

1 **Short title:** The function of NFU4 and NFU5 in *Arabidopsis*

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3 **Title: The Fe-S cluster assembly factors NFU4 and NFU5 are primarily required for**
4 **protein lipoylation in mitochondria**

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15 **One sentence summary:** A pair of evolutionarily conserved proteins involved in iron-sulfur
16 cofactor assembly have a specific role in lipoate biosynthesis for mitochondrial
17 dehydrogenases.

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19 **Footnotes:**

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41 authors analysed the data. J.B. wrote the article with figures and text contributions of all
42 authors.

43 **ABSTRACT**

44 Plants have evolutionarily conserved NFU-domain proteins that are targeted to plastids or
45 mitochondria. The ‘plastid-type’ NFU1, NFU2 and NFU3 in *Arabidopsis thaliana* play a role in
46 iron-sulfur (Fe-S) cluster assembly in this organelle, whereas the type-II NFU4 and NFU5
47 proteins have not been subjected to mutant studies in any plant species to determine their
48 biological role. Here we confirm that NFU4 and NFU5 are targeted to the mitochondria. The
49 proteins are constitutively produced in all parts of the plant, suggesting a housekeeping
50 function. Double *nfu4 nfu5* knockout mutants were embryonic lethal, and depletion of the
51 proteins led to growth arrest of young seedlings. Biochemical analyses revealed that NFU4
52 and NFU5 are required for lipoylation of the H proteins of the glycine decarboxylase complex
53 and the E2 subunits of other mitochondrial dehydrogenases, with little impact on Fe-S
54 cluster-containing respiratory complexes and aconitase. Consequently, the Gly-to-Ser ratio
55 was increased in mutant seedlings and early growth was improved by elevated CO₂. In
56 addition, pyruvate, 2-oxoglutarate and branched-chain amino acids accumulated in the *nfu4*
57 *nfu5* mutants, further supporting defects in the other three mitochondrial lipoylation-dependent
58 enzyme complexes. NFU4 and NFU5 interacted with mitochondrial lipoyl synthase (LIP1) in
59 yeast 2-hybrid and bimolecular fluorescence complementation assays. These data indicate
60 that NFU4 and NFU5 have a more specific function than previously thought, in providing Fe-
61 S clusters to lipoyl synthase.

62 **INTRODUCTION**

63 Iron-sulfur (Fe-S) clusters are inorganic cofactors with redox or regulatory functions. These
64 labile metal cofactors are essential for the function of more than hundred different proteins
65 involved in electron transfer, catalysis or regulatory processes in plants (Przybyla-Toscano et
66 al., 2021a). The biosynthesis of Fe-S clusters requires dedicated assembly proteins that are
67 conserved from bacteria to eukaryotes. Plants contain three Fe-S cluster assembly
68 pathways, the so-called ISC (iron-sulfur cluster) pathway in the mitochondria, the SUF (sulfur
69 mobilization) pathway in the plastids and the CIA (cytosolic iron-sulfur protein assembly)
70 pathway for the maturation of Fe-S proteins in the cytosol and nucleus (Couturier et al.,
71 2013; Balk and Schaedler, 2014). The mitochondrial ISC pathway provides Fe-S clusters for
72 the respiratory complexes I, II and III of the electron transport chain, aconitase in the

73 tricarboxylic acid cycle and several enzymes in biosynthetic pathways (Przybyla-Toscano et
74 al., 2021).

75 The proposed working mechanism of the ISC pathway is based on mutant studies in
76 Baker's yeast, *Saccharomyces cerevisiae*, as well as extensive *in vitro* studies using
77 orthologs from other eukaryotes (Lill and Freibert, 2020; Przybyla-Toscano et al., 2021b). In
78 brief, and using nomenclature of the plant proteins, the first step of Fe-S cluster assembly is
79 carried out by the cysteine desulfurase NFS1, which acquires a protein-bound persulfide (S^0)
80 from cysteine. The persulfide is transferred to the scaffold protein ISU1, where it is reduced
81 to sulfide (S^{2-}) and combined with Fe to form a Fe_2S_2 cluster. Next, a chaperone-
82 cochaperone couple helps move the cluster from ISU1 to one of several so-called transfer
83 proteins which facilitate incorporation of the correct type of cluster into client Fe-S proteins.
84 The precise molecular roles of these transfer proteins are not known, and research has
85 focussed on establishing their sequence of action and whether they are required for specific
86 Fe-S proteins. The mitochondrial glutaredoxin GRXS15 is likely to accept the Fe_2S_2 cluster
87 from ISU1, as shown for yeast GRX5 (reviewed in Lill and Freibert, 2020). *In vitro* studies
88 demonstrated that Arabidopsis GRXS15 can bind an Fe_2S_2 cluster and transfer it directly to
89 ferredoxin 1 (Moseler et al., 2015; Ströher et al., 2016; Azam et al., 2020b). (Moseler et al.,
90 2015; Azam et al., 2020b)(Moseler et al., 2015; Azam et al., 2020b)(Moseler et al., 2015;
91 Azam et al., 2020b)(Moseler et al., 2015; Azam et al., 2020b)(Moseler et al., 2015; Azam et
92 al., 2020b)(Moseler et al., 2015; Azam et al., 2020b)These glutaredoxins also mediate the
93 reductive fusion of two Fe_2S_2 clusters to form a Fe_4S_4 cluster on the heterodimeric ISCA1/2
94 proteins (Brancaccio et al., 2014; Azam et al., 2020a; Weiler et al., 2020). Further *in vitro*
95 studies showed that ISCA1/2 can transfer the Fe_4S_4 cluster to NFU4 or NFU5, which can
96 donate the cluster to aconitase 2 (Azam et al., 2020a). All mitochondrial NFU proteins
97 studied to date form homodimers with a Fe_4S_4 cluster bound to the CxxC motifs of each
98 protomer. The Fe-S cluster binding CxxC motif is also found in INDH (Iron-sulfur cluster
99 protein required for NADH Dehydrogenase) which has been proposed to specifically transfer
100 Fe-S clusters to respiratory complex I (Wydro et al., 2013).

101 The NFU (NifU-like) proteins are named after the *nifU* gene in the nitrogen fixation
102 (*nif*) regulon in *Azotobacter vinelandii* (Yuvaniyama et al., 2000). In fact, AvNifU has three
103 protein domains that usually exist as separate proteins in other organisms: the N-terminal
104 domain of AvNifU is homologous to ISU1, the central domain is a functional ferredoxin, and
105 the C-terminal domain is similar to what are generically called NFU proteins (Mühlenhoff and
106 Lill, 2000; Py et al., 2012). Based on phylogenetic analysis, four different types of NFU
107 proteins have been identified in bacteria, of which type II in alpha-proteobacteria has been
108 inherited by mitochondria (Py et al., 2012). In the green lineage, NFU proteins are also
109 present in plastids. They fall outside the bacterial classification system and are characterized
110 by a duplicated NFU sequence, of which the second sequence is degenerate and lacks the
111 CxxC motif. Of the five NFU proteins in Arabidopsis, NFU1, NFU2 and NFU3 belong to the
112 'plastid-type' and are indeed targeted to plastids (León et al., 2003). NFU4 (AT3G20970)
113 and NFU5 (AT1G51390) are type-II NFU proteins. Mitochondrial localization of Arabidopsis
114 NFU4 was supported by transient expression of a GFP fusion protein (León et al., 2003).
115 Furthermore, it was shown that *NFU4* and *NFU5* are able to complement the yeast *nfu1Δ*
116 and *nfu1Δisu1Δ* strains with respect to growth and biochemical phenotypes (León et al.,
117 2003; Uzarska et al., 2018), demonstrating that the plant genes are functional orthologs of
118 yeast *NFU1*. However, the physiological role of mitochondrial NFU proteins has not been
119 investigated in plants.

120 Deletion of *NFU1* in yeast impairs growth on acetate as carbon source and affects
121 specific Fe-S enzymes, such as aconitase, respiratory complex II (succinate dehydrogenase,
122 SDH), and also lipoyl-dependent enzymes (Melber et al., 2016). The severity of the *nfu1Δ*
123 phenotype depends on the yeast strain but is relatively mild in contrast to the essential
124 nature of *NFU1* in mammals. A small number of mitochondrial disorders in human patients
125 has been associated with rare mutations in the coding sequence of *NFU1* (Cameron et al.,
126 2011; Navarro-Sastre et al., 2011; Mayr et al., 2014). *NFU1* patients commonly display
127 infantile encephalopathy with symptoms such as hyperglycinaemia and lactic acidosis, which
128 in severe cases is fatal in the first year of life. Biochemical tests on muscle biopsies or
129 fibroblasts showed decreased activities of lipoylated enzymes and, in some but not all cases,
130 a decrease in aconitase and SDH activity.

131 In eukaryotes, there are four mitochondrial lipoyl-dependent enzyme systems,
132 pyruvate dehydrogenase (PDH), α -ketoglutarate dehydrogenase (KGDH), branched-chain α -
133 ketoacid dehydrogenase (BCKDH) and glycine decarboxylase complex (GDC), also referred
134 to as the glycine cleavage system. The PDH, KGDH and BCKDH complexes consist of three
135 different subunits (E1 - E3), of which E2 is lipoylated. The structure of GDC complex differs,
136 being composed of four proteins named L, P, T and H, with lipoyl bound to the H protein.
137 The lipoyl cofactor (6,8-dithiooctanoic acid) mediates the oxidative decarboxylation reactions
138 carried out by those enzyme complexes (Mayr et al., 2014). The 8-carbon fatty acid is
139 covalently bound to proteins via a lysine residue, forming a lipoamide, and contains two
140 sulfur atoms at C6 and C8 that are inserted by lipoyl synthase. The sulfur atoms are
141 provided by the auxiliary Fe₄S₄ cluster of lipoyl synthase. Therefore, this cluster needs to be
142 reassembled after every catalytic cycle. *In vitro* studies showed that the bacterial NfuA
143 protein enabled catalytic rates of lipoyl formation by its ability to reconstitute the auxiliary
144 cluster of lipoyl synthase (McCarthy and Booker, 2017).

145 Here we show that the Arabidopsis *NFU4* and *NFU5* proteins perform an essential
146 function in mitochondria. Analysis of several mutant lines showed that low levels of the *NFU*
147 proteins are sufficient for normal growth, but their near-complete depletion revealed a defect
148 in protein lipoylation, affecting substrate turnover by lipoate-dependent enzyme complexes
149 such as GDC, KGDH and BCKDH.

