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Physiological, biochemical and molecular aspects of mitochondrial complex I in plants

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Abstract

Respiratory complex I of plant mitochondria has to date been investigated with respect to physiological function, biochemical properties and molecular structure. In the respiratory chain complex I is the major entry gate for low potential electrons from matrix NADH, reducing ubiquinone and utilizing the released energy to pump protons across the inner membrane. Plant complex I is active against a background of several other NAD(P)H dehydrogenases, which do not contribute in proton pumping, but permit and establish several different routes of shuttling electrons from NAD(P)H to ubiquinone. Identification of the corresponding molecular structures, that is the proteins and genes of the different NADH dehydrogenases, will allow more detailed studies of this interactive regulatory network in plant mitochondria. Present knowledge of the structure of complex I and the respective mitochondrial and nuclear genes encoding various subunits of this complex in plants is summarized here. © 1998 Elsevier Science B.V.

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1. Plant complex I in its biochemical and physiological context

Plants deviate from other eukaryotes by the unique complexity of their mitochondrial respiratory chain. In addition to the basic setup of respiratory enzymes in mammals, including the proton-pumping complexes I, III and IV, four different rotenone-insensitive NAD(P)H dehydrogenases and an alternative ubiquinol oxidase, none of which translocate protons, are present in plants (Fig. 1) [1–3]. Two of the additional NAD(P)H dehydrogenases, one oxidising NADH and the other oxidising NADPH (and possibly also NADH), are directed towards the internal matrix space and can compete with complex I for NADH formed in the citric acid cycle. The complexity of the electron-input domain of the respiratory chain in plants creates special conditions and complications for complex I to carry out basal energy-linked NADH oxidation and to contribute to ATP synthesis.

The physiological demands for ATP supply by respiration vary substantially between plant tissues. As an example of extremely high requirements for respiratory efficiency, the pollen developing cells of higher plants are found to be highly sensitive to any

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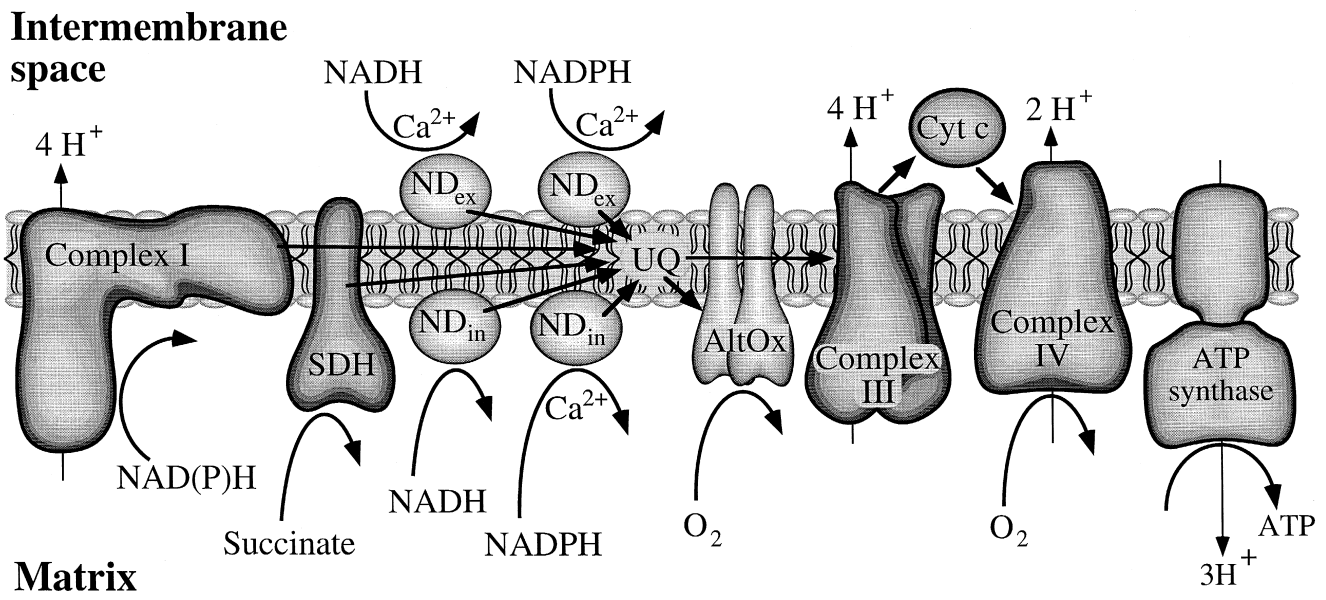

