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ARTICLE ADDENDUM

Chloroplastic iron-sulfur scaffold protein NFU3 is essential to overall plant fitness

Krishna Nath, James P. O'Donnell, and Yan Lu [iD]

Department of Biological Sciences, Western Michigan University, Kalamazoo, MI, USA

ABSTRACT

A previous study showed that Nitrogen-Fixing-subunit-U-type protein NFU3 may act an iron-sulfur scaffold protein in the assembly and transfer of 4Fe-4S and 3Fe-4S clusters in the chloroplast. Examples of 4Fe-4S and 3Fe-4S-requiring proteins and complexes include Photosystem I (PSI), NAD(P)H dehydrogenase, and ferredoxin-dependent glutamine oxoglutarate aminotransferases. In this paper, the authors provided additional evidence for the role of NFU3 in 4Fe-4S and 3Fe-4S cluster assembly and transfer, as well as its role in overall plant fitness. Confocal microscopic analysis of the fluorescently-tagged NFU3 protein confirmed the chloroplast localization of the NFU3 protein. Detailed analysis of chlorophyll fluorescence data revealed that a substantial increase in minimal fluorescence is the primary contributor to the decrease in PSII maximum photochemical efficiency observed in the *nfu3* mutants. The substantial increase in minimal fluorescence in the *nfu3* mutants is probably the result of an impaired PSI function, blockage of electron flow from PSII to PSI, and over-accumulation of reduced plastoquinone at the acceptor side of PSII. Analyses of seed morphology and germination showed that NFU3 is essential to seed development and germination, in addition to plant growth, development, and flowering. In summary, NFU3 has wide-ranging effects on many biologic processes and is therefore important to overall plant fitness. NFU3 may exert these effects by modulating the availability of 4Fe-4S and 3Fe-4S clusters to 4Fe-4S and 3Fe-4S-requiring proteins and complexes involved in various biologic processes.

Introduction

Iron-sulfur clusters are involved in various vital biologic processes including; photosynthesis, respiration, sulfur and nitrogen assimilation, amino acid and purine metabolism, chlorophyll and cofactor metabolism, hormone synthesis, DNA replication and repair, ribosome biogenesis, tRNA maturation, and translation.[1-3] Iron-sulfur clusters may contain different numbers of iron and sulfur ions. The 4 most common iron-sulfur clusters in plants are: classic 2Fe-2S, Rieske-type 2Fe-2S, 3Fe-4S, and 4Fe-4S clusters.[1-3] Three iron-sulfur assembly and transfer pathways have been identified in plants: the SUF (sulfur mobilization) pathway in plastids, the ISC (iron-sulfur cluster) pathway in mitochondria, and the CIA (cytosolic iron-sulfur) assembly pathway.[1-3] The SUF pathway is responsible for the assembly and transfer of classic 2Fe-2S, Rieske-type 2Fe-2S, 3Fe-4S, and 4Fe-4S clusters in plastids; the ISC pathway is in charge of assembly and transfer of classic 2Fe-2S, 3Fe-4S, and 4Fe-4S clusters in mitochondria; and the CIA pathway assembles and transfers classic 2Fe-2S and 4Fe-4S clusters in the cytosol.[1] In all 3 pathways, the process can be divided into 2 steps: (1) assembly of iron-sulfur clusters on an iron-sulfur scaffold protein by cysteine desulfurase, and (2) subsequent transfer of iron-sulfur clusters from the iron-sulfur scaffold protein to recipient apoproteins.[1-3]

Recently, it was found that Nitrogen-Fixing-subunit-U-type protein NFU3 acts as a scaffold protein in the assembly and transfer of 4Fe-4S and 3Fe-4S clusters in the chloroplast.[4] The recombinant NFU3 protein showed characteristics of 4Fe-4S and 3Fe-4S clusters and an *in vitro* activity of iron-sulfur cluster reconstitution. The loss-of-function *nfu3* mutants of Arabidopsis (*Arabidopsis thaliana*) had reduced amounts of 4Fe-4S-containing PSI core subunits PsaA, PsaB, and PsaC (where Psa stands for PSI) and 3Fe-4S-containing ferredoxin-dependent glutamine oxoglutarate aminotransferases (Fd-GOGATs). The ~90% reduction in the levels of 4Fe-4S-containing PSI core subunits resulted in a near-complete loss of PSI activity and a substantial decrease in F_v/F_m, the maximum quantum efficiency of PSII photochemistry.