150

151 **RESULTS**

152

153 ***NFU4* and *NFU5* are soluble mitochondrial matrix proteins**

154 A previous study showed that transient expression of a fusion protein of the first 116 amino
155 acids of *NFU4* with GFP was targeted to mitochondria in Arabidopsis protoplasts (León et
156 al., 2003). However, the localization of *NFU5* was not experimentally tested. Some evidence
157 for the presence of *NFU5* in mitochondria has been obtained by proteomics studies (Ito et
158 al., 2006; Tan et al., 2010; Fuchs et al., 2020), but has so far not been confirmed by other
159 approaches. Therefore, we cloned the predicted mitochondrial targeting sequences (MTS) of
160 *NFU4* and *NFU5* in frame with the *GFP* coding sequence. The length of the MTS of each
161 protein was determined by combining several *in silico* analyses such as target peptide
162 prediction algorithms, the distribution of acidic amino acids and alignment with eukaryotic
163 and prokaryotic homologs. Following this, amino acids 1 to 79 for *NFU4* and 1 to 74 for
164 *NFU5* were assigned as MTS. The fusion genes were placed under the control of a double
165 *CaMV* 35S promoter and transformed into Arabidopsis protoplasts. Both *NFU4*_{MTS}-GFP and
166 *NFU5*_{MTS}-GFP showed a punctate pattern of GFP which co-localized with MitoTracker dye,
167 but was distinct from the chloroplast autofluorescence (Supplemental Fig. S1A). Protein blot
168 analysis of total and mitochondrial protein fractions confirmed the enrichment of the native
169 *NFU4* and *NFU5* proteins in mitochondria (Supplemental Fig. S1B). Furthermore,
170 fractionation of the mitochondria into soluble matrix and membrane fractions indicated that
171 *NFU4* and *NFU5* are matrix proteins, similar to *ISU1* and *GRXS15* (Supplemental Fig. S1C).

172

173 ***NFU4* and *NFU5* proteins are abundant in all plant organs**

174 Antibodies raised against *NFU4* cross-reacted with *NFU5* and vice versa, which is not
175 surprising as the mature proteins share 90% amino acid identity. To determine which of the
176 two immune signals with similar gel mobilities belongs to *NFU4* and *NFU5*, and for
177 subsequent mutant studies, we obtained three mutant alleles each for *nfu4* and *nfu5* (Fig.
178 1A).

179 Quantitative reverse transcription PCR (RT-qPCR) showed a virtual absence of
180 *NFU4* transcripts in homozygous *nfu4-2* and *nfu4-4* single mutant plants, whereas ~10%
181 transcript remained in the *nfu4-1* mutant (Fig. 1B). Expression of *NFU5* was strongly
182 diminished in the *nfu5-1* line (Fig. 1B). The *nfu5-2* mutant had approximately 15% less *NFU5*
183 transcript compared to its respective wild type, consistent with insertion of the T-DNA in the
184 promoter, 251 nucleotides upstream of the ATG start codon. For the *nfu5-3* mutant, qPCR
185 analysis suggested that the transcript levels of *NFU5* were strongly increased, but this result
186 is likely due to the presence of an outward facing promoter sequence at the right T-DNA
187 border and the qPCR primers being downstream of this. Probing for full-length *NFU5*
188 transcript by RT-PCR showed that the transcript is lacking in the *nfu5-3* mutant (Fig. 1B,
189 right panel), as expected from the position of the T-DNA in exon 2 (Fig. 1A).

190 Protein blot analysis using anti-*NFU4* serum showed that the upper immune signal is
191 absent in the *nfu4-2* and *nfu4-4* mutants, and that the lower signal is missing in the *nfu5-1*
192 and *nfu5-3* mutant alleles (Fig. 1C). The native protein products of *NFU4* and *NFU5* could
193 thus be assigned and their electrophoretic mobilities match the calculated molecular weights
194 of 22.1 kDa for *NFU4* and 21.7 kDa for *NFU5* without their predicted MTS. The levels of
195 *NFU5* protein produced from the *nfu5-2* allele were assessed by semi-quantitative protein
196 blot analysis in plants homozygous for the *nfu4-2* allele, to rule out signal contribution from

197 *NFU4*. The *NFU5* protein level was ~15% less in the *nfu4-2 nfu5-2* double mutant compared
198 to wild type (Fig. 1D; Supplemental Fig. S2), matching the decrease in *NFU5* transcripts (Fig.
199 1B).

200 Publicly available RNA-seq data (bar.utoronto.ca) indicated that *NFU4* and *NFU5* are
201 expressed throughout the plant's life cycle and in all organs. Protein blot analysis using
202 protein extracts from roots, leaves, stems and reproductive organs confirmed that *NFU4* and
203 *NFU5* proteins are expressed in all organs, with slightly lower levels in stems and slightly
204 higher levels in flower buds and flowers, based on total protein (Fig. 2A). Densitometry and
205 quantification of the immune signals relative to known amounts of recombinant protein
206 indicated that *NFU4* was approximately 2-fold more abundant than *NFU5* (Fig. 2B), in
207 agreement with a recent quantitative analysis of all mitochondrial proteins (Fuchs et al.,
208 2020).

209 ***NFU4* and *NFU5* are redundant genes, but together are essential for embryo** 210 **development**

211 In order to evaluate the physiological importance of *NFU4* and *NFU5*, the available T-DNA
212 insertion lines were grown on soil under long-day conditions. The three different mutant lines
213 for *NFU4* or *NFU5* showed normal growth and development of the rosette leaves compared
214 to their respective wild-type plants (Fig. 3A). The growth rate of primary roots in young
215 seedlings, which is particularly sensitive to mitochondrial defects, was decreased by 8% in
216 the *nfu4-2* mutant but a decrease in the *nfu4-4* mutant was not statistically significant
217 (Supplemental Fig. S3A, B). Root growth in the *nfu5-1* mutant was ~30% decreased,
218 whereas *nfu5-2* root length was similar to wild type (Supplemental Fig. S3C, D), as expected
219 based on the weak nature of this mutant allele. In addition, the seedlings were challenged
220 with Paraquat (methyl viologen) in the medium, which strongly inhibited growth of an *E. coli*
221 *nfuAD* strain (Angelini et al., 2008) and is a well-known inducer of oxidative stress in
222 mitochondria (Cochemé and Murphy, 2008). A concentration of 10 nM Paraquat was chosen
223 based on experimental calibration, resulting in 20% inhibition of wild-type root growth.
224 However, Paraquat treatment did not specifically affect the mutants more than the wild-type
225 controls (Supplemental Fig. S3). Taken together, these observations indicate that aside from
226 a role in root elongation, individually neither *NFU4* nor *NFU5* perform a critical function under
227 normal growth conditions.

228 To generate double mutants, we made reciprocal crosses between *nfu5-1* and *nfu4-2*
229 or *nfu4-4* plants. In the F2 generation, double mutants could not be isolated despite
230 extensive screening by PCR. Therefore, the siliques of *nfu4-2^{-/-} nfu5-1^{-/+}* and *nfu4-4^{-/-} nfu5-1^{-/+}*
231 plants were dissected to analyse embryo development. Approximately one quarter of the
232 immature seeds were white (Fig. 3B, C), containing embryos arrested at the globular stage
233 (Fig. 3D). Based on these numbers it is reasonable to assume that the white seeds are
234 double knockout *nfu4 nfu5*, and that a complete lack of both *NFU4* and *NFU5* protein is
235 detrimental for embryo development.

236

237 **Functional relationship between *ISU1*, *NFU4* and *NFU5***

238 In yeast, the mild growth defect as a result of *NFU1* deletion (*nfu1Δ*) is enhanced in an
239 *isu1Δnfu1Δ* double mutant, underscoring the functional relationship of the *ISU1* and *NFU1*
240 proteins (Schilke et al., 1999). It should be noted that the yeast *isu1Δ* mutant is viable
241 because of a second *ISU* gene, *ISU2*, but evidently this cannot fully replace the function of
242 *ISU1*. Arabidopsis has three *ISU* paralogs, each of which can functionally substitute for the

243 yeast *ISU1* homolog in the *isu1Δnfu1Δ* strain (León et al., 2005). The *Arabidopsis ISU1* gene
244 is thought to be the main Fe-S scaffold protein in mitochondria, because *ISU2* and *ISU3*
245 have very low expression levels and are almost exclusively expressed in pollen grains (León
246 et al., 2005; Frazzon et al., 2007). In agreement with this, we found that the *isu1-2* allele, in
247 which 61 nucleotides directly upstream of the ATG are deleted due to a T-DNA insertion, is
248 embryonic lethal (Supplemental Table S1). The previously reported *isu1-1* allele, with a T-
249 DNA insertion at nucleotide -65 but with intact sequence up to the start codon, is viable
250 (Frazzon et al., 2007) (Supplemental Fig. S4A). *ISU1* protein levels in the *isu1-1* line are
251 decreased to ~20% of wild type (Supplemental Fig. S4B). In cell culture generated from the
252 roots of *isu1-1* seedlings, the levels of *NFU4*, *NFU5*, *INDH* and aconitase were normal, and
253 the activity of respiratory complex I was also comparable to wild type (Supplemental Fig.
254 S4B, C). Interestingly, *GRXS15* protein levels were strongly decreased in the *isu1-1* mutant.

255 Rosettes of *isu1-1* plants were phenotypically indistinguishable from wild type, but
256 seedlings had a 20% decrease in root elongation, which was exacerbated to 40%
257 impairment in the presence of Paraquat (Supplemental Fig. S4D, E). This phenotype may be
258 correlated with decreased *GRXS15* levels, since *grxs15* mutants have strongly impaired root
259 growth (Ströher et al., 2016). To investigate genetic interactions between *ISU1* and *NFU4* or
260 *NFU5*, the *isu1-1* line was crossed with *nfu4* and *nfu5* mutant alleles. Crosses involving any
261 of the *nfu5* alleles and *isu1-1* were unsuccessful, despite multiple attempts in reciprocal
262 combinations. In contrast, double *isu1-1 nfu4* mutants were isolated from the F2, and had a
263 shorter root length but not an enhanced growth defect (Supplemental Fig. S4D).

264 Thus, mild growth impairment of the primary root in seedlings with less than 20%
265 *ISU1* is not enhanced by deletion of *NFU4*. This is in agreement with the model that *NFU*
266 proteins function downstream from *ISU1* in Fe-S cluster transfer, and not as an alternative
267 assembly scaffold.

268

269 **Low amounts of *NFU5* alone are sufficient for normal mitochondrial function in** 270 **vegetative tissues**

271 The normal rosette growth of single *nfu4* and *nfu5* mutants does not preclude defects in Fe-
272 S enzyme activities. For example, up to 80% of complex I activity can be lost in *Arabidopsis*
273 without major growth penalties (Meyer et al., 2011). To analyse the levels and/or activities of
274 major ISC maturation factors and mitochondrial Fe-S enzymes as a consequence of loss of
275 *NFU4* and/or *NFU5*, mitochondria were purified from 2-week-old seedlings. Protein blot
276 analyses showed no differences in the levels of *ISU1*, *GRXS15*, *INDH* and aconitase
277 between *nfu4-2*, *nfu4-4* and *nfu5-1* and wild type (Supplemental Fig. S5A). Labelling with
278 antibodies against lipoate also showed no differences in the abundance of lipoylated
279 proteins (Supplemental Fig. S5B). To analyse the levels of mitochondrial respiratory
280 complexes, we generated cell cultures from primary roots, then purified mitochondria for
281 blue-native PAGE. Protein complexes were stained with Coomassie Blue (Supplemental Fig.
282 S5C), and complex I and complex II were additionally visualized by in-gel activity staining
283 (Supplemental Fig. S5D, E). Again, no differences in the levels or the activities of these
284 major Fe-S cluster-dependent complexes were detected in single mutants compared with
285 wild-type plants.