Fig. 1. The respiratory chain in the mitochondrial inner membrane of plants. The main respiratory chain is similar between plant and animal mitochondria, while plants additionally have several rotenone-insensitive NAD(P)H dehydrogenases in outside (NDex) and inside (NDin) orientations in the inner membrane. Plants furthermore have an alternative oxidase enzyme (AltOx), which is specifically enhanced in some plant tissues to generate heat. SDH, succinate dehydrogenase; Cyt *c*, cytochrome *c*.

genetic (i.e., mutational) perturbation of respiratory function in the mitochondria (see below). On the other extreme end, requiring little ATP production and thus respiratory chain capacity, are the thermogenic flowers of voodoo lilies, where massive amounts of starch are rapidly catabolised in order to elevate the temperature of the flower. This and analogous events of increased carbohydrate turnover, where released energy is dissipated as heat rather than used for efficient ATP generation, are in plants generally correlated with a massive induction of the alternative oxidase, which is controlled at transcriptional, protein and enzymatic activity levels [4,5]. Presently it remains unclear if and how these metabolic events are manifested at the respective protein levels and in the partitioning of matrix NADH between complex I and the non-proton-pumping NAD(P)H dehydrogenases, the genes of the latter enzymes are not yet characterised.

2. Enzyme activity and active redox centres

The activity and electron pathway in plant complex I have been analyzed with particular respect to identifying differences and similarities to the homologous enzymes of beef heart and *Neurospora*. In inside–out submitochondrial particles and with O_2 as terminal acceptor, the potato enzyme shows 5 times higher activity with NADH than with NADPH at pH 7.2. The latter activity may not be very significant in vivo since the Km for NADPH (above 1 mM) is much higher than for NADH (2.6 μM) [6]. Analysis of the electron acceptor preference shows that plant complex I generally reduces ferricyanide 20 to 40 times faster than short chained quinone analogues [7–9]. With respect to other characteristics of the enzyme activity, like pH dependency, inhibitor specificities and kinetics with ferricyanide, the plant complex I generally behaves similar to the respective enzymes of beef heart and *Neurospora* [1,2].

A highly specific assay for plant complex I activity appears to be the oxidation of the NADH analog deamino-NADH with the natural electron acceptor ubiquinone$_{10}$; in the actual experimental setup the terminal acceptor is O_2, e.g., in inside–out submitochondrial particles. This reaction is completely sensitive to the standard complex I inhibitor rotenone [6]. However, addition of hydrophilic ubiquinone$_1$ makes the activity partly rotenone-insensitive. Menz et al.

[10] consequently suggested a second, rotenone-insensitive quinone binding site which only reacts with hydrophilic quinone analogues. Alternatively the addition of quinone could change the specificity of a different NADH dehydrogenase in the respiratory chain as observed for external deamino-NADH oxidation [11]. The hydrophilic quinone could also connect a loosely attached soluble NADH:quinone oxidoreductase to the ubiquinone pool of the respiratory chain. In any case, although oxygen consumption with deamino-NADH is a specific assay for complex I in the mitochondrial membrane, great care has to be observed in the interpretation when using deamino-NADH under different conditions.

As electron carrying groups, the plant complex I contains FMN [9,12], and several FeS centres as detected by electron paramagnetic resonance (EPR) spectroscopy. In submitochondrial particles from *Arum maculatum*, Cammack and Palmer [13] could, based on g values and temperature dependencies, distinguish signals interpreted as centres N-1b, N-2 and superimposed centres N-3 and N-4. The refined techniques used by Lin et al. [7] distinctly resolved the signals of centres N-1b, N-2, N-3 and N-4 in the EPR spectra of isolated potato complex I. The signals observed are similar to the EPR spectra of *Neurospora* complex I with respect to g values and line symmetry. The midpoint potential of centres N-1b, N-2 and the superimposed N-3 and N-4 are -240, -110 and -275 mV, respectively [13], compatible with present models for the mechanism of complex I [14].

3. Protein composition of purified plant complex I

Mitochondrial complex I has been successfully isolated from several plant species, all of which are dicotyledons. The complex purified from beetroot (*Beta vulgaris*) has an apparent molecular mass of at least 700 kDa as estimated from gel filtration and native PAGE analyses [9]. About 30 subunits have been resolved by denaturing SDS-PAGE, several of which could be tentatively identified by their cross-reaction with antibodies against the respective polypeptides from *Neurospora* complex I, showing that some epitopes of the subunits are recognizably conserved in plants.

Complex I purified from mitochondrial membranes of broad bean (*Vicia faba*) is estimated to have a molecular mass comparable to the beetroot enzyme [8]. The N-terminal sequences of some of the individual subunits could be determined, these partial protein sequences being sufficient to identify several of the subunits by the primary sequence conservation between plants and fungi or animals, respectively.

