

1 **Faster responses of photosynthesis to light transitions increase biomass and grain yield in**
2 **transgenic *Sorghum bicolor* overexpressing Rieske FeS**

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21 photosynthesis induction.

22

23 **Abstract**

24 Sorghum is one of the most important crops providing food and feed in many of the world's harsher
25 environments. Sorghum utilises the C₄ pathway of photosynthesis in which a biochemical carbon
26 concentrating mechanism results in high CO₂ assimilation rates. Overexpressing the Rieske subunit of the
27 Cytochrome *b₆f* complex was previously shown to increase the rate of photosynthetic electron transport
28 and stimulate CO₂ assimilation in the model C₄ plant *Setaria viridis*. To test whether productivity of C₄ crops
29 could be improved by Rieske overexpression, we created transgenic *Sorghum bicolor* plants with increased
30 Rieske content. The transgenic plants showed no marked changes in abundance of other photosynthetic
31 proteins or chlorophyll content. Increases in yield of Photosystem II and CO₂ assimilation rate as well as
32 faster responses of non-photochemical quenching during transient photosynthetic responses were
33 observed as a result of an elevated *in vivo* Cytochrome *b₆f* activity in plants overexpressing Rieske. The
34 steady-state rates of electron transport and CO₂ assimilation did not differ between transgenic and control
35 plants, suggesting that Cytochrome *b₆f* is not the only factor limiting electron transport in sorghum at high
36 light and high CO₂. Nevertheless, more agile responses of photosynthesis to light transitions led to increases
37 in biomass and grain yield in plants overexpressing Rieske. Our results indicate that increasing Rieske
38 content could boost productivity of C₄ crops by improving the efficiency of light utilisation and conversion
39 to biomass.

40 **Introduction**

41 C₄ plants utilise a specialised photosynthetic pathway in which a metabolic C₄ cycle acts as a biochemical
42 carbon concentrating mechanism (Hatch, 1987). The C₄ cycle operates between mesophyll and bundle
43 sheath (BS) cells, and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the main enzyme of CO₂
44 fixation, is localised to the BS (Kanai and Edwards, 1999). Atmospheric CO₂ (in the form of HCO₃⁻) is first
45 fixed in mesophyll cells by PEP carboxylase (PEPC) into a C₄ acid (hence the term C₄ photosynthesis). C₄
46 acids diffuse to BS cells where they are decarboxylated to produce pyruvate and CO₂, providing high CO₂
47 partial pressure (pCO₂) around Rubisco (Furbank and Hatch, 1987). Higher carboxylation efficiency of
48 Rubisco in C₄ plants allows higher radiation use efficiency and increased biomass production compared to
49 C₃ plants (Long, 1999; Sage and Zhu, 2011). Because of their superior productivity, C₄ crops are becoming
50 increasingly important for food and bioenergy security. The global production of C₄ maize (*Zea mays*) often
51 surpasses the two key C₃ cereals, wheat and rice, and C₄ miscanthus (*Miscanthus × giganteus*) and
52 switchgrass (*Panicum virgatum*) are two of the currently leading dedicated biomass crops. This has created

53 considerable interest in identifying and testing strategies to improve productivity of C₄ crops (Sales et al.,
54 2021; von Caemmerer and Furbank, 2016).

55 While C₄ plants are more productive, running the C₄ cycle requires additional input of energy. Whilst C₃
56 plants need at least two NADPH and three ATP to fix one mol of CO₂, C₄ plants need two additional ATP
57 molecules to regenerate PEP from pyruvate in mesophyll cells (Edwards et al., 2001; Hatch, 1987). NADPH
58 and ATP are the products of light reactions of photosynthesis which include electron and proton transport
59 in the thylakoid membranes of chloroplasts. NADPH is produced during linear electron flow as electrons
60 originating from water split by Photosystem II (PSII) are transferred by the chain of cofactors, via
61 Cytochrome *b*₆*f* complex (Cyt*b*₆*f*) and Photosystem I (PSI), to NADP⁺. Cyt*b*₆*f* links oxidation of plastoquinol
62 with the translocation of protons to the lumen, a space enclosed by the thylakoid membrane, by operating
63 the Q-cycle (Malone et al., 2021). The transmembrane proton gradient (ΔpH) established across the
64 thylakoid membrane creates a proton motive force (*pmf*) that drives ATP synthesis via the ATP synthase
65 complex. In addition to linear electron flow, C₄ plants run active cyclic electron flow to produce additional
66 ATP (Ishikawa et al., 2016; Munekage and Taniguchi, 2016; Nakamura et al., 2013). Cyclic electron flow
67 returns electrons from the reducing side of PSI back to the plastoquinone (PQ) pool to repeat plastoquinol
68 oxidation by Cyt*b*₆*f* and build up additional *pmf* (Johnson, 2011). Thus, cyclic electron flow results in the net
69 production of ATP but not NADPH. ΔpH controls PSII activity by regulating the energy-dependent and
70 quickly reversible form of non-photochemical quenching (NPQ) (Li et al., 2002). The latter is a common
71 term for diverse reactions that help to reduce excitation energy reaching reaction centers of PSII (Malnoë,
72 2018). Establishing energy-dependent NPQ requires the PsbS protein that senses luminal pH and modifies
73 the light-harvesting complex II (LHCII) to dissipate a part of absorbed light as heat, as well as the conversion
74 of violaxanthin to zeaxanthin (Johnson et al., 2009; Li et al., 2004).

75 The vast majority of agriculturally important C₄ crops, like maize, sorghum (*Sorghum bicolor*), sugarcane,
76 miscanthus and several millets (e.g., *Setaria italica*), belong to the NADP-ME subtype of C₄ photosynthesis
77 which employs NADP⁺-dependent malic enzyme to decarboxylate the C₄ acid malate in BS chloroplasts
78 (Furbank, 2011). Because of the drastic differences in biochemistry of mesophyll and BS cells in NADP-ME
79 plants, electron transport chains of the two cell types are also largely different: mesophyll cells
80 predominantly run linear electron flow and BS cells – cyclic electron flow (Ermakova et al., 2021a;
81 Munekage, 2016). To coordinate the production of NADPH and ATP between cells, plants need to tightly
82 regulate the distribution of available light energy (Bellasio and Ermakova, 2021; Bellasio and Lundgren,
83 2016). Because of their higher ATP requirement, C₄ plants generally require higher solar radiation and are
84 typically found in tropical regions (Sage et al., 2011). At low irradiance however, slow operation of the C₄

85 cycle results in a lower $p\text{CO}_2$ in BS cells decreasing the efficiency of C₄ photosynthesis (Furbank and Hatch,
86 1987; Kromdijk et al., 2010). Therefore, increasing radiation use efficiency is one of the primary strategies
87 for increasing assimilation rates and productivity of C₄ plants.

88 Constitutive overexpression of the Rieske FeS subunit of *Cytb_{6f}* (hereafter Rieske), encoded by the nuclear
89 *petC* gene was shown to increase abundance of the whole complex in both mesophyll and BS cells of a
90 model NADP-ME grass *Setaria viridis* (Ermakova et al., 2019). This resulted in a higher quantum yield of
91 both photosystems and higher CO₂ assimilation rates at high light and high CO₂. However, the feasibility of
92 using Rieske overexpression for improving crop productivity required further assessment. Here we test
93 effects of Rieske overexpression on biomass and grain yield of a multipurpose C₄ crop, sorghum. We show
94 that sorghum plants with increased Rieske abundance use light more efficiently and accumulate more
95 biomass due to faster responses of photosynthesis to light transitions. Our results indicate that increasing
96 Rieske content is a promising strategy for stimulating yield of sorghum and other C₄ crops.