In this paper, the authors confirmed the chloroplast localization of the NFU3 protein, identified the causal parameters for reduced F_v/F_m in the *nfu3* mutants, and analyzed the morphology and germination rate of the *nfu3* seeds. These results demonstrated that NFU3 is essential to plant growth and development, flowering, seed development, and germination.

Analysis of the NFU3-Cerulean Fluorescent Protein (CFP) fusion protein confirmed the chloroplast localization of NFU3

According to the Arabidopsis eFP browser, NFU3 is highly expressed in leaves, flowers, and early stage embryos (Fig. S1).[5] Based on TargetP prediction,[6] NFU3 is located in the plastid. To test this prediction, the full-length NFU3 coding region without the stop codon (NFU3[1–708 bp], corresponding to NFU3[1–236 AA]) was inserted between the cauliflower mosaic virus 35S RNA promoter and the CFP coding sequence of a binary vector
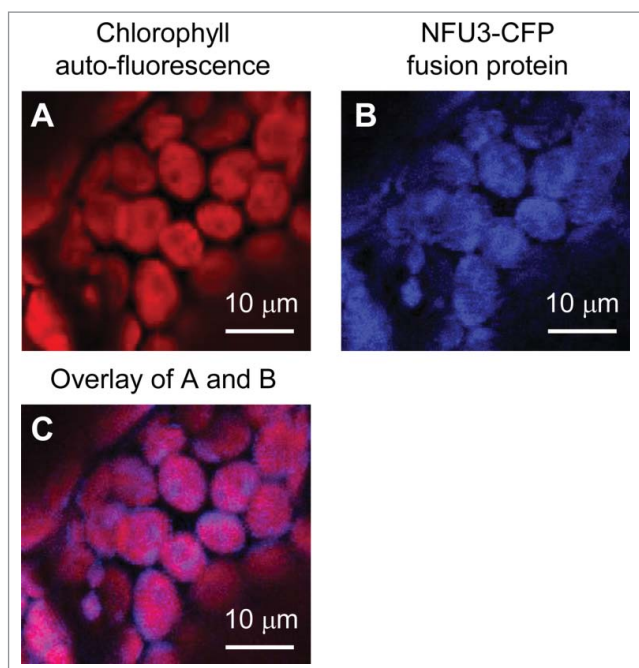

Figure 1. Localization of an NFU3–CFP fusion protein during transient expression in *Nicotiana benthamiana* leaf cells. (A) Chlorophyll auto-fluorescence of *N. benthamiana* mesophyll cells transiently expressing the NFU3-CFP fusion protein. Bars = 10 μm. (B) CFP fluorescence of *N. benthamiana* mesophyll cells transiently expressing the NFU3-CFP fusion protein. (C) Overlay of chlorophyll auto-fluorescence and CFP fluorescence of *N. benthamiana* mesophyll cells transiently expressing the NFU3-CFP fusion protein.

(pPH5ADEST-CFP) suitable for transient expression. The *Agrobacterium tumefaciens* culture containing the fusion protein construct (pPH5ADEST-NFU3-CFP) was used to inoculate *Nicotiana benthamiana* leaves, as described previously.[7] Transformed leaf tissues were observed using a Nikon C2$^+$ confocal laser scanning microscope and a CFI Apochromat 60x oil immersion objective. Chlorophyll red autofluorescence and NFU3-CFP fusion protein analysis confirmed that NFU3 is indeed located in the chloroplast (Fig. 1). Consistent with our results, confocal microscopic analysis of a fusion protein containing the N-terminal region of NFU3 and the full-length Green Fluorescent Protein (GFP) expressed in Arabidopsis protoplasts also demonstrated the chloroplast localization of NFU3.[8]