286 To obtain viable plants with strongly diminished *NFU4* and *NFU5* protein levels, mutant
287 alleles were combined via crossing. *nfu4-2 nfu5-2* plants lack *NFU4* protein and have ~85%
288 *NFU5* compared to wild type, whereas hemizygous *nfu4-2 nfu5-1/nfu5-2* plants have only

289 ~42.5% *NFU5* (Fig. 4A). The size and fresh weight of the rosettes of hemizygous plants
290 were similar to wild type (Fig. 4B; Supplemental Fig. S6). In root growth assays, the double
291 mutant seedlings performed similarly to the Col-4 parent. However, in the presence of
292 Paraquat, there was a small but significant decrease in root growth in the hemizygous
293 mutant (Supplemental Fig. S3E). Because of a lipoate biosynthesis defect in yeast and
294 mammalian *nfu* mutants, we further tested growth of the Arabidopsis *nfu4-2 nfu5-2* double
295 mutants under low CO₂ (150 ppm) which should bring out defects in GDC. However, no
296 obvious differences in either fresh or dry weight biomass were observed between mutant
297 and wild-type plants (Supplemental Fig. S6).

298 To follow up the growth assays, biochemical analyses were carried out on
299 mitochondria purified from cell culture of hemizygous *nfu4-2 nfu5-1/nfu5-2* and homozygous
300 *nfu4-2 nfu5-2* seedlings. Protein blot analyses showed no differences in the levels of the
301 mitochondrial Fe-S cluster assembly proteins ISU1, GRXS15 and INDH and the Fe-S
302 enzyme aconitase (Fig. 4A). The only observed difference was a decrease in the levels of
303 the H2 protein of GDC. The levels of respiratory complexes I and III in the viable *nfu4 nfu5*
304 double mutants were comparable to wild type (Fig. 4C). Taken together, these biochemical
305 studies show that *NFU4* and *NFU5* are largely redundant during vegetative development;
306 and suggest that small amounts of *NFU5* are sufficient to sustain key mitochondrial
307 functions.

308

309 **Depletion of *NFU4* and *NFU5* causes a pleiotropic growth defect and accumulation of** 310 **substrates of lipoate-dependent enzyme complexes**

311 To uncover the phenotypic effects of a complete lack of *NFU4* and *NFU5* in vegetative
312 tissues and circumvent embryo lethality, *NFU4* was placed under the control of the *ABI3*
313 promoter (Rohde et al., 1999; Despres et al., 2001) and transformed into hemizygous *nfu4-2*
314 ⁻ *nfu5-1/nfu5-2* plants (Fig. 5A). The *ABI3* promoter drives expression in developing and
315 germinating seeds and can be used to study essential genes (Despres et al., 2001). Positive
316 transformants were selected on hygromycin-containing plates, and three seedlings carrying
317 the *nfu5-1* knockout allele were identified by PCR for further study (independent lines 1, 4
318 and 10). In the next generation (T2), seedlings segregated in approximately 21 – 25% with a
319 severe growth phenotype and 75 – 79% with normal growth. Leaves of the mutant seedlings
320 turned white upon emergence, and growth was arrested at the second pair of true leaves
321 (Fig. 5B). PCR analysis confirmed that the mutant (m) seedlings lacked a functional copy of
322 *NFU5*, whereas the wild-type-like (wtl) siblings carried the *nfu5-2* allele with an intact *NFU5*
323 open reading frame (Fig. 5C). In agreement with the genotyping results, the mutant
324 seedlings lacked *NFU5* protein, whereas the *NFU5*-specific protein band was present in the
325 wtl seedlings (Fig. 5D). In both mutant and wtl segregants, the protein levels of *NFU4* were
326 either undetectable or very low, reflecting the combined effect of the *nfu4-2* allele and *ABI3*
327 promoter-driven *NFU4* expression.

328

329 In an attempt to overcome growth arrest, the segregating T2 generation was grown
330 under elevated CO₂. We reasoned that the observed photobleaching of the mutant seedlings
331 could be due to a defect in photorespiration. Under high CO₂, photorespiration is prevented
332 and impairment of GDC is tolerated, except when it is so low as to affect one-carbon
333 metabolism (Peterhansel et al., 2010). Indeed, when seedlings were germinated and grown
334 under high CO₂, segregating *nfu4 nfu5* mutant seedlings were more difficult to distinguish
335 from wtl siblings in the first two weeks, as double mutants remained green and started to
develop a third pair of true leaves (Fig. 6A). However, soon after transferring the seedlings

336 from agar plates to soil in the high CO₂ cabinet, the *nfu4 nfu5* mutants turned white and their
337 growth was arrested before the inflorescence was formed (Supplemental Fig. S7).

338 To investigate if GDC activity was decreased in the mutants, we measured the
339 concentration of glycine and serine as well as other free amino acids using LC-MS. Glycine
340 accumulated 8-fold in the mutant compared to wtl seedlings (Fig. 6B; Supplemental Table
341 S2). The Gly:Ser ratio of 3.5 in the mutant versus a ratio of 1 in wtl is indicative of impaired
342 GDC activity in the mutant (Timm et al., 2012; Reinholdt et al., 2021). It should be noted that
343 the serine levels we measured in control (wtl) seedlings were approximately 2-fold lower
344 than routinely measured in rosette leaves of Arabidopsis but the levels of other amino acids
345 were comparable to published values (reviewed in (Hildebrandt et al., 2015)).

346 Additionally, the branched-chain amino acids leucine and valine were significantly
347 increased in concentration compared with wtl, and the isoleucine concentration was elevated
348 although not significant (Supplemental Table S2). This suggests that the lipoyl-dependent
349 enzyme complex BCKDH has decreased functionality. Of the other amino acids, alanine and
350 phenylalanine levels were on average more than 7-fold increased in the mutant, but the
351 levels were very variable in the 3 biological replicates of the mutant. Similarly, arginine,
352 asparagine, lysine and tryptophan were >2-fold increased in the mutant, and aspartic acid
353 was >2-fold decreased, but not statistically significant because of large variation.

354 To identify changes in pyruvate and TCA cycle intermediates, we measured the
355 concentrations of selected organic acids by LC-MS/MS. The *nfu4 nfu5* mutant accumulated
356 10-fold more α -ketoglutarate than the wtl segregants and wild type, indicating that the
357 activity of KGDH is impaired (Fig. 6B; Supplemental Table S3). The concentration of
358 pyruvate in the mutant was 2-fold higher than in wild type seedlings and 1.5-fold higher than
359 in wtl. Citrate and malate concentrations were lower in the *nfu4 nfu5* mutant seedlings,
360 whereas succinate was elevated in both the mutant and wtl segregant compared to wild-type
361 seedlings (Fig. 6B; Supplemental Table S3). Together, these results indicate that *NFU4* and
362 *NFU5* are required for the function of GDC as well as the other major mitochondrial enzyme
363 complexes that depend on lipoate as a cofactor.

364 ***NFU4* and *NFU5* are required for lipoylation of GDC H proteins and E2 subunits of** 365 **mitochondrial dehydrogenase enzyme complexes**

366 To investigate a possible decrease in protein-bound lipoyl cofactor in the *nfu4 nfu5* mutant,
367 immunoblots of total plant protein extracts were labelled with antibodies against lipoate. This
368 showed a pattern similar to those previously published and proteins were assigned
369 accordingly (Ewald et al., 2014; Ströher et al., 2016; Guan et al., 2017). We observed that
370 *nfu4 nfu5* mutant seedlings lacked a signal with an electrophoretic mobility of 15 kDa
371 corresponding to the lipoylated H1 + H3 proteins of GDC (Fig. 6C, upper panel). The
372 mutants also showed a decreased signal at ~65 kDa, assigned as the E2b subunit isoform of
373 the PDH complex, compared to wild type (WT) and wild-type like (wtl) segregants. The
374 signal corresponding to the 82-kDa E2a subunit of PDH was slightly decreased in the mutant
375 compared to WT, but increased in wtl seedlings. The 45 kDa band was assigned to the E2
376 subunit proteins of plastid-localized PDH, which had similar levels of lipoylation in the mutant
377 and WT plants.

378 To probe whether non-lipoylated H proteins were present in the mutant lines, a
379 parallel blot of the same samples was probed with an antibody that recognizes H isoforms
380 from a range of plant species including the 3 isoforms in Arabidopsis (Ströher et al., 2016).
381 The signal from the H1 and H3 isoforms at 15 kDa was strongly decreased in the *nfu4 nfu5*
382 mutant, pointing to destabilization of these subunits due to a lack of bound lipoate. The

383 mature H2 protein has a theoretical mass similar to H1 and H3, but as noted before, it has a
384 lower electrophoretic mobility in SDS-PAGE (Fig. 6C, middle panel), possibly because of its
385 unusual low pI (Lee et al., 2008; Ströher et al., 2016). The two signals assigned as H2 likely
386 correspond to the lipoylated and non-lipoylated forms (Ströher et al., 2016). The major H2
387 isoform present in wtl and WT was barely detectable in *nfu4 nfu5* seedlings. These data
388 indicate that NFU4 and NFU5 are required for protein lipoylation in mitochondria, strongly
389 affecting the H subunits of GDC and to a lesser extent the E2 subunits of PDH.

390

391 **Aconitase and complex II activities are normal in seedlings depleted of NFU4 and** 392 **NFU5**

393 Since high CO₂ conditions only partially rescued the severe phenotype of *nfu4 nfu5* mutants,
394 we assessed the overall mitochondrial respiration rate by measuring O₂ consumption in
395 intact mutant and wtl seedlings in the dark. The *nfu4 nfu5* double mutant showed a 5-fold
396 decrease in respiration compared to wild-type seedlings and segregating wtl siblings (Fig.
397 7A), in line with defects in key mitochondrial enzyme complexes. In the yeast *nfu1Δ* mutant,
398 the activities of the Fe-S enzymes aconitase and SDH/complex II were decreased by ~25%
399 (Melber et al., 2016; Uzarska et al, 2018). Measurements of aconitase activity in total plant
400 extracts showed a decrease of ~50% in the *nfu4 nfu5* mutant compared to wild-type
401 seedlings and a decrease of 30% compared to wtl segregants, although the latter was not
402 statistically significant (Fig. 7B). However, in plants grown under high CO₂, aconitase activity
403 was 2-fold higher than under ambient CO₂ levels and similar in the mutant and wtl
404 segregants (Fig. 7B). This suggests that any decrease in aconitase activity may be a
405 secondary defect, caused by, for example, limited carbon flux through the tricarboxylic acid
406 cycle. This is in agreement with the observed decrease in citrate and malate (Fig. 6B).