97 **Results**

98 Sorghum plants transformed with the construct for Rieske overexpression (see Materials and Methods for
99 details) were selected based on kanamycin resistance and transferred to soil for growth in a glasshouse.
100 Sixteen T₀ plants were recovered and analysed for insertion number, transgene expression and leaf Rieske
101 abundance. The *ntpII* insertions and transcripts of *Brachypodium distachyon petC* (*BdpetC*) were confirmed
102 in ten T₀ plants (Fig. 1a and Fig. 1b). Plants 25, 26, 29 and 32 showed relatively higher Rieske abundance
103 per leaf area compared to wild type (WT) and escape plants without the T-DNA insertion (Fig. 1a). The T₁
104 progenies of those four plants were grown and analysed for insertion numbers and Rieske abundance. The
105 homozygous T₁ plants of lines 25 and 26 (4 insertions, Fig. 1c) had higher Rieske leaf content compared to
106 control plants (WT and null segregants). Furthermore, homozygous plants 25-11 and 26-11 showed
107 relatively lower NPQ compared to control and other T₁ plants when assayed at ambient light, in line with
108 the NPQ phenotype reported in *S. viridis* overexpressing Rieske (Ermakova et al., 2019). The progenies of
109 those two plants, as well as the progeny of the homozygous T₁ plant 32-14 with increased Rieske abundance
110 (Fig. S1), were used in further experiments, and are hereafter referred to as transgenic lines 25, 26 and 32.

111 T₂ plants of the three transgenic lines and azygous control plants were grown over summer in a glasshouse
112 with natural light. Abundance of photosynthetic proteins was analysed in leaf extracts loaded on leaf area
113 basis by immunoblotting with specific antibodies (Fig. 2a). Quantification of immunoblots demonstrated a
114 significant, about 40%, increase of Rieske content in all three transgenic lines compared to control plants.
115 Relative abundance of other electron transport components, such as the D1 protein of PSII, AtpB subunit

116 of ATP synthase, PsbS and Lhcb2 subunit of LHC II was largely unaltered in transgenic plants overexpressing
117 Rieske (Fig. 2b) as well as the relative Chl content (Table 1). The content of PEPC and Rubisco large subunit
118 (RbcL) did not differ between transgenic and control plants (Fig. 2b).

119 Sorghum plants with increased Rieske content were taller than the control plants at 5 weeks after
120 germination (Fig. 2c and Fig. 2d) and had more tillers ($0.036 < P > 0.09$, Table 1) and leaves (Fig. 2e). Whilst
121 the leaf thickness and leaf dry mass per area did not differ between the genotypes (Table 1), the total
122 aboveground biomass of lines 26 and 32 at harvest was higher compared to control plants (Fig. 2f). In
123 another experiment, when plants were grown in a glasshouse during late summer-autumn, Rieske-OE
124 plants of lines 25 and 26 had larger leaves compared to control plants (azygous and WT, Fig. S2) and
125 produced more seeds by weight and number than control plants (Fig. 2g and Fig. 2h).

126 Next, we analysed photosynthetic properties of sorghum plants overexpressing Rieske. First, we conducted
127 gas exchange and fluorescence analysis at different $p\text{CO}_2$ and irradiances. No significant differences in CO_2
128 assimilation rate or the effective yield of PSII (PhiPSII) were detected between the plants overexpressing
129 Rieske and control plants at constant irradiance of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and different $p\text{CO}_2$ (Fig. 3, left panels).
130 At ambient $p\text{CO}_2$, CO_2 assimilation rates and stomatal conductance were similar between the genotypes at
131 all irradiances (Fig. 3, right panels). The photochemical and non-photochemical yields of PSI and PSII
132 analysed at different irradiances were largely unaltered in transgenic plants compared to control plants
133 (Fig. 4), except for the yield of PSI (PhiPSI) being higher and the non-photochemical loss of PSI yield due to
134 the acceptor side limitation (PhiNA) being lower in lines 25 and 32 at $95 \mu\text{mol m}^{-2} \text{s}^{-1}$. These results indicated
135 that the steady-state rates of electron transport and CO_2 assimilation were largely not affected in plants
136 overexpressing Rieske. The maximum quantum efficiency of PSII (F_v/F_m), however, was significantly higher
137 in plants of line 32 compared to control plants (Table 1).

138 Energisation properties of the thylakoid membranes were tested by recording electrochromic shift signal
139 and absorbance changes at 535 nm. By the end of 3-min illumination intervals, *pmf* and proton conductivity
140 of the thylakoid membrane (g_{H^+}), reflecting the speed of *pmf* dissipation and thus ATP synthase activity, did
141 not differ between the plants overexpressing Rieske and control plants at any irradiance (Fig. 5a and Fig.
142 5b). To gain information about the kinetics of ΔpH build-up during the illumination, we recorded
143 absorbance changes at 535 nm which reflect both zeaxanthin formation and the LHCII modifications
144 induced by PsbS, therefore, providing information on the response of NPQ to ΔpH (Horton et al., 1991; Li
145 et al., 2004). All three transgenic lines overexpressing Rieske established NPQ significantly faster than

146 control plants in the beginning of illumination upon the shift from dark to $1600 \mu\text{mol m}^{-2} \text{s}^{-1}$, indicating a
147 transiently faster build-up of ΔpH due to the increased *Cytb_{6f}* activity (Fig. 5c).

148 Since transient increase of *Cytb_{6f}* activity could be detected upon changes in illumination, we analysed the
149 induction of photosynthesis in overnight-dark-adapted plants during the first 30 min of illumination with
150 actinic light of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 6). Because the steady-state CO_2 assimilation rate, PhiPSII and NPQ
151 did not differ between genotypes (Fig. 3 and Fig. 4), we normalised these parameters to minimum and
152 maximum values to facilitate comparison of the kinetics. During the induction of photosynthesis, CO_2
153 assimilation rates increased faster in sorghum plants overexpressing Rieske compared to control plants,
154 and between 18 and 26 min the rates were significantly higher in all three transgenic lines. The induction
155 kinetics of PhiPSII was similar to the kinetics of CO_2 assimilation rate. Transgenic lines reached the steady-
156 state faster and lines 25 and 26 had significantly increased PhiPSII between 18 and 26 min since the
157 beginning of illumination, compared to control plants (Fig. 6). Interestingly, during the first 9 min of
158 illumination plants overexpressing Rieske built-up NPQ faster than control plants (significant in line 25 and
159 26) and line 32 then relaxed NPQ significantly faster. Importantly, although the stomatal conductance was
160 significantly higher in all three lines overexpressing Rieske (Fig. 6d), increased CO_2 assimilation rates were
161 not caused by an increased CO_2 availability since the ratio of intercellular to ambient CO_2 partial pressures
162 (C_i/C_a) was unaltered (Fig. 6).