The nfu3 mutants had increased minimal, maximal and variable fluorescence

Previous studies have found that the calculated F_v/F_m ratio in the *nfu3* mutants was 13% lower than that observed in the wild type.[4] A low F_v/F_m ($F_v/F_m = [F_m - F_o]/F_m = 1 - F_o/F_m$) value could be the result of a low F_v (variable fluorescence) or a high F_o (minimal fluorescence) value. A low F_v value is indicative of an inability of PSII to perform primary photochemistry.[9] A high F_o value could be the result of an over-accumulation of reduced Q_A (bound primary plastoquinone electron acceptor of PSII) in the dark.[10] To identify the causal parameter(s) for reduced F_v/F_m in the *nfu3* mutants, we determined the minimal, maximal, and variable fluorescence of dark-adapted wild-type and *nfu3* plants (Fig. 2). We found that F_o in the *nfu3* mutants (0.21 on average) was 190% higher than that in the wild type (0.07), F_m in the *nfu3* mutants (0.75 on average) was 80% higher than that in the wild type (0.42),

and F_v in the *nfu3* mutants (0.54 on average) was 57% higher than that in the wild type (0.35). These data indicate that PSII in the *nfu3* mutants is not defective and that the substantial increase in F_o is a major contributor of the observed decrease in F_v/F_m in the *nfu3* mutants. The significant increase of the F_o value observed in the *nfu3* mutants suggests that reduced Q_A might be over-accumulated.[10] Consistent with this hypothesis, the redox state of PSII acceptor side (*1-qP*) in the *nfu3* mutants was much higher than that in the wild type.[4] Over-accumulation of reduced Q_A and increases in the redox state of the PSII acceptor side could be caused by a defect in intersystem electron transport or PSI.[11-13] The near-complete loss of PSI activity in the *nfu3* mutants strongly suggests that these observations are initially caused by the defects in PSI. In summary, these data provide additional support that, in the *nfu3* mutants, PSI suffers greatly from the lack of the 4Fe-4S cluster and the reduction in PSII activity is likely a secondary effect of the near-complete loss of PSI activity.[4]
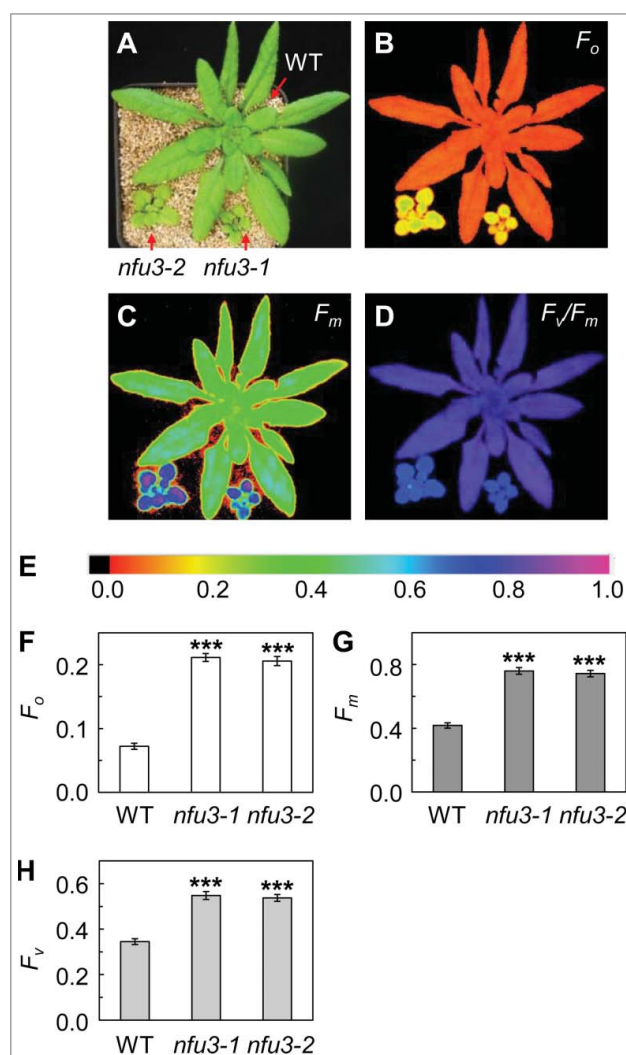
Figure 2. Minimal and maximal fluorescence were increased in the *nfu3* mutants. (A) Images of 4-week-old wild-type, *nfu3–1*, and *nfu3–2* plants. (B-D) False-color F_o, F_m, and F_v/F_m images of 4-week-old plants, respectively. (E) Color scale for chlorophyll fluorescence parameters F_o, F_m, and F_v/F_m in B-D. F-H, F_o, F_m, and F_v values of 4-week-old plants. Data are presented as mean ± SE (n = 4). The asterisk indicates a significant difference between the mutant and the wild type (WT) (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Plants used for chlorophyll fluorescence analysis were grown on a 12-h light/12-h dark photoperiod with an irradiance of 150 μmol photons m^{-2} s^{-1} during the light period.