The third plant species from which complex I has been purified is potato (*Solanum tuberosum*), from the tubers of which sufficient mitochondria can be prepared to yield enough of complex I for extensive N-terminal protein analysis [12] and EPR spectra (see also above). In gel filtration columns the complex elutes at higher apparent molecular mass (>800 kDa) than estimated from other approaches. Consistent with this observation is also blue native gel analysis of potato mitochondrial membranes, which suggests a molecular mass of about 1000 kDa for complex I [15].

In all three investigations the purity of the complex I enzyme was monitored by correlating the column elution patterns of all subunits to the enzymatic activity. The sum of the estimated molecular masses of the 32 individually resolved polypeptides in the purified potato complex gives a total molecular mass of 891 kDa for the complex [12], consistent with the range of the values determined by gel filtration and native gel electrophoresis. However, it has to be taken into account, that the latter techniques are somewhat error-prone due to differences in amount of bound detergent molecules and the overall shape of the enzyme in the respective solute space. The averaged and integrated size estimations of the different approaches suggest that the plant mitochondrial complex I is somewhat larger than the *Neurospora* complex of 700 kDa [16], and may be as large as the bovine complex of 890 kDa [17].

After reconstitution into phospholipid vesicles, the complexes purified from potato and beetroot mitochondria partially retain their rotenone-sensitivity. The observed rotenone-sensitivity was higher in the potato than in the beetroot preparation [9,12]. This difference may be correlated with the larger determined molecular mass, indicating a higher integrity, of the purified potato enzyme. Since rotenone binds at a quinone binding site in the membrane moiety of complex I, sensitivity to this inhibitor is an indicator

of the overall intactness of the isolated enzyme and the presence of all polypeptide constituents necessary for full catalytic function.

Partial N-terminal and internal protein sequences of the subunits purified from the isolated potato complex I [12] allowed the identification of several of these proteins. This information has rendered it possible to characterize several of the nuclear encoded subunits at the primary molecular level and to isolate their respective cDNAs and genes.

4. The mitochondrial genes for complex I subunits in plants

4.1. The sets of mitochondrial genes vary between different plant species

In plants usually nine subunits of the mitochondrial complex I are encoded in the mitochondrial genome (Fig. 2). These include all of the seven subunits coded by the mammalian mitochondrial genome and in addition also the genes for subunits seven and nine (*nad7* and *nad9*) [18–20]. These plant mitochondrial genes are the homologues of the mammalian nuclear genes for the 49 kDa and 30 kDa polypeptides, respectively. However, the precise

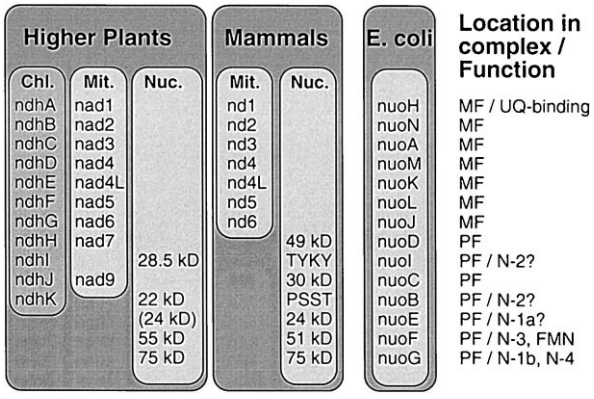

Fig. 2. Genes for the core subunits of complex I are found in the nuclear, mitochondrial and plastid genomes of plants. In plants two subunits are encoded in the mitochondrial (Mit.) genomes, which are in animals coded by the nucleus (Nuc.). Homologues to some of the 14 subunits of bacteria (*nuo* genes of *E. coli*), the minimal complex I structure, are in plants also found as genes in the chloroplast (Chl.; *ndh* genes). All the nuclear genes analysed so far in plant code for subunits of the mitochondrial complex I (24 kDa) is identified from an *Arabidopsis* cDNA, the actual molecular mass of the protein is not known. MF: Membrane fragment; PF: peripheral fragment; N-: FeS centres.