163 Discussion

164 Sorghum is one of the most important crops in the world which serves as a source of food, fodder and fuel.
165 Sorghum can withstand severe droughts allowing it to grow in regions where other major crops cannot be
166 grown, like Sub-Saharan Africa. However, in recent years, genetic progress in sorghum yield has stagnated
167 and not kept pace with increasing demand (Ananda et al., 2020). Therefore, it is critical to develop new
168 approaches for increasing sorghum productivity. Based on crop model predictions, up to 10% improvement
169 in sorghum yield could be harnessed from improving photosynthesis (Wu et al., 2019). According to the
170 biochemical model of C_4 photosynthesis, assimilation at low $p\text{CO}_2$ is limited by PEPC and CA activities and
171 mesophyll conductance to CO_2 , whilst assimilation at ambient and high $p\text{CO}_2$ is limited by Rubisco, electron
172 transport or the regeneration rate of Rubisco's substrate (von Caemmerer, 2000; von Caemmerer, 2021;
173 von Caemmerer and Furbank, 1999). Contribution of some of these factors to C_4 photosynthesis and plant
174 productivity was recently tested using transgenic approach in the model C_4 plant *S. viridis* (Alonso-
175 Cantabrina et al., 2018; Ermakova et al., 2022; Osborn et al., 2016), and improvements of C_4 photosynthesis
176 were shown in *S. viridis* and *Z. mays* with increased Rieske and Rubisco content (Ermakova et al., 2019;

177 Ermakova et al., 2021b; Salesse-Smith et al., 2018). Here we expand on our previous results and assess how
178 Rieske overexpression affects productivity of sorghum.

179 Rieske overexpression in sorghum provided increased *Cytb₆f* activity, confirmed by monitoring the build-
180 up of NPQ and dynamics of electron transport during the photosynthesis induction. A faster build-up of
181 NPQ during dark-light transition in transgenic plants indicated larger ΔpH due to increased *Cytb₆f* activity
182 (Fig. 5c). Observing this transient increase is possible because NPQ engages on the scale of seconds to
183 minutes (Müller et al., 2001). However, by the end of 3-min illumination periods, *pmf*, which is a sum of
184 ΔpH and $\Delta\psi$ (the membrane potential), did not differ between genotypes (Fig. 5a). This was consistent with
185 the largely unchanged electron transport parameters and CO_2 assimilation rates (Fig. 3 and Fig. 4) – all
186 indicating that steady-state rates of electron transport were unaltered in sorghum plants overexpressing
187 Rieske. Similar observations were made with tobacco plants overexpressing Rieske (Heyno et al., 2022). In
188 C_3 plants, this phenomenon could be explained by a conserved relationship between ΔpH and NPQ which
189 would reduce PSII activity in case if electron transport rate exceeds the capacity of dark reactions of
190 photosynthesis to consume ATP and NADPH, typically due to a limited availability of CO_2 (Kanazawa and
191 Kramer, 2002). C_4 photosynthesis, however, is less limited by CO_2 due to the C_4 cycle concentrating CO_2
192 around Rubisco, and an increase of electron transport rate is projected to provide proportional increase in
193 assimilation (von Caemmerer and Furbank, 2016). Increasing Rieske content in *S. viridis* was sufficient to
194 enhance electron transport rates, resulting in higher photosynthesis at non-limiting CO_2 and high light
195 (Ermakova et al., 2019). One possible explanation for the difference observed between *S. viridis* and
196 sorghum overexpressing Rieske is that C_4 crops underwent a selection for photosynthetic traits which could
197 have altered a balance between electron transport components. For example, translational efficiency of
198 *Lhca6*, a subunit of the light-harvesting complex I that facilitates a formation of PSI supercomplex involved
199 in cyclic electron flow (Otani et al., 2018), was significantly enhanced during maize domestication (Zhu et
200 al., 2021). Changes to the ratio of cyclic to linear electron flow likely resulted in additional or different
201 factors limiting electron transport. Better understanding of photosynthetic changes that C_4 crops
202 underwent during domestication will help to uncover additional targets for accelerating steady-state
203 electron transport and photosynthesis rates (Hu et al., 2018; Tao et al., 2020a; Zhu et al., 2021). Increased
204 assimilation rates detected in maize with increased Rubisco content at non-limiting CO_2 and high light could
205 also be indicative of an altered relationship between electron transport and Rubisco limitations in C_4 crops
206 (Salesse-Smith et al., 2018).

207 Interestingly, the largest difference in electron transport and assimilation between plants overexpressing
208 Rieske and control plants was detected during the induction of photosynthesis. Induction, or activation of

209 photosynthesis during dark-to-light transition, is a complex process that, in C₃ plants, requires opening of
210 stomata, activation of Rubisco and other enzymes and a build-up of metabolite concentrations (Deans et
211 al., 2019; Slattery et al., 2018). Faster activation of photosynthesis was identified as one of the desirable
212 traits in crop plants that could allow up to 20% increase in total assimilation (Acevedo-Siaca et al., 2020;
213 Long et al., 2022). Activation of C₄ photosynthesis is further complicated by the distribution of electron
214 transport and metabolic reactions between mesophyll and BS cells and a necessity to coordinate activities
215 of C₄ and C₃ cycles (Furbank and Taylor, 1995; Kromdijk et al., 2014). Our results support previous works
216 suggesting that, due to the operation of carbon concentrating mechanism, activation of C₄ photosynthesis
217 is less limited by stomatal conductance compared to C₃ photosynthesis (Furbank and Walker, 1985; Usuda
218 and Edwards, 1984). Indeed, in our experiments, C_i/C_a never dropped below 0.25 (Fig. 6e) which is
219 equivalent to C_i ≥ 100 µmol mol⁻¹ sufficient to saturate assimilation (Pignon and Long, 2020). Instead, the
220 observed increase of CO₂ assimilation rates during the light-induced activation of photosynthesis in
221 sorghum overexpressing Rieske was underpinned by the higher PhiPSII (Fig. 6). It is conceivable that
222 activation of photosynthetic enzymes provides a strong sink for ATP and NADPH, and an increased Cytb_{6f}
223 activity could transiently stimulate electron transport to activate enzymes and build-up metabolite
224 gradients faster. In line with this, activation of Rubisco that uses ATP was suggested as one of the major
225 factors limiting C₄ photosynthesis during the induction (Wang et al., 2021).

226 Sorghum plants with increased Rieske content had increased biomass and grain yield. Transgenic plants
227 produced more leaves and had larger leaves during the vegetative growth phase and accumulated more
228 biomass by the end of growth season, compared to control plants (Fig. 2, Fig. S2). Moreover, transgenic
229 plants produced about 20% more grain by weight and number (Fig. 2g and Fig. 2h) indicating that higher
230 yield of transgenic plants was largely attributed to setting more seeds. Grain yield and grain number highly
231 correlate in sorghum, and a supply of assimilates during the seed setting largely determines grain number
232 (Craufurd and Peacock, 1993; van Oosterom and Hammer, 2008). Although the conditions of induction
233 experiment (Fig. 6) were designed to maximise differences between control and transgenic plants and did
234 not occur in glasshouse conditions, a cumulative effect of transiently or marginally increased electron
235 transport rates could explain the observed improvements in biomass and yield of transgenic plants.
236 Similarly, small changes in CO₂ assimilation rates due to accelerated NPQ relaxation were shown to result
237 in significant increase in biomass of tobacco grown in the field (Kromdijk et al., 2016). Taken together, our
238 results show that increasing Rieske content in sorghum improves the light use efficiency and stimulates
239 biomass production therefore presenting a promising trait to be introduced into commercial sorghum

240 varieties. Exploring variation of Rieske content in sorghum variety panels (Tao et al., 2020b) could inform
241 breeding programs for developing higher yielding sorghum with improved photosynthesis.