407 To measure complex II activity, mutant and wtl segregants were pooled for small-
408 scale mitochondrial preparations. Of the different enzyme assays for complex II, succinate to
409 ubiquinone reduction (SQR) using 2,6 dichloroindophenol as electron acceptor gave the
410 most robust results in relatively impure mitochondrial preparations (León et al., 2007). The
411 activity was measured before and after addition of the complex II inhibitor 2-
412 thenoyltrifluoroacetone (TTFA) to distinguish complex II activity from other succinate
413 reduction reactions. The TTFA-sensitive succinate turnover was similar in all three
414 genotypes, i.e. *nfu4 nfu5* mutant, wtl segregants and true wild type (Fig. 7C). Thus, both
415 aconitase and SDH had a normal operational capacity in *nfu4 nfu5* mutant seedlings, in
416 contrast to the decrease in activities of these enzymes in yeast *nfu1Δ* mutants.

417 **NFU4 and NFU5 proteins interact with the mitochondrial lipoyl synthase LIP1**

418 Previously, Arabidopsis NFU4 and NFU5 were shown to interact with the late-acting ISC
419 proteins ISCA1a and ISCA1b (Azam et al., 2020a). To test if NFU4 and NFU5 can interact
420 with downstream client Fe-S proteins, we carried out yeast 2-hybrid assays with
421 mitochondrial lipoyl synthase (LIP1), biotin synthase (BIO2) and the major aconitase protein
422 localized to mitochondria (ACO2). Biotin synthase is similar to lipoyl synthase in that an
423 auxiliary Fe-S cluster is destroyed to donate a sulfur atom in the last step of biotin synthesis,
424 except that this cluster is a Fe₂S₂ cluster rather than a Fe₄S₄ cluster. Mitochondrial targeting
425 sequences were removed from the coding sequences, which were cloned behind the
426 activation domain (AD) or DNA binding domain (BD) of the Gal4 transcriptional activator.
427 Yeast growth on medium without histidine indicated that the *HIS3* reporter gene is
428 transcribed as a consequence of a direct protein interaction between NFU4/5 and LIP1 (Fig.
429 8A). The growth persisted under more stringent conditions with 3-amino-1,2,4-triazole (3AT),

430 a competitive inhibitor of the *HIS3* gene product, suggesting that the interaction between
431 NFU4, or NFU5, with LIP1 is relatively strong. However, no interactions between NFU4/5
432 and BIO2, nor with ACO2, were detected in these assays.

433 To assess NFU4/5-LIP1 interactions in plant cells, we additionally performed bimolecular
434 fluorescence complementation (BiFC) assays in Arabidopsis protoplasts using the LIP1
435 coding region fused upstream of the N-terminal domain of the YFP protein, and NFU
436 proteins fused upstream of the C-terminal region of YFP (LIP1-N and NFU-C, respectively, in
437 Fig. 8B and Supplemental Fig. S9). Positive BiFC signals indicated that LIP1 is in close
438 proximity to NFU4 and NFU5 within plant cells, and that the LIP1 interaction with NFU4 or
439 NFU5 occurs in the mitochondria as highlighted by the overlap of the YFP fluorescence with
440 MitoTracker dye (Fig. 8B, merged panels). None of the proteins expressed alone could
441 restore YFP fluorescence as previously shown (Azam et al., 2020a). Together, these results
442 indicate that NFU4 and NFU5 interact equally well with LIP1 in the mitochondria, and likely
443 assist with the insertion or repair of Fe-S clusters in LIP1.

444

445

446 DISCUSSION

447 The physiological function of the mitochondrial NFU proteins has thus far not been studied in
448 plants, except for one study in sweet potato which found that the gene encoding a
449 mitochondrial NFU was upregulated under salt stress in a salt-tolerant variety (Wang et al.,
450 2013). Here we show that the mitochondrial NFU proteins perform a key function in lipoate-
451 dependent metabolism in the model plant *Arabidopsis*, most likely by providing Fe-S clusters
452 to lipoyl synthase as shown for bacterial NfuA.

453 *Arabidopsis* has two paralogous genes, *NFU4* and *NFU5*, each encoding a functional
454 mitochondrial NFU protein of relatively high abundance (Figs. 1 – 3). While no obvious
455 growth phenotypes were observed in *nfu4* or *nfu5* single mutants, some of our findings
456 suggest that *Arabidopsis* *NFU4* and *NFU5* may not be fully redundant. Single *nfu4* and *nfu5*
457 mutants had impaired primary root growth in young seedlings (Supplemental Fig. S3).
458 Moreover, during reproductive development, *nfu5* mutants could not be crossed with the
459 *isu1-1* mutant as male or female parent, despite a functional copy of *NFU4* (Supplemental
460 Fig. S4). Possibly, cell-specific expression of *NFU4* and *NFU5* in some specialized cell
461 types, or differences in protein stability, could underlie these observations. Related to this is
462 the question of whether *NFU4* and *NFU5* can form heterodimers or exist exclusively as
463 homodimers. The yeast 2-hybrid assay testing for *NFU4*-*NFU5* interaction was negative
464 (Azam et al., 2020a), which is not due to technical issues since the same constructs showed
465 positive interactions with other mitochondrial proteins such as ISCA1/2 (Azam et al., 2020a)
466 and LIP1 (Fig. 8A). Thus, *NFU4* and *NFU5* are likely functioning as homodimers, with mostly
467 overlapping but perhaps not identical physiological functions, which will be interesting to
468 delineate in future studies.

469 The double knockout mutant of *NFU4* and *NFU5* in *Arabidopsis* is embryonic lethal
470 (Fig. 3B-D), demonstrating that mitochondrial NFUs are essential in plants. The single *NFU1*
471 homolog is also essential in mammals but the yeast gene is non-essential. How could this
472 difference be explained? Lipoyl synthase is the main client Fe-S protein in all three types of
473 organisms and an essential enzyme in higher eukaryotes, but not in yeast (Cronan, 2016).
474 This is because lipoyl enzyme complexes can be bypassed, especially when yeast is grown
475 on glucose and switches to fermentation. In bacteria, loss of lipoyl synthase (LipA) can be
476 overcome by supplementation with lipoate in the medium (McCarthy and Booker, 2017), but
477 this cannot rescue yeast *lip5* mutants (Sulo and Martin, 1993). Whether plant mutants in
478 lipoyl synthase can be chemically complemented by external lipoate has not been
479 investigated to our knowledge, but we found that addition of lipoate to the medium did not
480 rescue the growth of *nfu4 nfu5* mutant seedlings in any way (Supplemental Fig. S10). The
481 relatively high abundance of *NFU4* and *NFU5* compared to other Fe-S cluster maturation
482 proteins would fit with a role of NFU proteins in reassembling the auxiliary cluster of LIP1,
483 which is constantly turned over to insert two sulfurs into lipoyl cofactor. Particularly
484 mitochondria in leaf mesophyll cells have a very high demand of lipoate for the H proteins of
485 GDC.

486 To obtain *nfu4 nfu5* mutant lines with a clear phenotype, we used a strategy based
487 on the transcriptional depletion of *NFU4* in a *nfu4 nfu5* genetic background (Fig. 5), after
488 other approaches gave non-viable plants or no visible growth defects (e.g. Fig. 4). While the
489 depletion approach was stringent and robust, the seedlings did not develop further than the
490 2-leaf stage, as reported for other essential genes (Rohde et al., 1999; Despres et al., 2001).
491 This limited biochemical assays, especially since it was not possible to isolate mitochondria
492 from the double mutant for enzyme assays. However, protein blot analysis showed a striking

493 decrease in lipoylated proteins, especially the H proteins, and metabolite analysis provided
494 further evidence of blocks in specific mitochondrial processes. While some variation between
495 biological replicates of the mutant, for example in amino acid levels, could have been due to
496 the precise timing of sampling relative to growth arrest, several highly significant changes
497 were noted that have also been observed in other mutants in the lipoate biosynthesis
498 pathway. Specifically, accumulation of glycine and elevated serine has so far been observed
499 in all mutants affected in lipoyl cofactor biosynthesis and its octanoyl precursor (Ewald et al.,
500 2014; Guan et al., 2017; Fu et al., 2020; Guan et al., 2020). Weaker mutant alleles (e.g.
501 RNAi lines or non-essential genes) are primarily affected in the lipoylation of H proteins,
502 whereas stronger alleles also showed decreased lipoylation of E2 subunits of PDH and
503 KGDH. Interestingly, mutation of *GRXS15*, the mitochondrial glutaredoxin associated with
504 the Fe-S cluster assembly pathway, also primarily affects lipoyl biosynthesis but not other
505 Fe-S cluster-dependent processes (Ströher et al., 2016; Moseler et al., 2021). In-depth
506 metabolomics analysis of a *grxs15* K83A mutant line showed accumulation of glycine,
507 branched-chain amino acids and their keto-acids, a 5-fold increase in pyruvate but no
508 significant difference in α -ketoglutarate (Moseler et al., 2021). Decreased lipoylation of the H
509 proteins coincided with lower abundance of the polypeptides, as seen in *nfu4 nfu5* mutants
510 (Fig. 6C).

511 *NFU1* mutations in human cells and yeast are known to cause defects in specific Fe-
512 S enzymes, namely complex II and aconitase (Mayr et al., 2014; Melber et al., 2016). In
513 Arabidopsis we saw no marked impairment of the activities of these enzymes in either single
514 mutants or the *nfu4 nfu5* double mutant (Figs 4, S5 and 7). However, metabolite analysis of
515 young leaves showed accumulation of succinate to similar levels in both *nfu4-2* and *nfu4-2*
516 *nfu5-1* seedlings, suggesting that NFU4 may have a specific role in supporting succinate
517 dehydrogenase function. Similarly, aconitase activity was slightly but significantly decreased
518 in the double mutant compared to wild type when grown under standard conditions. By
519 contrast, yeast 2-hybrid assays were negative for a protein interaction between NFU4 and
520 ACO2 (Fig. 8). Interestingly, *in vitro*, ACO2 can receive an Fe-S cluster from NFU4 or NFU5
521 (Azam et al., 2020a), but this may simply reflect a possible thermodynamic transition, and
522 not a physiological event. Alternatively, if ACO2 can receive its cluster directly from ISCA1/2
523 *in vivo*, this would bypass NFU4/5. It is also possible that the decreased aconitase and
524 complex II activities represent an indirect effect of strong impairment of PDH. Further
525 investigations are necessary to establish if NFU proteins do play a minor role in Fe-S cluster
526 assembly on other proteins than lipoyl synthase.

527 The mitochondrial NFU proteins differ from their plastid counterparts in both structure
528 and function. The plastid NFU proteins have an extra C-terminal domain which is a copy of
529 NFU but lacking the CxxC motif. The function of this second domain is as yet unclear.
530 Mutant analysis has shown that NFU1, NFU2 and NFU3 have partially overlapping functions
531 required for the stability of Photosystem I and other plastid Fe-S proteins (Touraine et al.,
532 2004; Yabe et al., 2004; Touraine et al., 2019; Berger et al., 2020; Roland et al., 2020).
533 Interestingly, one of the proteins found to interact with NFU2 using yeast 2-hybrid and BiFC
534 assays was the plastid isoform of lipoyl synthase LIP1p (Berger et al., 2020). The levels of
535 lipoyl cofactor on the plastid PDH E2 subunits have not been analysed to date. Plastid PDH
536 is an essential enzyme in fatty acid synthesis, and defects in this pathway have pleiotropic
537 effects on photosynthesis (Bao et al., 2000; Ke et al., 2000).