number of active genes located in the mitochondrial DNA of a given plant species can vary from the gene complement determined in potato and *Arabidopsis thaliana*. In the liverwort, *Marchantia polymorpha*, NAD7 is encoded by a nuclear gene, which has been created by copy and transfer of the coding sequence from the mitochondrial genome into the nucleus [21]. In the mitochondrial genome of this moss only a non-functional pseudogene is found, characterized by several in-frame stop codons, which render the RNA of this genomic region untranslatable. Furthermore, two of the introns cotranscribed with the degenerated remnant gene are not removed from the RNA molecules, most likely due to deleterious nucleotide mutations which prohibit correct splicing of these introns [22]. Thus in *Marchantia*, the NAD7 polypeptide is provided by a nuclear gene, in which the former mitochondrial coding region is now preceded by a mitochondrial target sequence. This ensures import of the protein into the mitochondrial compartment. Although in vascular plants such dramatic differences in the location of genes for complex I subunits have not (yet) been identified, we cannot exclude such divergencies just by extrapolation from the two most intensively investigated plants, *Arabidopsis* and potato, that their gene distribution indeed holds true for all of the vascular or even the flowering plants. It is interesting to note that in the largest set of 12 *nad* genes in the mitochondrial genome of the protozoan *Reclinomonas americana* [23] only missing two of the minimal complex subunits are not included, the 24 kDa and the 51 kDa subunits.

4.2. Mitochondrial complex I genes require trans-splicing

In plants expression of the mitochondrially encoded genes for subunits of complex I involves several specific features of gene expression, notably *cis*- and *trans*-splicing and RNA editing [24–26]. While some of the subunits, such as NAD3 are encoded as continuous open reading frames in the mitochondrial genome [27], other subunits particularly NAD1, NAD2 and NAD5 are encoded by highly complex and disrupted gene structures scattered around the genome [20,28–31]. From these the respective open reading frames have to be reconstituted at the RNA

level by connecting different exons in *cis*- and *trans*-splicing reactions. The various RNA molecules in plant mitochondria can be monocistronic transcripts (e.g., *nad4*), or polycistronic transcripts, in which complex I subunits are cotranscribed with other coding regions. The *nad3* gene for example is cotranscribed with the gene for *rps12* in most flowering plant species [32]. From this precursor, processing generates monocistronic mRNAs to allow proper translation of each of the two reading frames.

The requirement to connect exons of *nad1*, *nad2* and *nad5* by *trans*-splicing from different mRNA molecules in plant mitochondria has been documented in several vascular plant species. Intriguingly, beyond these three complex I genes none of the other mitochondrial genes appears to need *trans*-splicing. The evolutionary origin of the physically disrupted genes of *nad1*, *nad2* and *nad5* has been found to be intricately connected to genomic recombinations. Undisrupted, *cis*-connected pendants for all of these *trans*-splicing introns have been found in various species of ferns, suggesting that these introns have once been *cis*-splicing [33]. During evolution of the flowering plants these 'normal' group II introns were disrupted by genomic recombinations, which in vivo could be compensated for by recreating the proper splicing-competent intron structure even from two separate RNA molecules.

The intron content in individual genes can vary and even between comparatively closely related species of flowering plants introns can become lost or rearranged, resulting in variable gene structures. The *nad4* gene for example is disrupted by three *cis*-splicing introns in turnip (*Brassica campestris*), while in green salad (*Lactuca sativa*) only one and in spinach (*Spinacia oleracea*) just two of these introns are found [34]. In the non-vascular plant *Marchantia polymorpha* group I introns have also been identified in mitochondrial *nad* genes. In this liverwort, the locations and evolutionary origins of the group II introns are usually distinct from those in higher plants [35].

4.3. Extensive RNA editing in plant mitochondrial complex I mRNAs

The reading frames coding for the different subunits of complex I like nearly all of the mRNAs in plant mitochondria require extensive RNA editing to code for the correct proteins. In the open reading frame of *nad1* in wheat mitochondria even the AUG translation initiation codon is created from an ACG codon by RNA editing [28]. Although unedited and partially edited mRNA sequences have been found to be associated with plant mitochondrial ribosomes [36], and although antibodies against partially edited proteins detect such polypeptides in polysomal fractions [37], only the protein derived from fully edited mRNA appears to be integrated into the mature complex I. When the NAD9 polypeptide is purified from isolated potato complex I, partial peptide sequences correspond only to the fully edited mRNA, although 50% of the total steady state mRNA population are unedited at the relevant position in vivo [19]. A minority of the protein sequence may still be derived from partially edited RNA molecules and remains undetectable by this approach. However, the most likely scenario predicts that although variant polypeptides may be synthesized indiscriminately from partially edited mRNA molecules, because of their deviating sequence and thus altered secondary structure such proteins may not be competent for functional integration into the complex during the assembly process. It is thus presumed, although not formally proven, that the mRNA population created by RNA editing in plant mitochondria generally does not correspond to a functionally or developmentally regulated variation in proteins. Considering that many of the un-edited sites would preclude the integration of evolutionarily conserved amino acids, of which several have been implicated to be functionally essential, it is theoretically not very likely that proteins predicted by partially or completely unedited mRNA molecules could yield competent protein subunits.