242 Conclusion

243 In sorghum, effects of Rieske overexpression on stimulating electron transport were more apparent during
244 transient photosynthetic responses suggesting that other photosynthetic components co-limit the steady-
245 state electron transport rate. However, faster responses of electron transport and CO₂ assimilation to light
246 transients resulted in plants overexpressing Rieske accumulating more biomass and producing more grain.
247 Our results indicate that increasing Rieske content, relative to other electron transport components, is a
248 promising way to boost productivity of C₄ crops and ensure food and energy security.

249 Materials and Methods

250 Generation and selection of transgenic plants

251 The gene construct for Rieske overexpression was created using the Golden Gate cloning system (Engler et
252 al., 2014), as described in Ermakova et al. (2019). The first expression module containing the selection
253 marker was occupied by *neomycin phosphotransferase II* (*ntpII*) driven by the *Z. mays ubiquitine1* promotor.
254 The second expression module contained the coding sequence of *petC* from *Brachypodium distachyon*
255 (*BdpetC*) under the control of the *Z. mays ubiquitine1* promotor. The construct was transformed into
256 *Agrobacterium tumefaciens* strain AGL1 and then into *Sorghum bicolor* Tx430 according to (Gurel et al.,
257 2009). Transgenic plants were analysed for the *ntpII* copy number by digital PCR (iDNA genetics, Norwich,
258 UK). Azygous plants were used as control in all experiments. Transcript abundance of *BdpetC* was estimated
259 by qPCR as described in Ermakova et al. (2019). Electron transport at ambient light intensity and leaf
260 properties (relative chlorophyll and leaf thickness) were assayed with MultispeQ (Kuhlgert et al., 2016) and
261 analysed using the PhotosynQ platform (<https://photosynq.com>).

262 Plant growth conditions

263 Plants were grown in a glasshouse at ambient irradiance, 28 °C day, 20 °C night and 60% humidity. Plants
264 were shuffled every 2-3 days to reduce any positional growth effects. The youngest fully expanded leaves
265 of the 4-5 weeks old plants were used for all physiological analyses. Photos of plants, leaves and tiller count
266 and height measurements were done during the fully emerged leaves stage, 5 weeks after germination.

267 Gas exchange and fluorescence analyses

268 Rates of CO₂ assimilation were measured over a range of *p*CO₂ and irradiance using a portable gas-exchange
269 system LI-6800 (LI-COR Biosciences, Lincoln, NE). Chlorophyll fluorescence was assessed simultaneously
270 with a Fluorometer head 6800-01 A (LI-COR Biosciences). Leaves were first equilibrated at 400 ppm CO₂ in

271 the reference side, $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$, leaf temperature 28°C , 60% humidity and flow rate $500 \mu\text{mol s}^{-1}$.
272 CO₂ response curves were conducted under the constant irradiance of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ by imposing a
273 stepwise increase of $p\text{CO}_2$ from 0 to 1600 ppm at 3 min intervals. Light response curves were measured at
274 the constant $p\text{CO}_2$ of 400 ppm in the reference cell under a stepwise decrease of irradiance from 0 to
275 $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 3 min intervals. Red-blue actinic light (90%/10%) was used in all measurements. The
276 effective yield of PSII (PhiPSII) was assessed at the end of each step upon the application of a multiphase
277 saturating pulse ($8000 \mu\text{mol m}^{-2} \text{s}^{-1}$) and calculated according to Genty et al. (1989).

278 Induction of photosynthesis was analysed on dark-adapted overnight plants. First, leaves were clipped into
279 LI-6800 chamber in darkness and the minimum and maximum levels of fluorescence were recorded upon
280 the application of a saturating pulse. After that, leaves were illuminated with actinic light of $1000 \mu\text{mol}$
281 $\text{m}^{-2} \text{s}^{-1}$, and gas-exchange and fluorescence parameters were recorded every 1 min for 30 min. NPQ was
282 calculated according to Bilger and Björkman (1990). All parameters were normalised for min and max values
283 to facilitate comparison of the kinetics.

284 **Electron and proton transport**

285 Fluorescence parameters informing on the activity of PSII and absorbance at 820 nm reflecting the
286 formation of oxidised P700, the reaction centre of PSI, were analysed simultaneously by the Dual-PAM-100
287 (Heinz Walz, Effeltrich, Germany). Measurements were done using red actinic light and 300-ms saturating
288 pulses of $10000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Leaves were first dark-adapted for 30 min to record the minimal and maximal
289 levels of fluorescence and calculate F_v/F_M , the maximum quantum yield of PSII. Then, a saturating pulse
290 was applied after a pre-illumination with strong far-red light to record the maximal level of P700⁺ signal
291 and, after the pulse, the minimal level of P700⁺ signal. Next, leaves were illuminated for 10 min with an
292 actinic light of $378 \mu\text{mol m}^{-2} \text{s}^{-1}$. After that, photosynthetic parameters were assessed over a range of
293 irradiances from 0 to $2043 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 2 min intervals by applying a saturating pulse at the end of each
294 step. The effective quantum yield of PSII (PhiPSII), the yield of non-photochemical quenching (PhiNPQ) and
295 the yield of non-regulated non-photochemical reactions within PSII (PhiNO) were calculated according to
296 Kramer et al. (2004). The effective quantum yield of PSI (PhiPSI), the non-photochemical yield of PSI caused
297 by the donor side limitation (PhiIND) and the non-photochemical yield of PSI caused by the acceptor side
298 limitation (PhiNA) were calculated according to Klughammer and Schreiber (2008).

299 The electrochromic shift signal (ECS) was monitored as the absorbance change at 515-550 nm using the
300 Dual-PAM-100 equipped with the P515/535 emitter-detector module (Heinz Walz). The absorbance signal
301 at 535 nm was monitored simultaneously. Leaves were first dark-adapted for 40 min and the amplitude of
302 ECS induced by a single turnover flash was recorded. Dark-interval relaxation kinetics of ECS was then

303 recorded after 3-min intervals of illumination with red actinic light of increasing irradiance. Proton motive
304 force (*pmf*) was estimated from the amplitude of the rapid decay of ECS signal upon light-dark transition,
305 normalised for the ECS induced by the single turnover flash. Proton conductivity of the thylakoid membrane
306 through ATP synthase was calculated as an inverse time constant obtained by the fitting of first-order ECS
307 relaxation kinetics after Sacksteder and Kramer (2000).

308 **Protein isolation and Western blotting**

309 Total protein extracts were isolated from 0.7 cm² frozen leaf discs as described in Ermakova et al. (2019)
310 and separated by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane and probed with
311 antibodies against various photosynthetic proteins in dilutions recommended by the producer: Rieske
312 (AS08 330, Agrisera, Vännäs, Sweden), D1 (Agrisera, AS10 704), PGR5 (Agrisera, AS163985). Quantification
313 of immunoblots was performed with Image Lab software (Biorad, Hercules, CA).

314 **Statistical analysis**

315 The relationship between mean values of different groups was tested by one-way ANOVAs with Dunnett's
316 or Tukey's *post-hoc* test or by two-tailed heteroscedastic *t*-test, as indicated in figure legends.

317

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325 **Author Contributions**

326 SvC, ME and RTF designed the research; SB generated transgenic plants; ME, RW, SB and ZT performed
327 research; ME and RW analyzed data; ME wrote the paper.

328

329 **Short legends for Supporting Information**

330 **Fig. S1.** Immunodetection of Rieske in the T₁ progeny of line 32.

331 **Fig. S2.** Length and width of top fully expanded leaves from WT, azygous and Rieske overexpressing plants.