538 In summary, our functional study of the plant mitochondrial NFU proteins reveals
539 their importance for lipoyl cofactor biosynthesis and narrows down the position of these
540 proteins in the downstream pathway of Fe-S cluster assembly.

541

542 MATERIALS AND METHODS

543 Plant material and growth conditions

544 The following T-DNA insertion mutants were obtained from Arabidopsis stock centres
545 (ecotype in brackets, see Supplemental Table S1 for details): *isu1-1*, SALK_006332 (Col-0);
546 *isu1-2*, GK_424D02 (Col-0); *nfu4-1*, SALK_035493 (Col-0); *nfu4-2*, SALK_061018 (Col-0);
547 *nfu4-4*, SAIL_1233_C08 (Col-0); *nfu5-1*, WiscDsLoxHs069_06B (Col-0); *nfu5-2*, SK24394
548 (Col-4); *nfu5-3*, GT_3_2834 (Ler). Homozygous plants were selected using genotyping PCR
549 and the T-DNA insertion site was verified by sequencing from the left T-DNA border. For
550 *isu1-1* and *isu1-2*, the right border and contingent genomic DNA was also sequenced.
551 Primers are listed in Supplemental Table S4. The *nfu5-1* allele needed to be outcrossed to
552 remove an unrelated leaf phenotype.

553 Seeds were sown onto Levington's F2 compost, or they were surface sterilised using
554 chlorine gas and spread on ½-strength Murashige and Skoog (MS) medium containing 0.8%
555 (w/v) agar. After vernalization for 2 days at 4°C, plants were grown under long-day
556 conditions (16 hours light, 8 hours dark) at 22°C and light intensity of 180-200 $\mu\text{mol m}^{-2} \text{s}^{-1}$
557 unless otherwise indicated. The generation and propagation of callus cell culture was
558 performed as previously described (May and Leaver, 1993).

559 Gene expression analysis

560 Total RNA was extracted using the Plant RNeasy kit (Qiagen), followed by DNase treatment
561 (Turbo DNase kit, Agilent). The integrity of RNA in all samples was verified using agarose
562 gels, and RNA purity was analysed by comparing 260/230 nm and 260/280 nm absorbance
563 ratios (Nanodrop 2000, Thermo Fisher). RNA was quantified using a Qubit 2.0 fluorometer
564 (Thermo Fisher). RNA (4 μg) was reverse transcribed to cDNA using Superscript III (Thermo
565 Fisher). RT-qPCR reactions were made using SensiFAST master-mix (Bioline), in 20 μl
566 volumes, each with 20 ng of cDNA. Reactions were measured in a Bio-Rad CFX-96 real-
567 time PCR system and cycled as per the Bioline protocol. Data were analysed using the Bio-
568 Rad CFX Manager 3.1 software, and were normalized using primer efficiency. The house-
569 keeping gene *SAND* (*AT2G28390*) was used as reference gene (Han et al., 2013).

570 Protein extraction, gel electrophoresis and protein blot analysis

571 To extract proteins, plant tissues were ground in cold buffer containing 50 mM Tris-HCl pH
572 8.0, 50 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride, then centrifuged for
573 10 min to remove debris. Mitochondrial proteins from seedlings or cell culture were isolated
574 according to (Sweetlove et al., 2007). Blue-native polyacrylamide gel electrophoresis (BN-
575 PAGE) was carried out as described by (Wydro et al., 2013). For protein blot analysis, total
576 protein extracts or purified mitochondria were separated by SDS-PAGE and transferred to
577 nitrocellulose membrane by semi-dry electroblotting. To separate NFU4 and NFU5, 17%
578 (w/v) acrylamide was used in the gels. Blots were labelled with antibodies diluted 1:5000 for
579 anti-NFU4 and 1:2500 for anti-NFU5, followed by secondary anti-rabbit IgG conjugated to
580 horseradish peroxidase, and detected by chemiluminescence.

581 To produce antibodies for NFU4 and NFU5, recombinant proteins were expressed
582 and purified as described in (Zannini et al., 2018). These were used to raise polyclonal
583 antibodies in rabbits by the Agro-Bio company (La Ferté Saint Aubin, France). Antibodies
584 against the following proteins have been published: ISU1 (León et al., 2005); GRXS15
585 (Moseler et al., 2015); INDH and PDH E1 alpha (Wydro et al., 2013); and TOM40 (Carrie et

586 al., 2009). Antibodies against aconitase (AS09 521) and GDC-H protein (AS05 074) were
587 from Agrisera, Vännäs, Sweden; Antibodies against lipoate (ab58724) were purchased from
588 Abcam, Cambridge, UK and monoclonal antibodies against actin (clone mAbGEa, product
589 number MA1-744) were from ThermoFisher Scientific.

590 **Free amino acids and organic acids**

591 Unbound, water soluble amino acids were extracted according to (Winter et al., 1992), with
592 minor modifications. Approximately 30 mg of whole seedling were homogenised in 60 µl of
593 20 mM HEPES pH 7.0, 5 mM EDTA and 10 mM NaF. After addition of 250 µl of
594 chloroform:methanol (1.5:3.5 volumes) and incubation on ice for 30 min, the water-soluble
595 amino acids were extracted twice with 300 µl of HPLC-grade H₂O. The aqueous phases
596 were combined and stored at -80°C until further analysis. Samples were filtered and diluted
597 50x in water. Ten µl of each sample was derivatized using the AccQ tag kit following
598 manufacturer's instructions (Waters, UK) and 2 µl was used for injection. Derivatized amino
599 acids were separated on a 100 mm × 2.1 mm, 2.7 µm Kinetex XB-C18 column
600 (Phenomenex, USA) in an Acquity UPLC using a 20 min gradient of 1 to 20 % acetonitrile
601 versus 0.1 % formic acid in water, run at 0.58 ml.min⁻¹ at 25°C. The UPLC was attached to a
602 TQS tandem mass spectrometer (Waters, UK) instrument for detection of the correct
603 masses and quantitative measurement.

604 The organic acids citrate, α-ketoglutarate, malate, pyruvate and succinate were
605 measured using a recently developed method (Marquis et al., 2017). In brief, 30 mg of whole
606 seedlings were homogenised in 900 µl methanol:H₂O (50:50 by volume, ice cold). After
607 centrifugation at 15000 x g for 10 min at 4°C, the supernatant was evaporated in a GeneVac
608 EZ-2 Plus (SP Industries, USA) and stored at -80°C until further analysis. Samples were then
609 resuspended in 15 µl of water and derivatised with 50 µl of 10 mM 4-bromo-N-
610 methylbenzylamine in acetonitrile and 25 µl of 1 M 1-ethyl-3-(3-dimethylaminopropyl)
611 carbodiimide hydrochloride in acetonitrile:water (90:10 by volume) for 90 min at 37°C.
612 Samples were run on an Acquity UPLC equipped with a Xevo TQS tandem mass
613 spectrometer (Waters, UK) and a 100×2.1mm 2.6µm Kinetex EVO C18 column
614 (Phenomenex). The following gradient of 1% (v/v) formic acid in acetonitrile versus 1% (v/v)
615 formic acid in water, at 0.6 ml/min, 40°C was used: (0 min) 40%, (1 min) 40%, (2 min) 45%,
616 (6.5 min) 95%, (7 min) 95%, (7.5 min) 100%, (8.5 min) 100%, (8.6 min) 40%, (12 min) 40%.

617

618 **Oxygen electrode and enzyme assays**

619 Oxygen consumption rates of intact seedlings were measured using an Oxygraph
620 (Hansatech, King's Lynn, UK). Seedlings were transferred from ½ MS agar plates to 2 ml
621 water in the oxygen electrode chamber and kept dark during the measurement. Aconitase
622 activity was determined in total cell extracts in a coupled reaction with isocitrate, as
623 described (Ströher et al., 2016). Succinate:ubiquinone reductase (SQR) activity of complex
624 II, including preparation of mitochondria-enriched fractions from seedlings, was essentially
625 as described by León et al (2007), except that 10 mM 2-thenoyltrifluoroacetone (TTFA)
626 instead of 1 mM was used as specific complex II inhibitor. Addition of 10 mM TTFA in
627 dimethyl sulfoxide (10 µl in a 1-ml reaction) inhibited SQR activity by 50-60%, but there was
628 no inhibition with dimethyl sulfoxide alone.

629

630 **Yeast 2-hybrid assays**

631 Open reading frames corresponding to the presumed mature forms of NFU4, NFU5, LIP1,
632 BIO2, ACO2 (primers in Supplemental Table S4) were cloned into the pGADT7 and pGBKT7

633 vectors (Clontech) between the *NdeI* or *NcoI* and *BamHI* restriction sites in order to express
634 N-terminal fusion proteins with the GAL4 DNA binding domain (BD) or with the GAL4
635 activation domain (AD), respectively, under the control of the *ADH1* promoter. The plasmids
636 were co-transformed by heat shock treatment into the GAL4-based yeast 2-hybrid reporter
637 strain CY306, which is deficient for cytosolic thioredoxins (*MAT α* , *ura3-52*, *his3-200*, *ade2-*
638 *101*, *lys2-801*, *leu2-3*, *leu2-112*, *trp11-901*, *gal4-542*, *gal80-538*, *lys2::UASGAI1-TATAGAL1-*
639 *HIS3*, *URA3::UASGal4* 17MERS(x3)-TATACYC1-LacZ, *trx1::KanMX4*, *trx2::KanMX4*
640 (Vignols et al., 2005). Transformants were selected on yeast nitrogen base (YNB) medium
641 (0.7% yeast extract w/o amino acids, 2% glucose, 2% agar) without tryptophan and leucine
642 (-Trp, -Leu). Two clones were selected and interactions were observed as cells growing on
643 YNB medium in the absence of histidine (-His-Trp-Leu) at 30°C. The strength of the
644 interactions was evaluated by challenging growth in the presence of 2 or 5 mM of the
645 competitive inhibitor of *HIS3* gene product 3-amino-1,2,4-triazole. Images were taken 5 days
646 after spotting on plates (7 μ l/dot at an optical density of 0.05 at 600 nm). Absence of auto-
647 activation capacities of the *HIS3* reporter gene by all constructs used in this study was
648 systematically assayed after co-transformation of yeast cells by individual constructs
649 producing a fusion protein with the corresponding empty pGADT7 or pGBKT7 vectors.