5. Nuclear genes for subunits of complex I

5.1. The genes of the core complex subunits are identified in plants

Nuclear genes for subunits of complex I have been identified in potato and *A. thaliana* (Table 1) starting from N-terminal protein sequence information for complex I subunits of bean and potato [8,12]. The genes most intensively investigated are those for the

Table 1
The subunits of complex I characterised in plants

Subunit		Plant species[a]	Identification
bovine	plant		
ND1	NAD1	A.t.	gene, cDNA
ND2	NAD2	A.t.	gene, cDNA
ND3	NAD3	A.t.	gene, cDNA
ND4	NAD4	A.t.	gene, cDNA
ND5	NAD5	A.t.	gene, cDNA
ND6	NAD6	A.t.	gene, cDNA
49 kDa	NAD7	A.t., S.t.	isolated protein = > cDNA
30 kDa	NAD9	A.t., S.t.	isolated protein = > cDNA
75 kDa	76 kDa	S.t.	isolated protein = > cDNA
51 kDa	55 kDa	S.t.	isolated protein = > cDNA
24 kDa	(cDNA)	A.t.	sequence comparison
23 kDa (TYKY)	28.5 kDa	S.t., A.t.	isolated protein = > cDNA
20 kDa (PSST)	22 kDa	S.t., A.t.	isolated protein = > cDNA
ACP	ACP	S.t., A.t.	Western blot, this publication

The ND and NAD subunits of beef heart and potato, respectively, are mitochondrially encoded. Remaining subunits are nuclear-encoded in the respective species. Apart from the acyl carrier protein (ACP), all subunits are part of the minimal core. Nomenclature of bovine subunits is as in Walker, 1992 [17].

[a]For the mitochondrially encoded subunit only the reference to the completely sequenced mitochondrial genome in A.t. is given [20], although each subunit has also been analyzed in several other plant species.

S.t. = *Solanum tuberosum*; A.t. = *Arabidopsis thaliana*.

76 kDa subunit [38], the NADH-binding 55 kDa subunit [39], the 28.5 kDa [40] and the 22 kDa [41] subunits in potato and in *Arabidopsis*. Sequence comparisons to the respective bovine subunits show the motifs for FMN and NADH binding and for liganding of FeS cluster N-3 are conserved in the 55 kDa subunit of potato [39]. The putative motifs for FeS cluster liganding in the bovine 75 kDa, the TYKY and PSST subunits are likewise conserved in the respective potato 76 kDa, 28.5 and 22 kDa subunits [38,40,41]. Additionally an apparently complete cDNA homologous to the 24 kDa subunit of the bovine flavoprotein moiety has been identified from the *Arabidopsis* EST database (Table 1 and data not shown). Together with the nine mitochondrial encoded subunits, these five nuclear genes make up the plant counterpart of the bacterial minimal complex I which consists of 14 subunits [42,43]. The basic subunit organization of complex I thus appears to be highly conserved between bacteria, animals, fungi and plants, since in all these kingdoms the centrally located 14 subunits are present and evolutionarily conserved.

It is accordingly expected that in plants, more nuclear genes will be identified in the various EST databases especially of *Arabidopsis* and rice by similarity to the mammalian homologues. Table 2 shows the non-minimal subunits of bovine complex I conserved in plants as identified by comparison to experimentally determined N-terminal protein sequences and/or by similarity to entries in the cDNA sequences of the EST programs. These identified nuclear encoded subunits are all found as multiple entries in the respective EST databases. It is presumed that the plant homologues to the respective bovine subunits not included in Table 2 are most likely either less well conserved or completely absent from the plant mitochondrial complex I.

5.2. The acyl-carrier protein is associated with the mitochondrial complex I in plants

Similarities of subunit compositions between different species include the example of an unrelated enzymatic activity, the acyl-carrier-protein (ACP) [44]. This protein is associated with complex I in fungi, animals [45,46] and is also found to copurify with this protein complex in plants (Fig. 3; L. Grohmann and H.-P. Braun, unpublished results). The structural connection between ACP and complex

Table 2
Homologues to accessory subunits of bovine complex I identified by partial sequence similarities in plants

Subunit	Identification	Species	Reference accession
39 kDa	38.5 kDa	S.t.	[12]
	EST-clone	A.t.	TC8585
19 kDa	EST-clone	A.t.	TC8214
18 kDa	18 kDa	S.t.	[12]
	EST-clone	A.t.	Q − ATTS1561
13 kDa	EST-clone	A.t.	P − 3853
B22	EST-clone	A.t.	TC9210
B18	EST-clone	A.t.	P − 20091
B14	EST-clone	A.t.	P − 3802
B13	22.5 kDa	S.t.	[12]
	EST-clone	A.t.	TC11832
B8	EST-clone	A.t.	TC10147

Identification in potato (*Solanum tuberosum*, S.t.) was done by N-terminal sequencing of isolated subunits of purified complex I. Accession numbers refer to ESTs and assemblies in the TIGR database of *Arabidopsis thaliana* (A.t.). Nomenclature of bovine subunits is as in Table 1.