332

333 **Figure legends**

334 **Fig. 1.** Selection of transgenic sorghum lines overexpressing Rieske. **a.** Immunodetection of Rieske in leaves
335 of T_0 plants. **b.** Transcript abundance of *B. dystachion petC* (*BdpetC*) in T_0 sorghum plants. **c.**
336 Immunodetection of Rieske in T_1 progenies of lines 25 and 26. **(a and c)** Samples were loaded on leaf area
337 basis, and the titration series of WT samples was used for relative quantification. Insertion numbers indicate
338 a copy number of *ntpII* obtained by digital PCR. Asterisks indicate the plants which progenies were used in
339 further experiments. **d.** Quantum yield of non-photochemical quenching (PhiNPQ) measured at ambient
340 irradiance in T_1 progenies of lines 25 and 26. WT and azygous plants were used as control. Each point
341 represents a technical replicate.

342 **Fig. 2.** Protein analysis and growth of sorghum lines overexpressing Rieske. **a.** Immunodetection of
343 photosynthetic proteins in leaves of control and transgenic plants: Rieske (*Cytb6f*), D1 (PSII core), AtpB (ATP
344 synthase), PsbS (energy-dependent non-photochemical quenching), Lhcb2 (light-harvesting complex II),
345 RbcL (large subunit of Rubisco), PEPC (PEP carboxylase). Samples were loaded on leaf area basis, and the
346 titration series of the Control sample #1 was used for relative quantification. **b.** Quantification of
347 immunoblots relative to control plants. Mean \pm SE, $n = 3$ biological replicates. **c.** Phenotype of plants 5
348 weeks after germination. **(d, e, f)** Height, leaf number and plant biomass, $n = 5$ biological replicates. **(g, h)**
349 Total weight and number of seeds produced per plant, $n = 18$ biological replicates for Control (WT and
350 azygous plants), $n = 20$ for Rieske-OE (lines 25 and 26). Asterisks indicate statistically significant differences
351 between transgenic and control plants based on one-way ANOVA or *t*-test ($^{**}P < 0.05$ or $^{*}P < 0.1$).

352 **Fig. 3.** Gas exchange and fluorescence analysis of control and transgenic sorghum plants overexpressing
353 Rieske at different $p\text{CO}_2$ (left panels, measured at $1500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or irradiance (right panels,
354 measured at ambient $p\text{CO}_2$). PhiPSII, the effective quantum yield of PSII; gsw, stomatal conductance to
355 water vapor. Azygous plants were used as control. Mean \pm SE, $n = 5$ biological replicates. No statistically
356 significant differences were found between transgenic and control plants (one-way ANOVA and Dunnett's
357 post-hoc test at $P < 0.05$).

358 **Fig. 4.** Electron transport parameters estimated at different irradiance from leaves of control and transgenic
359 sorghum plants overexpressing Rieske. PhiPSII, the effective quantum yield of PSII; PhiNPQ, the yield of
360 non-photochemical quenching; PhiNO, the yield of non-regulated non-photochemical reactions within PSII;
361 PhiPSI, the effective quantum yield of PSI; PhiND, the non-photochemical yield of PSI due to the donor side
362 limitation; PhiNA, the non-photochemical yield of PSI due to the acceptor side limitation. Azygous plants
363 were used as control. Mean \pm SE, $n = 5$ biological replicates. Asterisks indicate statistically significant

364 difference between line 25 (black) or line 32 (grey) and control plants (one-way ANOVA and Dunnett's post-
365 hoc test at $P < 0.05$).

366 **Fig. 5.** Analysis of the thylakoid membrane energisation in control plants and transgenic sorghum lines
367 overexpressing Rieske. **(a and b)** Proton motive force (pmf) and proton conductivity of the thylakoid
368 membrane (g_{H^+}) at different irradiance analysed using electrochromic shift signal. Mean \pm SE, $n = 4$
369 biological replicates. **c.** Absorbance changes at 535 nm recorded upon the shift from dark to $1600 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The absorbance at the beginning and end of the 3-min illumination period was normalised to 0 and 1,
370 respectively, to facilitate comparison of the kinetics. Averages of 4 biological replicates are presented.
371 Azygous plants were used as control. Asterisks indicate intervals of statistically significant difference
373 between transgenic and control plants (one-way ANOVA and Dunnett's post-hoc test at $P < 0.05$).

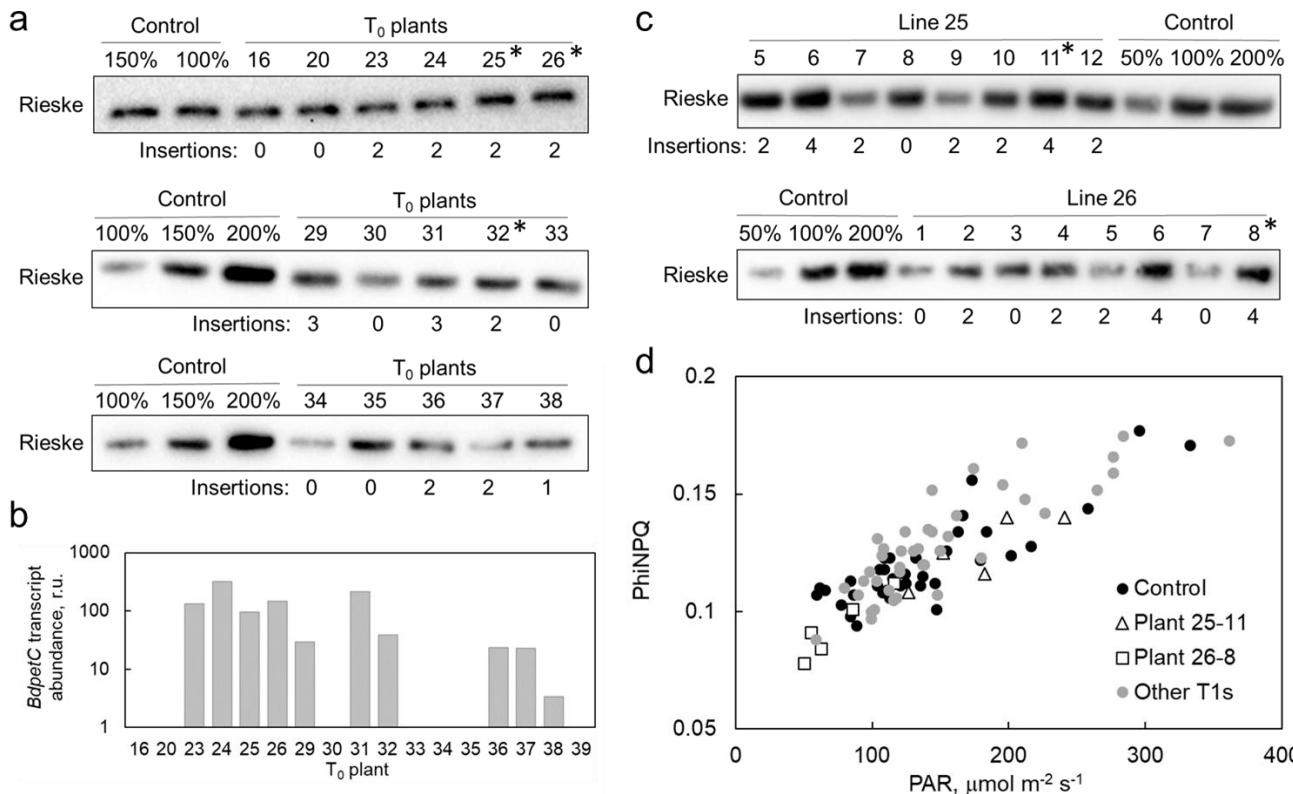
374 **Fig. 6.** Induction of photosynthesis during the first 30 min of illumination with actinic light of $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in control plants and transgenic sorghum lines overexpressing Rieske. PhiPSII, the effective quantum
375 yield of PSII; NPQ, non-photochemical quenching; gsw, stomatal conductance to water vapor; C_i/C_a , the
376 ratio between the intercellular and ambient CO_2 partial pressures. The values of each parameter, except
377 for C_i/C_a , at the beginning and end of the 30-min illumination were normalised to 0 and 1, respectively, to
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379 replicates. Asterisks indicate intervals of statistically significant differences between transgenic and control
381 plants (one-way ANOVA and Dunnett's post-hoc test at $P < 0.05$).