650 **Confocal microscopy**

651 For localization of GFP-fusion proteins, full-length open reading frames (ORFs) of *NFU4* and
652 *NFU5*, or the predicted MTS (amino acids 1-74 for *NFU5* and 1-79 for *NFU4*) were amplified
653 from *A. thaliana* leaf cDNA and cloned between *NcoI* and *BamHI* sites of pCK-GFP-C65T
654 (2x *CaMV* 35S promoter, C-terminal GFP (Reichel et al., 1996). For BiFC, full-length ORFs
655 of *NFU4*, *NFU5* and *LIP1* were amplified and cloned upstream of the C-terminal and N-
656 terminal regions of the mVenus YFP protein into the pUC-SPYCE and pUC-SPYNE vectors
657 (Walter et al., 2004, abbreviated -C and -N in figures, respectively) using the restriction sites
658 *XbaI* and *XhoI* or *SmaI*. Primers for amplification are listed in Supplemental Table S4.

659 Leaf protoplasts were prepared from 21- to 28-day old *Arabidopsis* seedlings,
660 transfected according to (Yoo et al., 2007) and imaged after 20 - 24 h. Prior to confocal
661 analyses, the protoplasts were incubated with 20 nM MitoTracker® Orange CMXRos
662 (ThermoFisher) to fluorescently label the mitochondria.

663 Image acquisition for *NFU*-GFP localization was performed using a Zeiss LSM780
664 confocal laser scanning microscope, and a water-corrected C-Apochromat 40 \times objective
665 with a numerical aperture (NA) of 1.2. Signals were detected according to the following
666 excitation/emission wavelengths: GFP (488 nm/494–534 nm), MitoTracker (561 nm/569–613
667 nm) and chloroplast autofluorescence (488 and 561 nm/671–720 nm). Pictures were
668 analyzed using ImageJ software (<https://imagej.nih.gov/ij/>).

669 Image acquisition for BiFC was performed on a Leica TCS SP8 confocal laser-
670 scanning microscope using a water x40 objective. Wavelengths for excitation/emission were:
671 mVenus (514 nm/520–550 nm), MitoTracker (560 nm/580-620). Images were obtained using
672 LAS X software and processed with Adobe Photoshop CS3 at high resolution. All confocal
673 images shown here were captured without Z-stack intensity projection.

674 **Accession Numbers**

675 Accession numbers are as follows: AT3G20970, *NFU4*; AT1G51390, *NFU5*; AT4G22220,
676 *ISU1*; AT2G20860, *LIP1*.

677

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685 PHIV Platform for assistance with confocal microscopy.

686

687

688

689 **FIGURE LEGENDS**

690 **Figure 1. Genetic analysis of Arabidopsis mutants in *NFU4* and *NFU5*.**

691 **A.** Gene models of *NFU4* and *NFU5* and the positions of T-DNA insertions. Black bars
692 represent exons, grey bars are the 5' and 3' untranslated regions of the transcript. Triangles
693 represent T-DNA insertions, their orientation is marked with an arrow to indicate the outward
694 facing left border primer. The position of the T-DNA relative to the ATG start codon is
695 indicated by the number of the nucleotide next to the left-border sequence.

696 **B.** Transcript levels of *NFU4* and *NFU5* in leaf tissue of wild type (Col-0, Col-4 or Ler) and
697 the indicated T-DNA insertion lines, determined by RT-qPCR (graphs) or standard RT-PCR
698 (right). For RT-qPCR, values are the average of 3 biological samples \pm SE.

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709 **B.** Specific affinity of the polyclonal antibodies raised against *NFU4* and *NFU5*.
710 Luminescence signals of known amounts of recombinant proteins were compared with
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718 lines. Scale bars: 0.5 mm.

719 **C.** Frequency of normal and aborted embryos in *nfu4 nfu5-1/+* plants. *** $p < 0.0001$ for 1:3
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721 **D.** An aborted and healthy embryo from the silique of a *nfu4-2 nfu5-1/+* plant. Plant tissue
722 was cleared with Hoyer's solution and imaged with DIC microscopy. Scale bars: 50 μ m.

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726 type (Col-0), hemizygous and *nfu4-2 nfu5-2* double mutants as indicated. Antibodies against
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728 glutaredoxin GRXS15, complex I assembly factor INDH, aconitase (ACO), the H protein
729 subunit of the glycine decarboxylase complex (GDC), E1 α subunit of pyruvate
730 dehydrogenase (PDH) and the translocase of the outer membrane TOM40.

731 **B.** Growth phenotype of 4-week-old wild-type and *nfu4-2 nfu5* plants. Scale bar: 0.5 cm

732 **C.** Blue-Native PAGE of mitochondrial complexes I, III and V stained with Coomassie Blue
733 (left panel) and by NADH/NBT activity staining for complex I (right panel) in the indicated
734 plant lines.

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739 embryogenesis but switched off after seed germination. The observed segregation numbers
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746 independent lines, showing the absence or presence of a functional NFU5 sequence using
747 primers AM84 and AM85.

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752 **A.** Representative images of *nfu4 nfu5* double mutant segregants (m) and sibling wild-type
753 like (wtl) seedlings, grown on $\frac{1}{2}$ MS agar plates under ambient CO₂ and 1% CO₂ in the
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755 cm. Additional images in Supplemental Fig. S7.

756 **B.** Concentrations of selected free amino acids and organic acids in 3-week-old seedlings of
757 the indicated genotypes. Values represent the average of 3-6 biological replicates \pm SD. * $p <$
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759 Supplemental Tables S2 and S3 for complete data sets.

760 **C.** Protein blot analysis for lipoyl cofactor (top) and H protein isoforms of glycine
761 decarboxylase complex (bottom), in wild type (WT), *nfu4 nfu5* (m) and wtl segregants. See
762 also Supplemental Fig. S8.

763

764 **Figure 7. NFU4 and NFU5 are not required for the Fe-S enzymes aconitase and complex**
765 **II.**

766 **A.** Respiration in intact seedlings measured using a liquid-phase oxygen electrode. WT, wild
767 type (Col-0); m, *nfu4-2 nfu5-1* mutant expressing *ABI3prom:NFU4*; wtl, wild-type-like *nfu4-2*
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770 **B.** Aconitase activity in total cell extracts in seedlings grown under ambient and 1% CO₂ for 3
771 weeks. Values represent the mean \pm SD ($n = 3-4$). * $p <$ 0.05 (Student *t*-test).

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774 complex II inhibitor TTFA was added at a concentration of 0.1 mM, and only the TTFA-
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784 **B.** Bimolecular Fluorescence Complementation to test interaction between NFU4/NFU5 and
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786 YFP, and the plasmids transformed into Arabidopsis protoplasts. Results are representative
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788 transformation event. Images are provided with (Fig. 8B) and without (Supplemental Fig. S7)
789 maximal Z-stack intensity projections. Scale bars: 10 μm .

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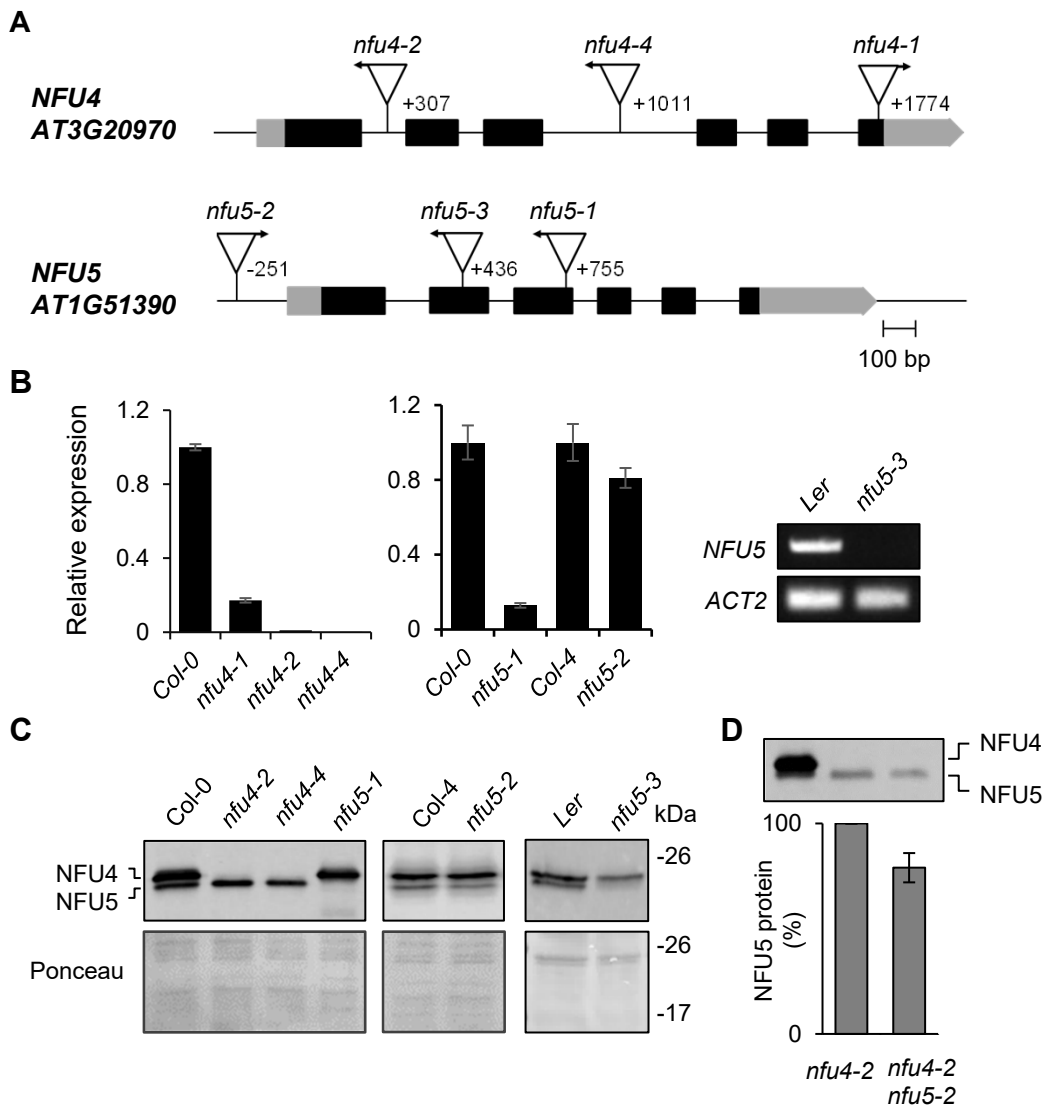


Figure 1. Genetic analysis of Arabidopsis mutants in *NFU4* and *NFU5*

A. Gene models of *NFU4* and *NFU5* and the positions of T-DNA insertions. Black bars represent exons, grey bars are the 5' and 3' untranslated regions of the transcript. Triangles represent T-DNA insertions, their orientation is marked with an arrow to indicate the outward facing left border primer. The position of the T-DNA relative to the ATG start codon is indicated by the number of the nucleotide next to the left-border sequence.

B. Transcript levels of *NFU4* and *NFU5* in leaf tissue of wild type (Col-0, Col-4 or Ler) and the indicated T-DNA insertion lines, determined by RT-qPCR (graphs) or standard RT-PCR (right). For RT-qPCR, values are the average of 3 biological samples \pm SE.

