes against v3.0 (July 2002) of the Arabidopsis protein set released by The Institute for Genomic Research and mitochondrial-encoded protein sets from NCBI. Transmembrane domains were identified using sliding window hydrophobicity analysis (19 AA) with the augmented Wimley–White scale at http://blanco.biomol.uci.edu/mpex, CONH was set to 0.0.

3. Results

3.1. BN-PAGE separation of F_1F_O ATP synthase subunits

Mitochondria can be isolated at a high level of purity from Arabidopsis cell cultures and their protein components analysed [13,16]. In our separation of Arabidopsis membrane proteins using BN-PAGE three complexes are highlighted that contain subunits of the Arabidopsis F_1F_O ATP synthase (Fig. 1). Previous analyses of this sort in plant mitochondria have typically identified an intact F_1F_O complex and a dissociated F_1 complex. The apparent mobility and general polypeptide composition of the intact F_1F_O and F_1 complexes in Fig. 1 are similar to those previously observed in potato [7], suggesting masses of 550–600 kDa for F_1F_O and 350–400 for F_1. In addition we observe another complex with an apparent mass of 150–200 kDa that contains three protein bands of masses similar to components found in F_1F_O but absent from F_1. This complex is thus likely to represent F_O.

3.2. MS identification of F_1F_O ATP synthase subunits

We then attempted to identify each of the subunits of the intact complex and the two subcomplexes and thus to potentially identify unknown ATP synthase subunits physically associated with known components in native complexes. Protein bands comprising F_1F_O subunits and similar components from the putative F_1 and F_O subcomplexes were excised, in-gel digested with trypsin and the resultant peptides extracted. Samples were analysed by MS/MS and spectral data matched to theoretical digests of the Arabidopsis predicted proteome using Mascot. This analysis revealed that four of the F_1F_O protein bands (1, 2, 5 and 8) and all the F_1 protein bands (10, 11, 12, 13) contained peptides derived from the Arabidopsis orthologs of the α, β, γ, δ, and ε F_1 components from yeast and mammals (Table 1). In addition, the F_1F_O complex contained a 29 kDa protein matching the F_Ad subunit gene (At2g21870) and a 26 kDa protein matching the gene encoding OSCP (At5g13450). Neither of these proteins were identified in the putative F_1 or F_O subcomplexes suggesting disassociation during the separation of F_1 and F_O. The F_1F_O complex also contained three protein bands with apparent molecular masses of 21, 17 and 7 kDa that were also found in the putative F_O complex. Peptides derived from the nuclear encoded d subunit (At3g52300) and mitochondrial-encoded orf25 (NP_085524) were found in the 21 kDa bands of spots 6 and 14. The 17 kDa bands (spots 7 and 15) contained peptides derived from the mitochondrial encoded orfB (NP_085508).

The 7 kDa bands (spots 9 and 16) stained weakly with Coomassie and were identified as the lipid binding protein subunit 9 (NP_085561). Subunit 9 is known to bind Coomassie weakly and contains very few predicted peptides after trypsin digestion that are amenable to MS detection. In recent mammalian research using MS based detection of ATP synthase components this subunit was only identified by size on SDS-PAGE [17]. We have identified two, very abundant singly charged peptides from subunit 9 samples with apparent