382 **Table 1.** Properties of control plants and transgenic sorghum lines overexpressing Rieske. Azygous plants
383 were used as control. Mean \pm SE, $n = 5$ biological replicates. Asterisks indicate statistically significant
384 differences between transgenic and control plants (one-way ANOVA and Dunnett's *post-hoc* test at
385 $P < 0.05$). LMA, leaf mass per area; F_v/F_M , the maximum quantum efficiency of PSII.

	Control	Line 25	Line 26	Line 32
Relative Chl	60.1 ± 1.9	57.6 ± 1.9	60.9 ± 1.8	60.5 ± 1.8
Leaf thickness, mm	0.63 ± 0.05	0.63 ± 0.03	0.60 ± 0.03	0.63 ± 0.04
LMA, g (dry weight) m^{-2}	119.42 ± 4.78	115.73 ± 4.08	113.92 ± 4.72	122.32 ± 2.65
Tiller number, plant $^{-1}$	1.4 ± 0.27	2.4 ± 0.45	2.0 ± 0.36	2.0 ± 0.36
F_v/F_M	0.802 ± 0.002	0.802 ± 0.006	0.803 ± 0.003	$0.810 \pm 0.001^*$

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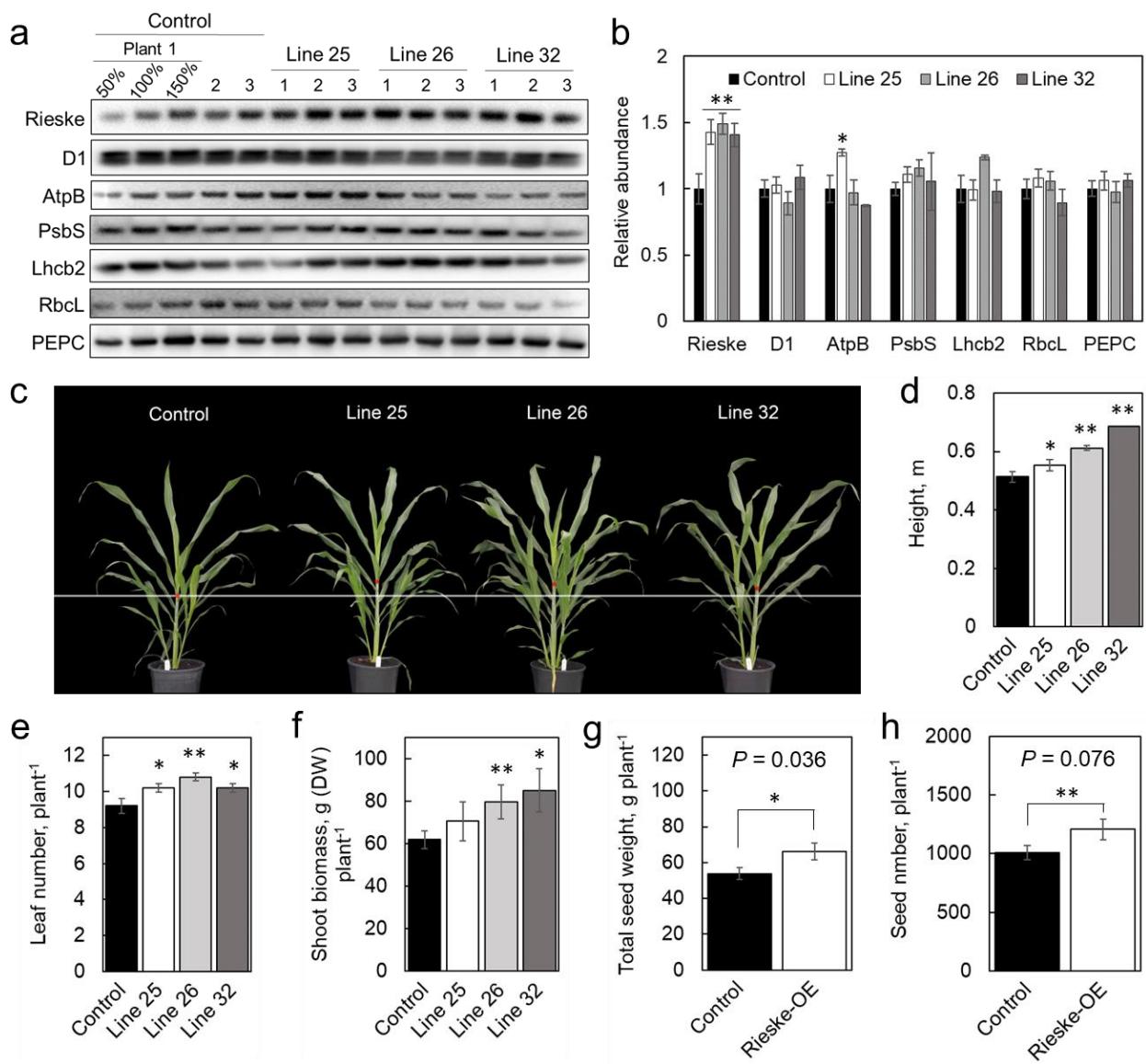