I has thus been conserved through a large evolutionary distance, suggesting an important benefit for cellular function. The physiological implications most likely include an energetic advantage by coupling the two enzymatic processes of the respiratory chain complex and the lipid carrier. Complex III in plant mitochondria has a similar dual function, containing both characterized subunits of the matrix processing peptidase [47]. The copurification of both acylated and deacylated forms of the mitochondrial ACP with complex I from potato as well as from *Arabidopsis* in blue native gel analyses indeed confirms the conserved function of the ACP in the fatty acid synthesis pathways of plant mitochondria (L. Grohmann and H.-P. Braun, unpublished results).

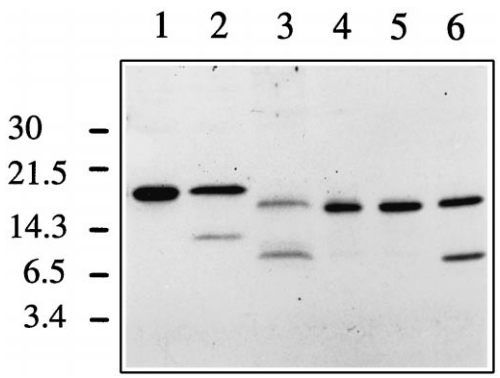

Fig. 3. The acyl-carrier protein (ACP) is associated with complex I in plant mitochondria. A western blot of *Arabidopsis* and potato mitochondrial protein fractions and of isolated complex I respectively was probed with an antibody against the *Arabidopsis* mitochondrial ACP [44]. Lanes 1 and 2 are deacylated and untreated total mitochondrial proteins from *A. thaliana*, respectively. Lanes 3 and 4 are untreated and deacylated mitochondrial protein factions from potato and lanes 5 and 6 are deacylated and untreated mitochondrial complex I proteins from potato. The molecular masses of protein standards coelectrophoresed in this 15% SDS-PAGE gel are given in the left margin.

5.3. A complex I-like assembly in plastids?

The identification and assignment of nuclear genes for plant mitochondrial complex I subunits is complicated by the possible presence of a complex I type enzyme in chloroplasts [48,49]. Homologues to some of the core mitochondrial complex I subunits have been identified as gene sequences in the plastid genomes (Fig. 2). Altogether eleven complex I-like genes have been found in flowering plant plastid genomes [50], but so far no additional nuclear encoded subunits of the corresponding enzyme complex have been reported. The chloroplast genes are generally only distantly related to the genes for mitochondrial complex I subunits, but can nevertheless be clearly identified. Although expression has been demonstrated for these genes [51], the physiological role and enzymatic function of the encoded proteins are not entirely known [48,49]. A possible function could be as a component of a photophosphorylating electron pathway around photosystem I, while recent

evidence suggests a possible function as an NADH: plastochinone oxidoreductase [49,52]. This scenario is supported by the observation that in sorghum, a C-4 plant, the quantities of 3 plastid NDH proteins are strongly increased in bundle sheath cells [53] and indirectly also by the apparent absence of further nuclear encoded subunits homologous to the respective mitochondrial subunits, which would be required for an analogous NADH-dehydrogenase function in the plastid [39].

However, it is possible that such additional subunits are present, but have not yet been identified because of a lower similarity in primary sequence. Alternatively, a gene for a subunit of the mitochondrial complex I could also code for a plastid subunit and target the respective protein into both organelles. Sequence similarities identified in the EST databases thus require additional biochemical and physiological data for clear assignments. The 55 kDa and the 76 kDa subunits of potato mitochondrial complex I are encoded by nuclear genes without homologues on the plastid genome (Fig. 2). However, the encoded protein products are only imported into mitochondria, but not chloroplasts [38,39,54]. More detailed investigations are required to resolve this question of a potential mitochondrial complex I-like structure and function in plastids.