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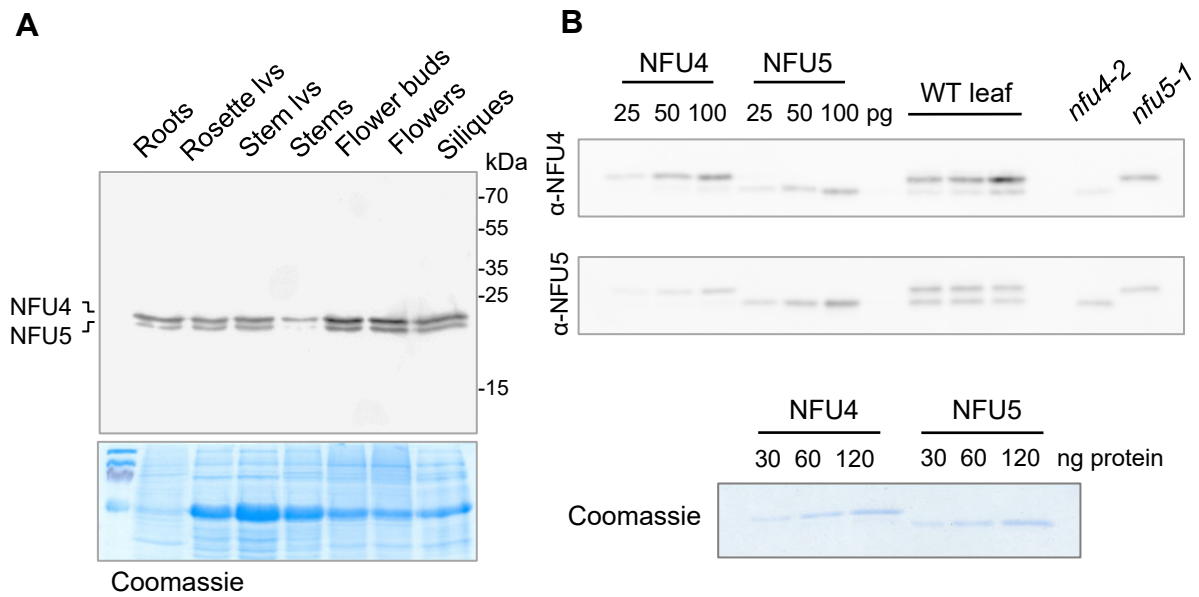


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B. Specific affinity of the polyclonal antibodies raised against NFU4 and NFU5. Luminescence signals of known amounts of recombinant proteins were compared with signals in purified mitochondria from wild-type (WT) leaves and from cell culture of *nfu4-2* and *nfu5-1* mutants. Each antiserum cross-reacts with the other isoform (90% amino acid identity), but has a stronger affinity for the protein it was raised against.

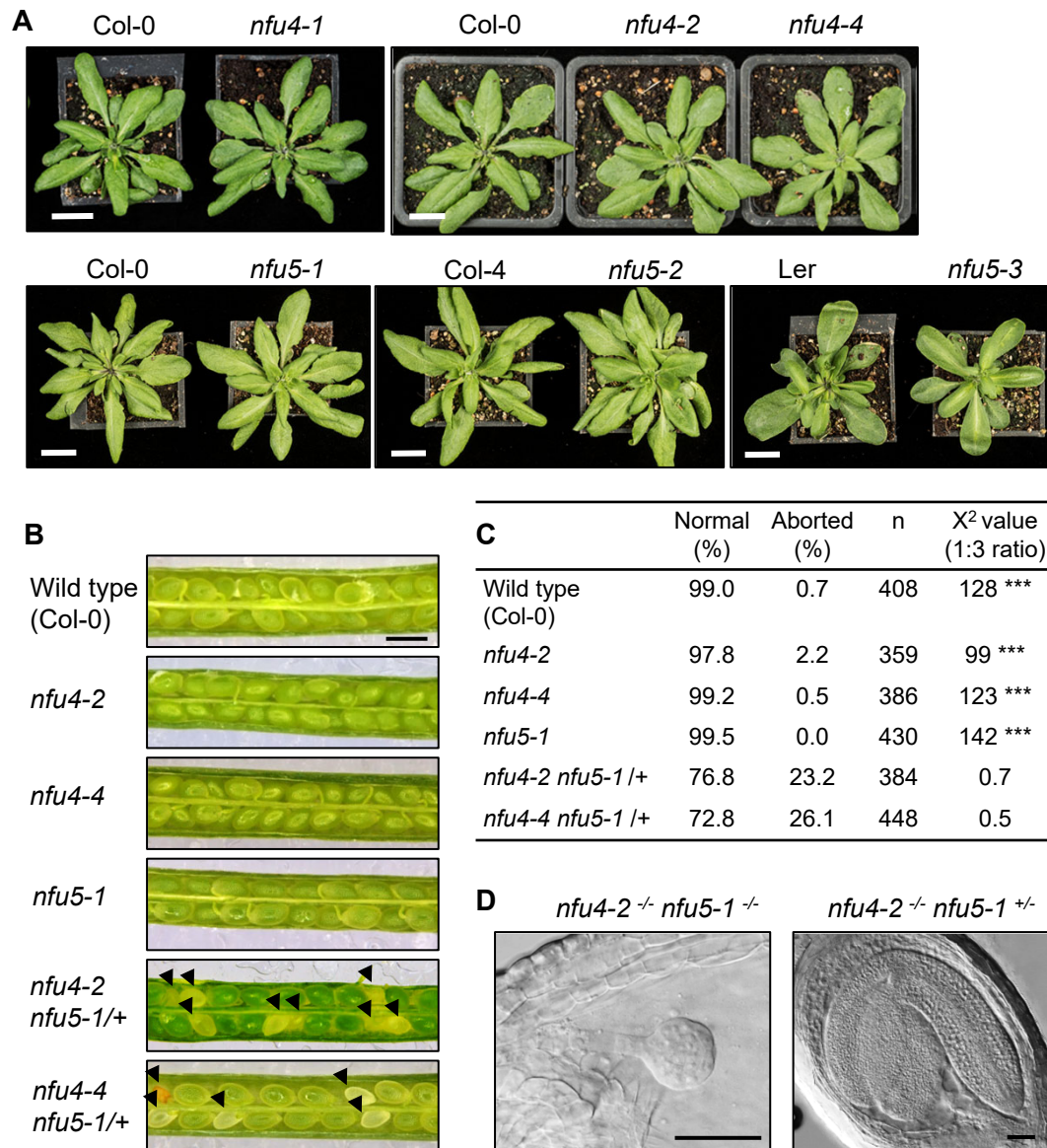


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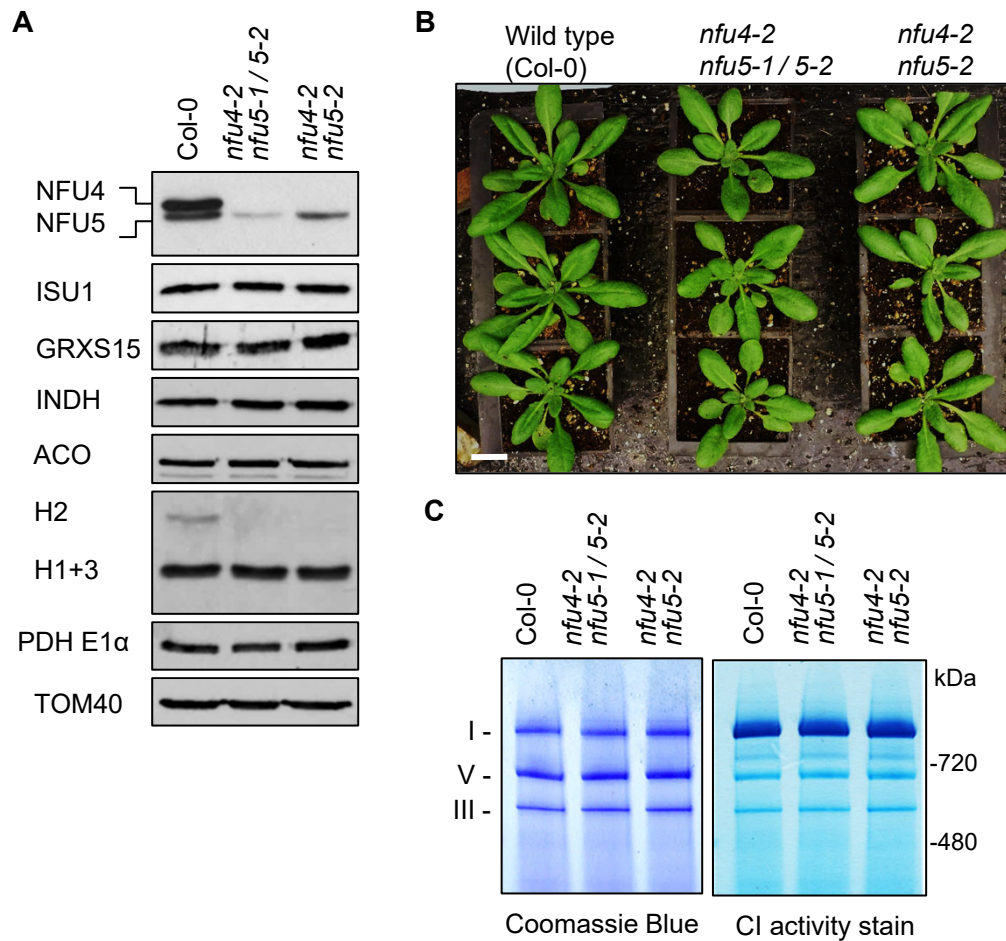


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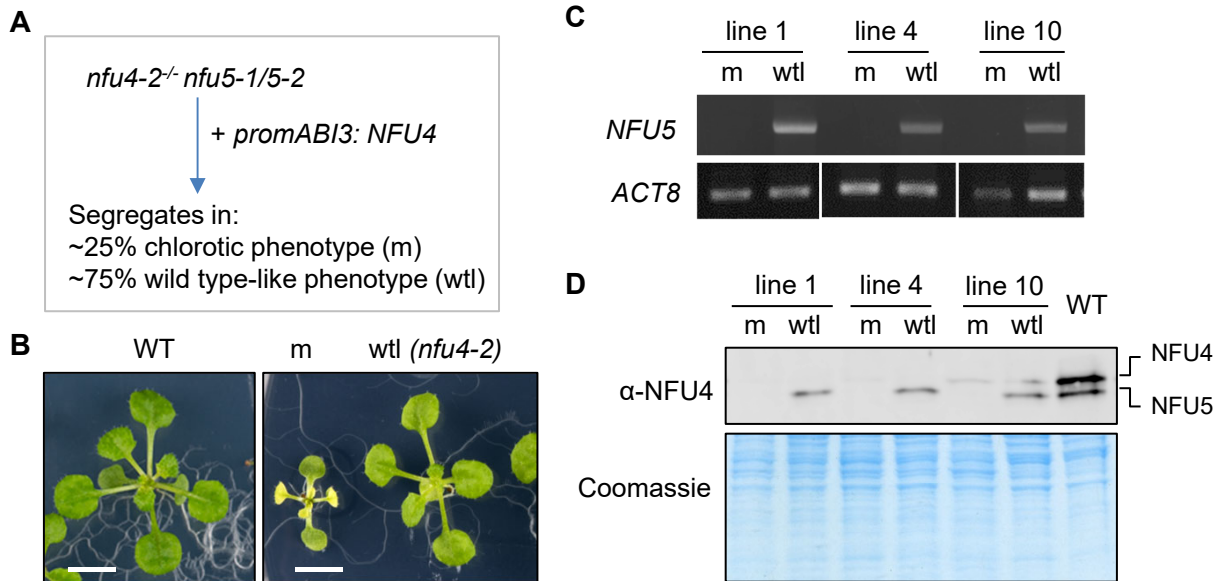