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390 of T₀ plants. **b.** Transcript abundance of *B. dystachion petC* (*BdpetC*) in T₀ sorghum plants. **c.**
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392 basis, and the titration series of WT samples was used for relative quantification. Insertion numbers indicate
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394 further experiments. **d.** Quantum yield of non-photochemical quenching (PhiNPQ) measured at ambient
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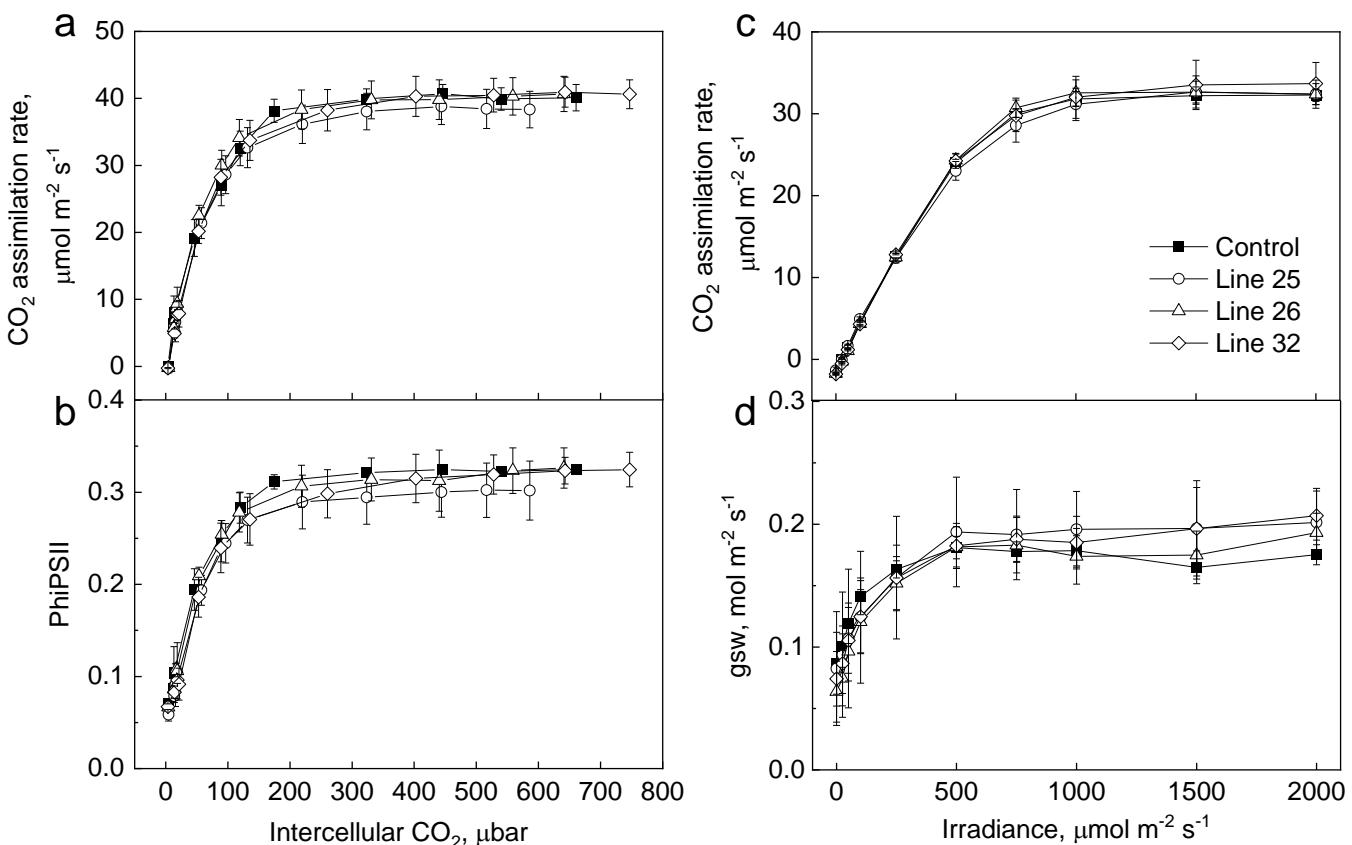
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405 immunoblots relative to control plants. Mean \pm SE, $n = 3$ biological replicates. **c.** Phenotype of plants 5
406 weeks after germination. **(d, e, f)** Height, leaf number and plant biomass, $n = 5$ biological replicates. **(g, h)**
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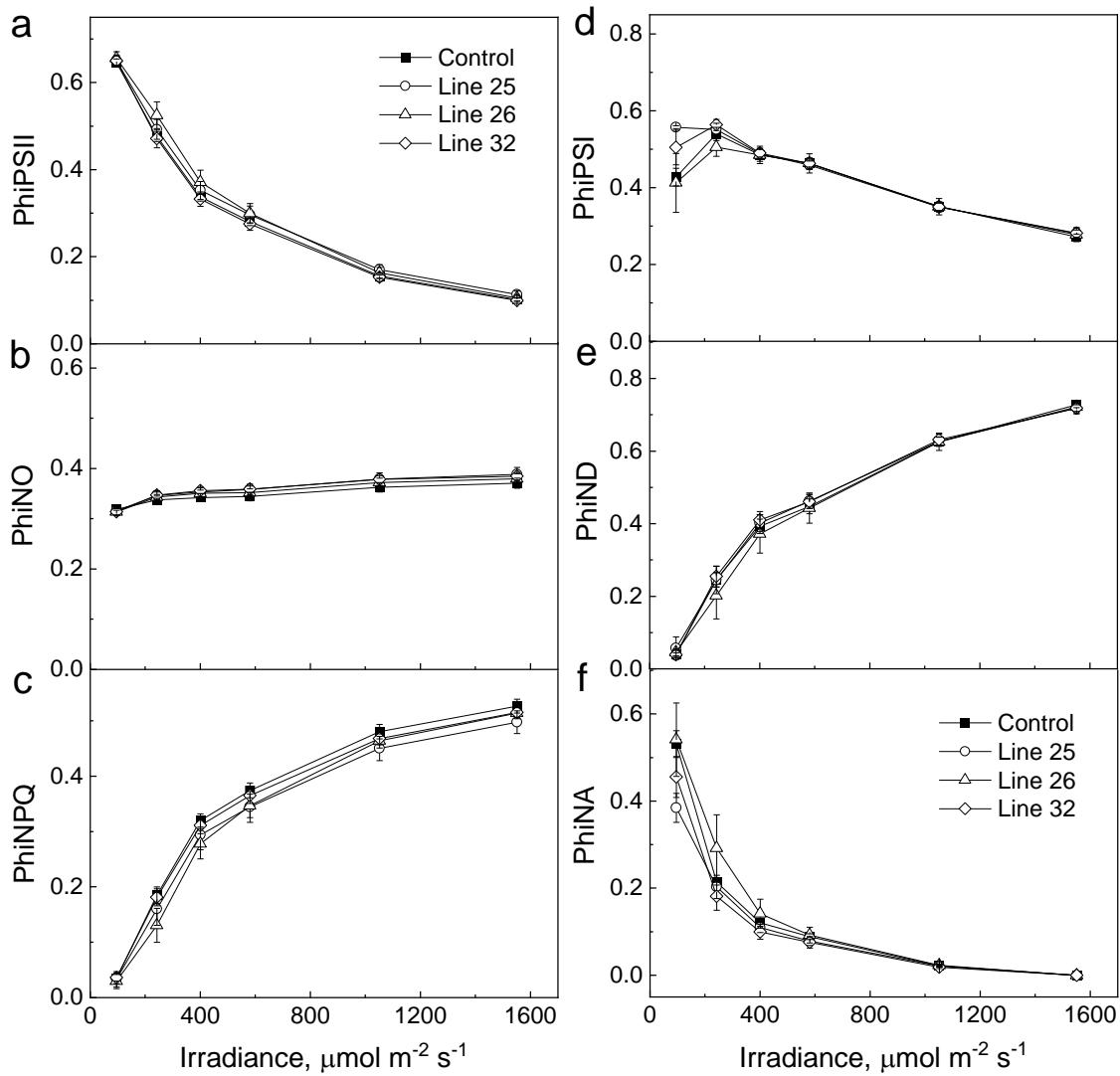
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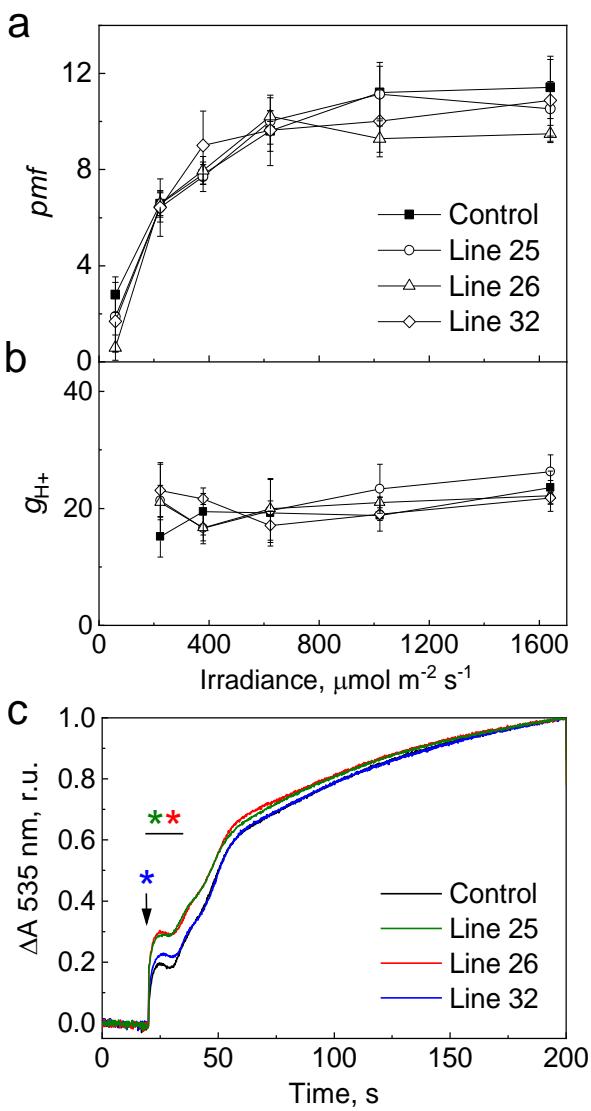
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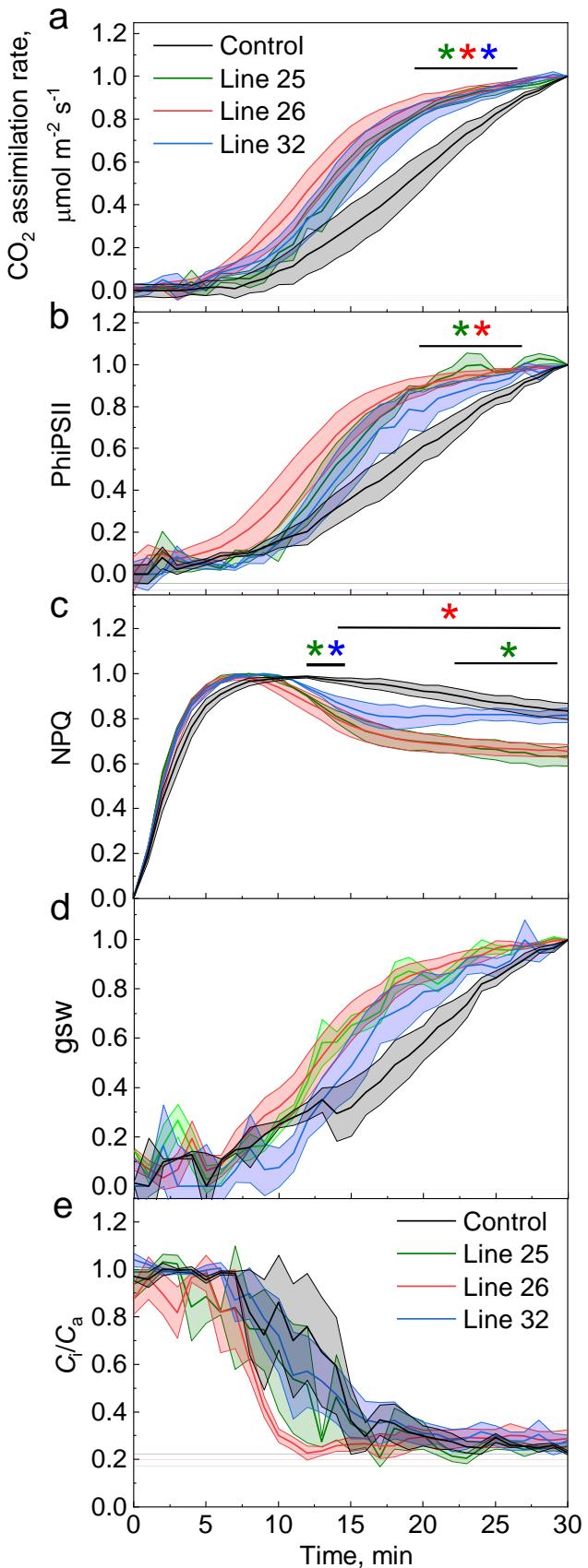