5.4. Structure of nuclear genes

The conserved regions of the nuclear encoded subunits are restricted to the mature protein region, while the N-terminal extensions which are cleaved off during import into the mitochondrial compartment differ substantially from the mammalian or fungal presequences. The nuclear genes for complex I subunits in plants investigated so far contain a number of small spliceosomal introns, which are characterized by a comparatively high A + T content. Both the genes for the 22 kDa PSST subunit and the 55 kDa NADH-binding subunit are interrupted by only single introns each [39,41]. The intron in the 55 kDa subunit gene is located next to the ATG, thus separating the first codon from the rest of the reading frame. The gene for the 28.5 kDa iron–sulfur protein contains seven introns in *Arabidopsis* as well as in potato [40]. Their sizes differ between 60 and 1700 nucleotides and not in all instances is their A + T content higher than in the adjacent exons, suggesting additional criteria for intron recognition in plants at least in these instances in the 28.5 kDa subunit genes. None of the introns is located at the border of the mitochondrial import sequences and the mature protein, suggesting that if exon shuffling did contribute to attach these presequences, their traces have been lost in evolution.

6. Mutations in complex I genes disturb pollen maturation

6.1. Mutations in mitochondrial genes

Mutations in mitochondrial genes occur in plants mostly through genomic recombinations, which often at the same time destroy an existing gene and create novel, often mosaic genes [53,54]. Several mitochondrial mutations have been described, which involve mitochondrial complex I genes, mostly in maize and tobacco [55–57,63,64]. These mutations generally cannot be maintained as pure homoplasmic lines but must be fed with the respective proteins synthesized from intact gene copies in some of the cells in the vicinity, such as in the *nad4* non-chromosomal stripe mutants of maize [56]. These observations confirm that in plants complex I functionality is essential for normal cellular development, including chloroplats maturation and pollen development in anthers. Maintenance of mitochondrial complex I gene mutations in tobacco such as a non-functional *nad7* disruption similarly requires a background of stoichiometrically lower copy numbers of intact mitochondrial gene arrangements [58]. The mutants show slowed vegetative and floral development and a male-sterile phenotype, but also metabolic compensation to some extent as evidenced by the induction of non-phosphorylating pathways of the respiratory chain.

Less existential alterations in mitochondrial complex I gene activity do not visibly affect somatic growth, but manifest the phenotype of cytoplasmic male sterility. Normal pollen development requires the full mitochondrial capacity at some stage, including the fully functional complex I. Disturbance of complex I gene expression such as altered transcript levels for *nad7* in mitochondrial genome mutations induced by protoplast culture thus results in male

sterility [57]. Upon fertilization with pollen from a normal plant, the sterility trait is inherited maternally to the offspring.

6.2. Suppression of nuclear complex I genes by transgenic antisense experiments

To investigate the phenotypic effect of disturbances in expression of nuclear complex I genes, the functional roles of the coding regions for complex I subunit genes were probed in antisense experiments. In the first such experiments reported to date, antisense repression of the 55 kDa subunit has been achieved through reduction by 30% of the respective mRNA of the wild type levels [59]. Of more than 60 individual transformant lines investigated for antisense constructs of the 55 kDa NADH-binding subunit, none contained less than 30% of the normal mRNA, suggesting that this amount is required to provide a minimal level of the respective subunit. This minimal quantity may be crucial to entertain mitochondrial respiratory function necessary for ATP production during vegetative growth and development. Efficient mitochondrial ATP generation is essential during the phases of germination, when carbohydrate supply is restricted before photosynthesis is fully activated. However, even in the light, mitochondria are the main suppliers of cytoplasmic ATP [60] and thus crucially important for cellular survival. Higher levels of repression may thus be selected against already at the embryonic stage, suggesting that such antisense plants may escape experimental detection and may have to be carefully nurtured to maturation.

In spite of the considerably lowered mRNA levels in the transgenic antisense plants and a corresponding reduced quantity of the 55 kDa protein subunits, no effect on somatic viability and vegetative development could so far be observed. Even when expressing only 50% of the normal 55 kDa protein levels, plants