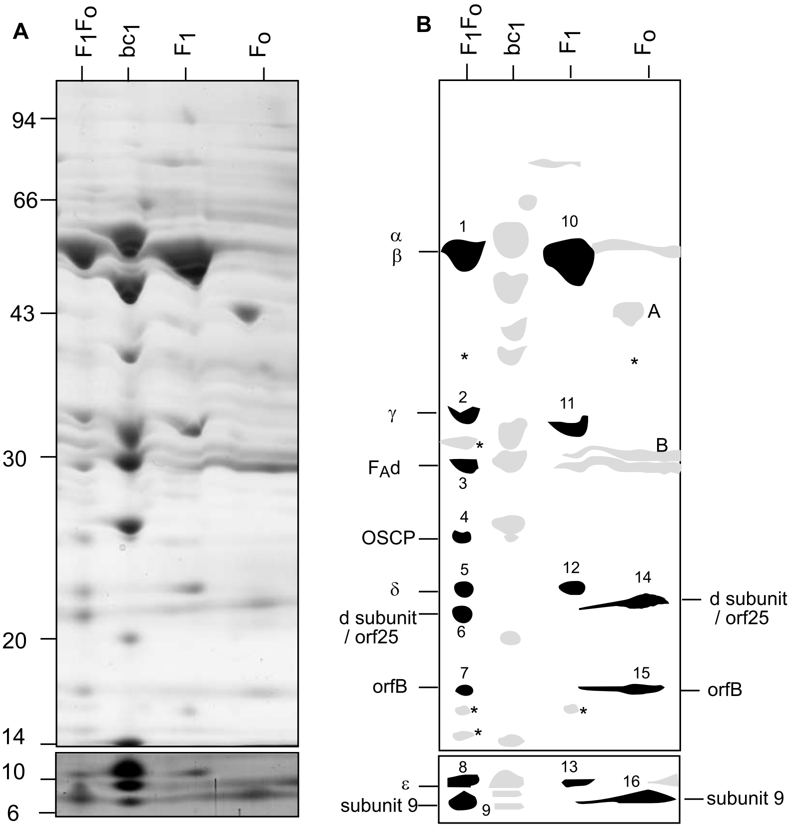


Fig. 1. BN-PAGE separation of Arabidopsis mitochondrial F_1F_O ATP synthase. A: Coomassie stained image of complexes (vertical) and component subunits (horizontal) of intact membrane protein complexes including F_1F_O, and F_1 and F_O subcomplexes. Apparent molecular mass of subunits is shown in kDa. B: Cartoon of stained image highlighting the 16 protein spots identified as components of ATP synthase by protein sequencing. The annotation bc_1 indicates the position of the ubiquinone:cytochrome *c* oxidoreductase (bc_1 complex) of the respiratory chain which has been confirmed by MS. The upper panel shows a Tris–glycine buffered gel and the lower panel the low molecular mass subunits resolved by Tris–tricine gel electrophoresis of a duplicate sample. Numbers on left hand panel are molecular masses in kDa, numbers in right hand panel indicate identified proteins presented in Table 1. Asterisks indicate potential subunits of the F_1F_O synthase that could not be identified due to the absence of peptides ionising for MS analysis. The protein spot labelled A is glutamate dehydrogenase and the streaks labelled B are both VDAC as determined by MS.

molecular masses of 628.35 and 647.32 which match by MS analysis to peptides NPSLAK and MLEGAK, respectively, in the Arabidopsis ATP9 sequence. The second peptide is not a predicted trypsin product but starts 11 amino acids from the currently annotated N-terminus of the protein. However, the presence of this second peptide strongly suggests the first methionine of this peptide represents the true N-terminus of the Arabidopsis subunit 9 and that in silico predictions are incorrect. The potato subunit 9 N-terminal sequence determined by Jänsch et al. [7], also commences MLEGAK, confirming that this start site is probably common in plants.

Several protein spots were present in the gel shown, and in some other replicate gel separations, from which no MS/MS spectra could be obtained due to absence of peptides following gel elution (asterisks, Fig. 1). Thus we have been unable to determine whether these represent true additional components or low-level breakdown products of proteins in Table 1. The large protein spot present above but not in-line with components of F_O (labelled A in Fig. 1) is glutamate dehydrogenase, and the streaks of protein material (labelled B in Fig. 1) are both VDAC (the voltage dependent anion channel) based on MS/MS analysis (data not shown). Neither are components of the F_1F_O ATP synthase and their presence near these subunits is probably coincidence. We have been unable to identify the product of the mitochondrial encoded *ATP6* gene in either intact F_1F_O or the F_O subcomplex. This absence is likely due to the extreme hydrophobicity of this protein and its resultant peptides that in all probability preclude its identification by MS. Extensive attempts to identify ATP6 peptides in the 35–45 kDa range in both the F_1F_O and F_O complexes, despite the very low Coomassie staining of this area, has failed to identify any such products.