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438

439



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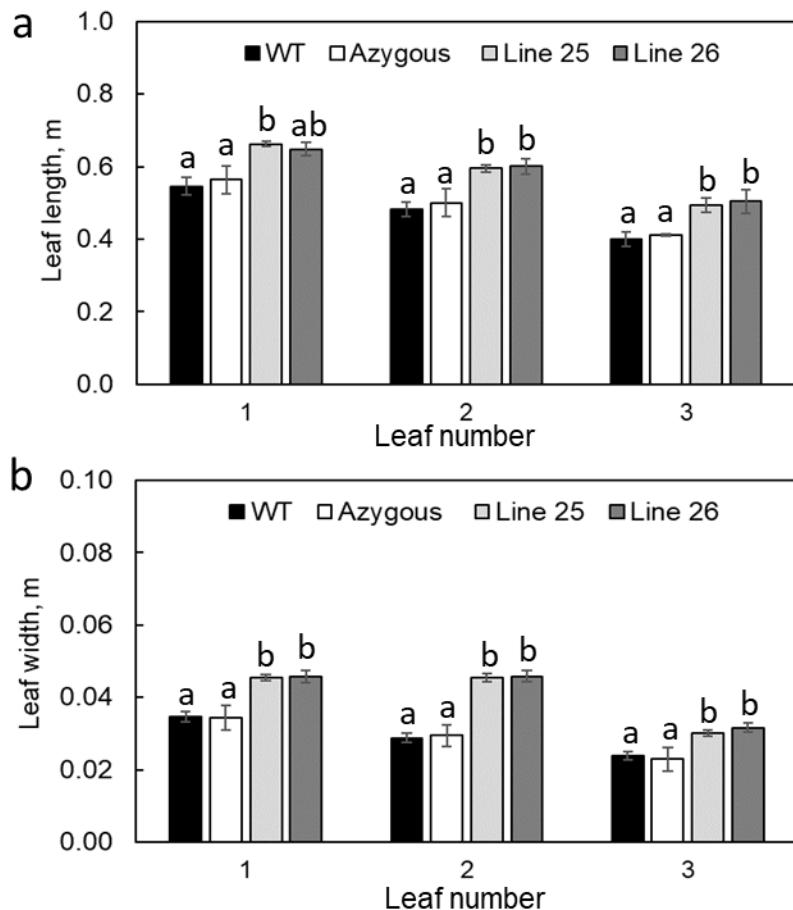
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Supplementary figures



461 **Fig. S1.** Immunodetection of Rieske in the T₁ progeny of line 32. Samples were loaded on leaf area basis,
462 and the WT sample was used as a control. Insertion numbers indicate a copy number of *ntpII* obtained by
463 digital PCR. Asterisk indicates homozygous plant 32-14 which T₂ progeny was used in further experiments.

464



465 **Fig. S2.** Length and width of top fully expanded leaves from WT, azygous and Rieske overexpressing plants.
466 Mean \pm SE, $n = 5$ biological replicates. Letters indicate statistically significant differences between groups
467 (one-way ANOVA and Tukey post-hoc test, $P < 0.05$).

468

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