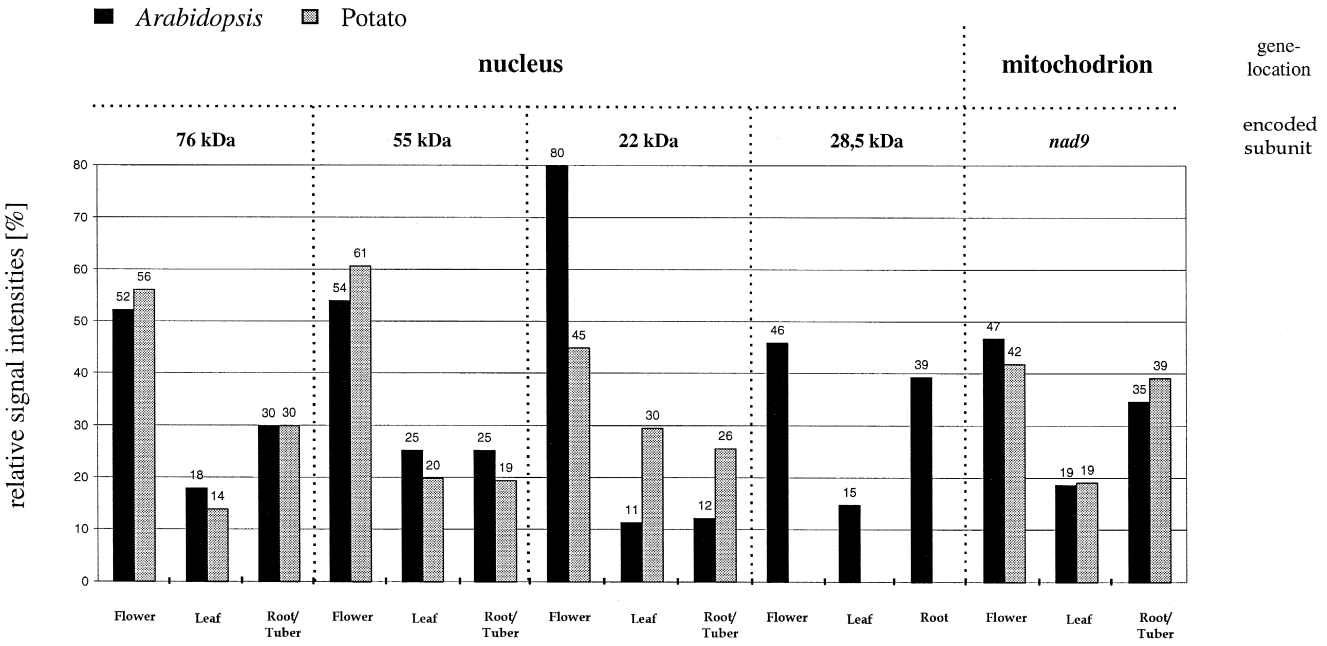

Fig. 4. Expression pattern of nuclear and mitochondrial genes of complex I subunits are similar in different tissues. Transcript accumulation was determined by hybridizing gene-specific probes to total RNA preparations from flowers, leaves and roots of *Arabidopsis* (black columns) and flowers, leaves and tubers from potato (grey columns). Data are assembled from Refs. [38,39,41] [A. Rasmusson, V. Heiser, L. Grohmann, unpublished results].

appear to be perfectly healthy and not depressed by a decreased capacity of respiration. The residual complex I activity is apparently sufficient to maintain respiratory chain function as far as required during normal growth. However, examination of pollen maturation and pollen viability shows that the remaining mitochondrial respiratory chain capacity is not sufficient to allow normal pollen development. Considerably less pollen grains are produced and examination of their viability furthermore shows that in the plants with the highest reduction of the 55 kDa complex I subunit hardly any functionally competent pollen is produced [59]. This connection between male fertility and mitochondrial functions has also been observed in natural mutations of plant mitochondrial genomes, which primarily affect the ability to produce viable pollen, but do not significantly disturb somatic growth patterns (see above). The transgenic antisense plants thus likewise show that fully intact complex I as well as overall fully competent mitochondrial functions are required during pollen maturation in flowering plants.

Indeed, sporogenesis is the highest energy consuming process in plants. It is known that the number of mitochondria per cell increases in anthers, particularly in tapetal cells [61]. This increase allows cells to produce enough energy necessary to develop viable pollen grains. Expression experiments show that in wild type plants the nuclear coded complex I genes as well as the nuclear Complex III genes are up-regulated in flowers particularly in anthers (Fig. 4) [39–41,62]. This upregulation is mainly mediated at the transcriptional level [E. Zabaleta, unpublished results]. Similarities in the expression patterns of nuclear complex I genes (Fig. 4) suggest a coordinate control through a common regulatory pathway, possibly through common *trans*-acting factors recognizing the respective promoter regions of these genes.

Loss-of-function as well as gain-of-function experiments using different regions of the nuclear complex I promoters in transgenic *Arabidopsis* plants indicate that positive *cis*- or enhancer-elements involved in anther/pollen specific expression are present in these promoters [E. Zabaleta, unpublished results]. Moreover, the presence of sequences homologous to pollen-specific boxes supports the involvement of common *trans*-acting factors, which could furthermore be involved in sensing and responding to the metabolic state of mitochondria and of the cell. Efforts to characterize these *trans*-acting factors are currently under way.

7. Outlook

The availability of antisense plants for specific complex I subunits may be of applied use since these gene suppressions do not affect normal plant growth. Such antisense induced male sterility may be controlled and reverted by specific introduction of additional sense sequences or by specific suppression of the antisense gene by crosses with corresponding transgenic lines in hybrid seed production processes. The feasibility of such a control of male fertility will have to be practically evaluated with the respective plant lines.

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