4. Discussion

The components of the yeast and mammalian mitochondrial ATP synthase complex have been extensively studied, while much less is known about the plant complex. Several studies over the past decade have purified and partially sequenced components from spinach [6], potato [7,8] and Arabidopsis [9,16]. This previous work has mainly identified F_1 subunits, only some of the corresponding genes from these species have been sequenced, and the high degree of similarity of these components to the yeast and mammalian complexes have been highlighted. The composition of the plant mitochondria F_O, in contrast, is largely unknown. It has long been assumed that the clear orthologs of subunit 6 and subunit 9 encoded in plant mitochondrial genomes are components of F_O, but other partners have been elusive. One reason why the plant F_O subunits have remained undetermined is

Table 1
Identification of separated F_1F_O protein spots from Arabidopsis mitochondria

Spot	Name	MM gel	Peptides (No.)	Coverage (%)	MOWSE score	Gene locus	MM predicted
F_1F_O							
1	α	55	14	26	437	NP_085571	54.9
	β	55	21	48	666	At5g08670/80/90	59.7
2	γ	34	18	41	443	At2g330400	35.4
3	F_Ad	29	4	13	85	At2g21870	27.6
4	OSCP	26	20	45	376	At5g13450	26.3
5	δ	23	2	7	46	At5g47030	21.5
6	d	21	14	61	420	At3g52300	19.5
	orf25	21	4	22	102	NP_085524	21.5
7	orfB	17	3	17	71	NP_085508	18.2
8	ε	9	3	30	42	At1g51650	7.8
9	9	7	2	17	19	NP_085561	8.3
F_1							
10	α	55	26	46	651	NP_085571	54.9
	β	55	21	37	641	At5g08670/80/90	59.7
11	γ	34	21	47	464	At2g33040	35.4
12	δ	23	2	12	78	At5g47030	21.5
13	ε	9	2	30	50	At1g51650	7.8
F_O							
14	d	21	13	60	300	At3g52300	19.5
	orf25	21	3	14	108	NP_085524	21.5
15	orfB	17	3	17	73	NP_085508	18.2
16	9	7	2	17	20	NP_085561	8.3

MS/MS spectra derived from trypsinized peptides of proteins were matched using Mascot against a translated Arabidopsis database. Table headings: Spot, spot number in Fig. 1B; Peptides, number of peptides matching to predicted protein sequence ($P < 0.05$); MM gel, observed molecular mass; MM predicted, predicted molecular mass of matched sequence; Coverage, percentage of protein sequence covered by matched peptides; MOWSE score, molecular weight search score used at Mascot for identification confidence. ATP synthase β subunit products derived from At5g08670, At5g08680 and At5g08690 could not be differentiated by MS/MS spectra obtained.

that they may display low levels of sequence identity compared to their eukaryotic counterparts. By directly identifying most of the Arabidopsis ATP synthase subunits here, several new components of plant F_O have been highlighted by physical association with these other well-documented subunits and a case can now be made for their role as structural orthologs of known F_O components.

4.1. Similarity of orfB, orf25 and F_Ad to known F_O subunits

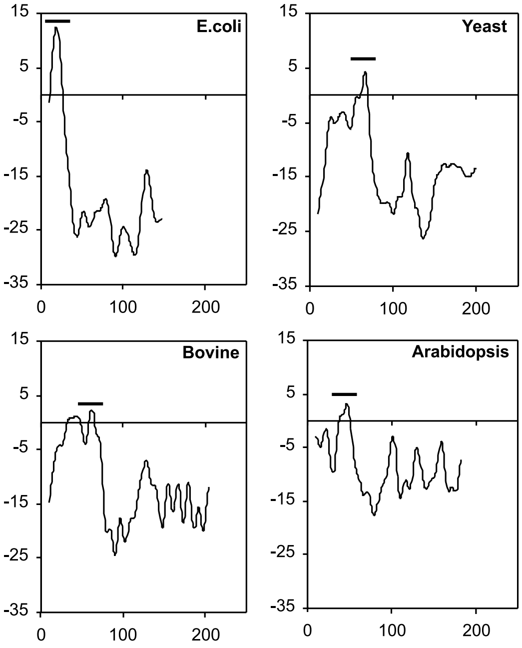
The case for orfB being the plant homologue of subunit 8, originally made based on sequence comparisons by Gray et al. [11], is further strengthened by this work. The role of the yeast subunit 8, and the mammalian ortholog AL6, as core components in the F_O of these organisms is well documented [3,4]. We have clearly shown that the gene product of Arabidopsis *orfB* is indeed in the plant F_1F_O complex and in fact appears tightly bound to other known F_O subunits (Fig. 1, Table 1).