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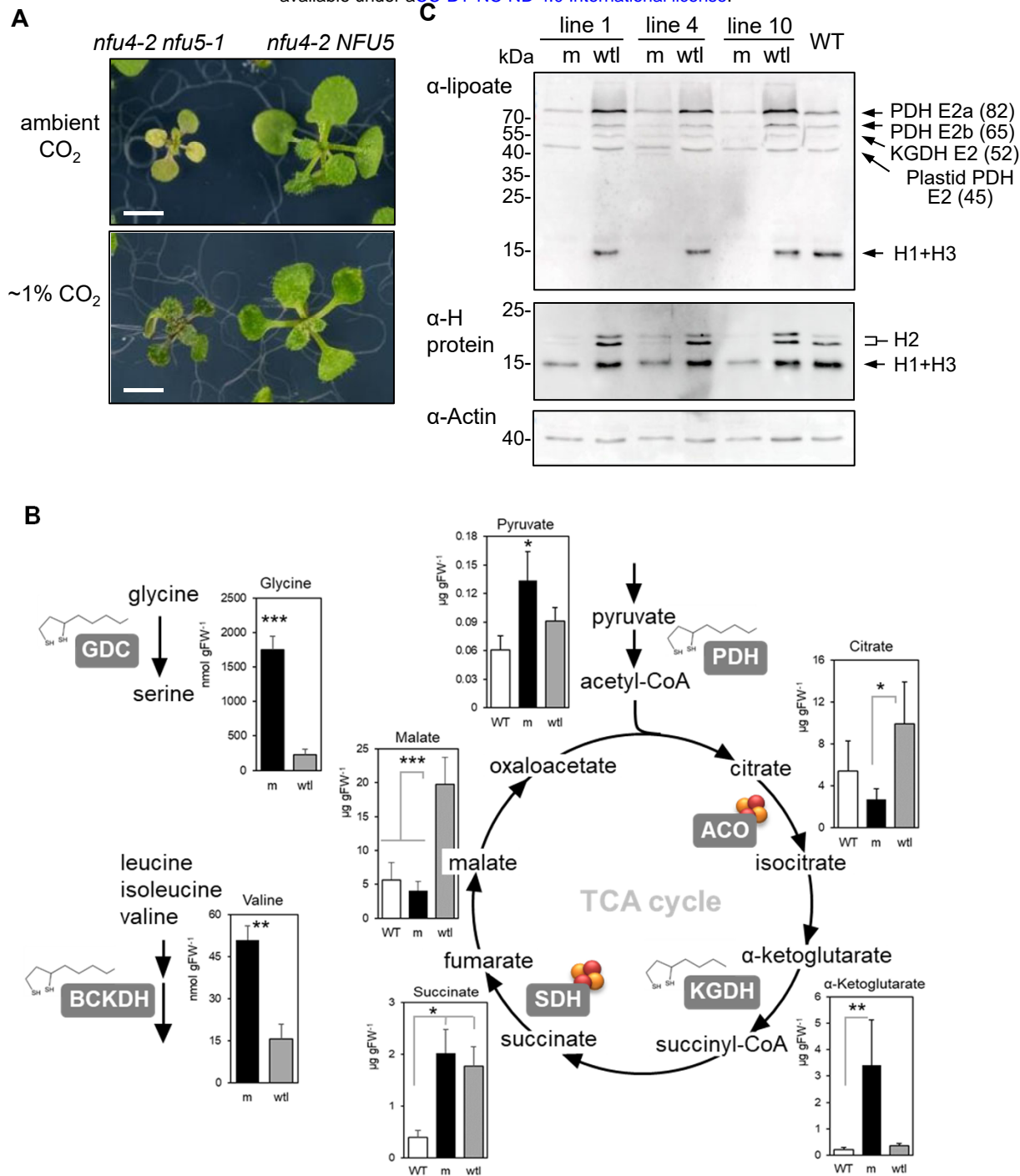


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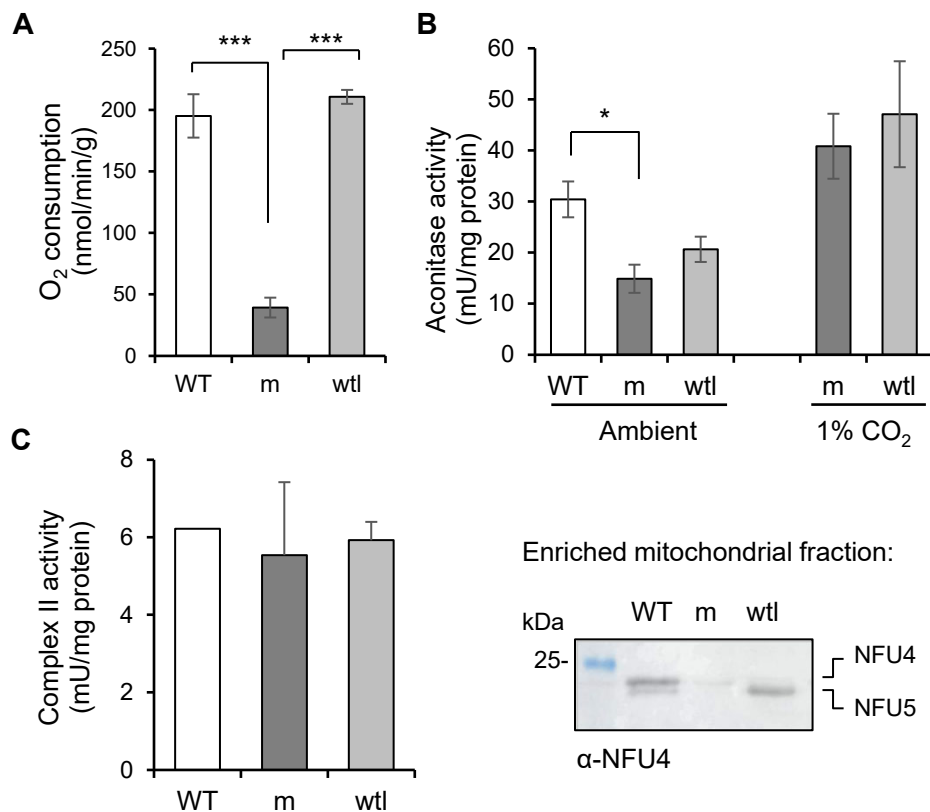


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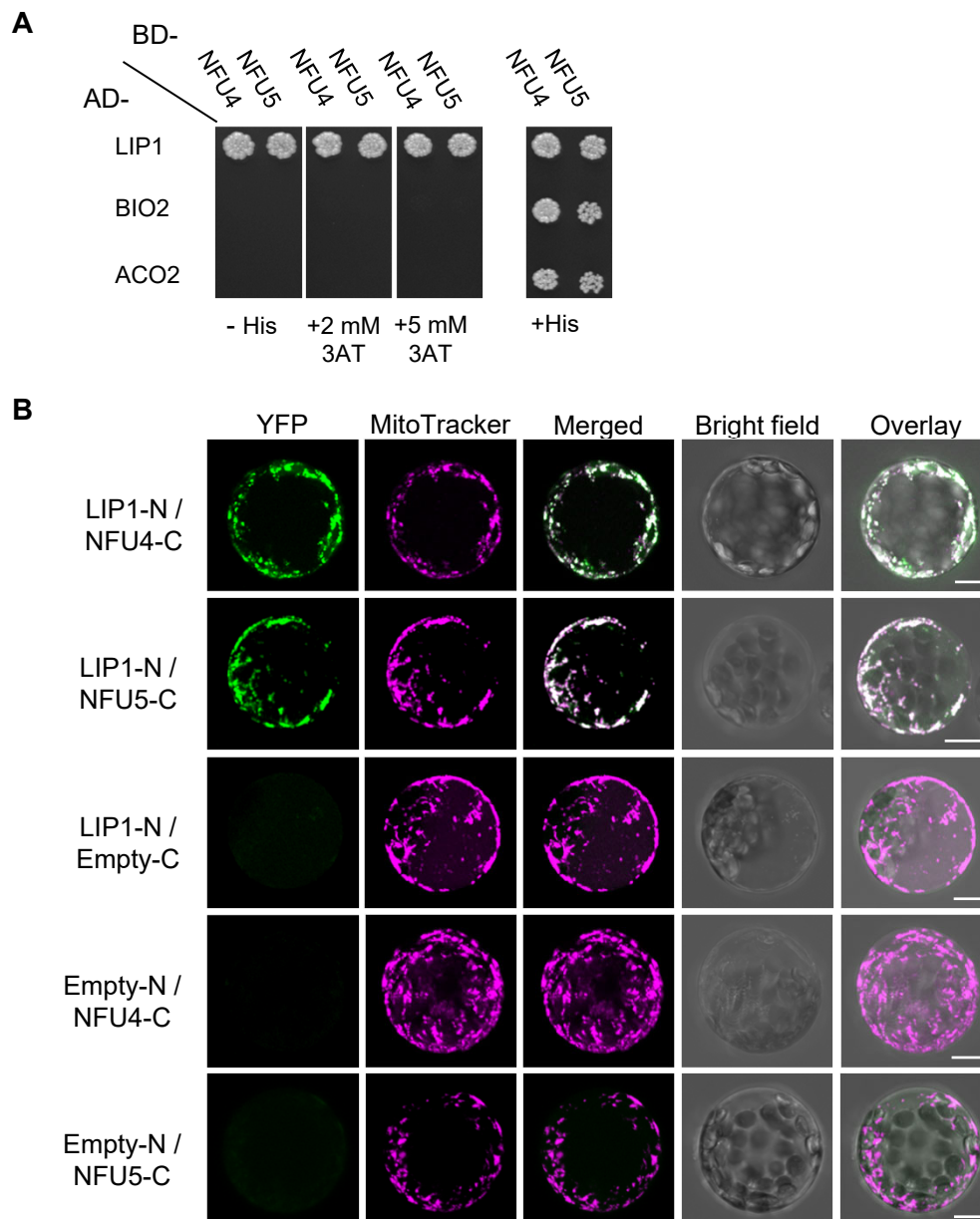


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