The *orf25* was first sequenced in the maize mitochondrial genome [18] and has subsequently been identified in a wide variety of species [19]. *Orf25* is translated into a membrane protein and has been identified in tobacco and wheat mitochondria by immunoreaction [20]. However, the functional identity of this protein has been a mystery. This current work highlights the fact that this protein product is a subunit of the plant F_1F_O and is found tightly bound to other F_O components. But what is orf25? The most likely explanation is that orf25 represents the plant counterpart of the mammalian b subunit and yeast subunit 4 which are also referred to as subunit b in *E. coli*. This explanation is based on a number of pieces of evidence. The b subunits from *E. coli*, yeast and mammals are structurally related as 20–25 kDa proteins in F_O, containing a hydrophobic N-terminal membrane anchor of ∼30 amino acids attached to a largely hydrophilic protein [1]. The b subunit is the foundation of the stator stalk. However, b subunits contain very low sequence homology between prokaryotic and eukaryotic forms. Even within known eukaryotic sequences, the degree of identity is often very low [1]. Orf25s from plants have a similar structure, they are 20–25 kDa in length and contain a hydrophobic N-terminus (Fig. 2a). There is only very low sequence identity between plant orf25s and other known b subunits, but this level of similarity is no lower than that apparent between other established b subunits from prokaryotes and eukaryotes (Fig. 2b).

From our analysis it appears that plant orf25 proteins likely represent the first eukaryotic b subunits to have remained mitochondrial-encoded. Plant mitochondrial genomes generally encode more proteins than other mitochondrial genomes and include several ribosomal proteins, additional subunits of complex I, and notably the F_1 α subunit of the ATP synthase [21,22]. The diverse presence of *orf25* in higher plant mitochondrial genomes [19], its identification in the liverwort mitochondrial genome of *Marchantia* [23] and in the mitochondrial genome of the green alga *Prototheca wickerhamii* [24] all strongly suggest that the retention of this putative F_O b subunit in the plant mitochondrial genome is common.

The plant F_Ad subunit does not have a clear mammalian or yeast counterpart. The current assignment as a plant d subunit, while possible, is not likely given the clear recent identification of a plant d subunit with significant sequence identity to the yeast subunit (Table 1, [13,16]). This leaves open the possibility that the F_Ad represents a different structural component of the stator stalk. Its highly hydrophilic nature makes it unlikely to be a structural ortholog of the proteins encoded by ATP14 or ATP17–21, however, it may be involved in a manner analogous to the F_1 inhibitor stabilisers STF1/STF2. Such an assignment as either an alternative d-subunit like protein or involvement in F_1 inhibitor activities, would be consistent with the F_Ad being part of the intact F_1F_O, but

A



B

		Arabidopsis	Yeast	Bovine	E.coli	
% Identity		14	16	13	100	**E.coli**
		15	20	100		**Bovine**
		14	100			**Yeast**
		100				**Arabidopsis**

Fig. 2. Comparison of b subunits of F_O between prokaryotic and eukaryotic species. A: Hydropathicity plots (Wimley–White method) of b subunit sequences showing the region of predicted transmembrane anchoring (as a bar), *x*-axis is amino acid residue number from N-terminus, *y*-axis is hydropathicity (kcal/mol). B: Sequence identity between b subunits based on CLUSTALX alignments. Amino acid sequences were from *E. coli* (P00859), bovine (P13619), *Saccharomyces cerevisiae* (P05626) and Arabidopsis (NP_085524).

absent from the smaller F_1 and F_O complexes (Fig. 1), and the very hydrophilic nature of the protein in plants.

4.2. *Further links between F_O subunits and plant cytoplasmic male sterility (CMS)*

Much of the research focused around *orfB* and all of the work on *orf25* over the last 15 years has been undertaken without any knowledge of their function or molecular association. Previous research has highlighted these open reading frames as 'hotspots' for chimeric rearrangements, changes in RNA editing patterns, or alterations in transcription rate or transcript stability in plants leading to CMS (for example [20,25–27]). Similar changes leading to CMS phenotypes have also been documented for subunit 6 and subunit 9 (for example [28–31]). The common physical locality of all four of these proteins (orfB, orf25, subunit 6 and subunit 9) as subunits of the plant F_O may be important in unravelling a common mechanism for a range of pollen abortion phenotypes in these species.

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