The nfu3 mutants displayed retarded growth and delayed flowering and seeding

The *nfu3* mutants were much smaller than wild-type plants at the same age (Fig. 2A), indicative of retarded growth. After 6 weeks under a 12-h-light /12-h-dark photoperiod, the Columbia wild-type plants flowered, while the *nfu3* mutants continued to stay in a vegetative growth stage (see Fig. 1E in ref. 4). Consistent with retarded growth and delayed flowering, the amount of seeds produced by the *nfu3* mutants was approximately 15% of those produced by the wild type (see Fig. 1G in ref. 4). These observations strongly suggest that NFU3 is essential to the normal growth, flowering, and seeding of Arabidopsis plants. Similar defects in growth, flowering, and seeding were also observed in the loss-of-function Arabidopsis mutants of NFU2, which have previously been shown to be required for the assembly and transfer of 4Fe-4S and classic 2Fe-2S clusters in the chloroplast.[14,15]

The nfu3 seeds had reduced weight and reduced germination

Loss-of-function mutants of several other genes involved in the assembly and transfer of iron-sulfur clusters showed abnormal seed morphology and germination;[2,3,16-18] therefore we analyzed the morphology and germination of the *nfu3* seeds (Fig. 3). The tip of the *nfu3* siliques tended to be thin and filled with small, dark brown, and wrinkled seeds (Fig. 3A–B). Consequently, the average *nfu3* seed weight was approximately 48% of wild-type seed weight (Fig. 3C). The small, dark brown, and wrinkled seed morphology has also been observed in other Arabidopsis mutants with defects in iron-sulfur assembly and transfer. For example, loss-of-function mutants of plastid-targeted iron-sulfur scaffold protein SufD/NAP6 and dual (mitochondrion and cytosol)-targeted DnaJ-like co-chaperone protein HscB displayed the same seed morphology.[16,17] SufD/NAP6 forms a SufBC$_2$D complex with SufB and SufC and fulfills the scaffold function in the assembly and transfer of 4Fe-4S, 3Fe-4S, and Rieske-type 2Fe-2S clusters in the chloroplast.[1-3,16] HscB interacts with DnaK-like chaperone protein HscA and iron-sulfur scaffold protein IscU1; therefore, HscB was proposed to participate in the assembly and transfer of iron-sulfur clusters in mitochondria and the cytosol.[1-3,17]

We separated the wrinkled *nfu3* seeds from the non-wrinkled *nfu3* seeds, determined their germination rates, and compared the germination rates between the 2 groups as well as with the wild type. The germination rate of the wrinkled *nfu3* seeds (10–15%) was substantially lower than those of the wild-type seeds (98%) and the non-wrinkled *nfu3* seeds (84–87%) (Fig. 3D). It is not surprising that the small, dark brown and wrinkled *nfu3* seeds germinated poorly, as the wrinkled *sufd/nap6* seeds also had a poor germination rate.[16] It is interesting that the germination rate of the non-wrinkled *nfu3* seeds was 11–14% lower than that of the wild-type seeds.

NFU3 is essential to overall plant fitness

The *nfu3* mutants suffer from a series of growth and developmental defects, likely explained by the role of NFU3 in modulating the availability of 4Fe-4S and 3Fe-4S clusters to 4Fe-4S and 3Fe-4S-requiring proteins and complexes. 4Fe-4S and
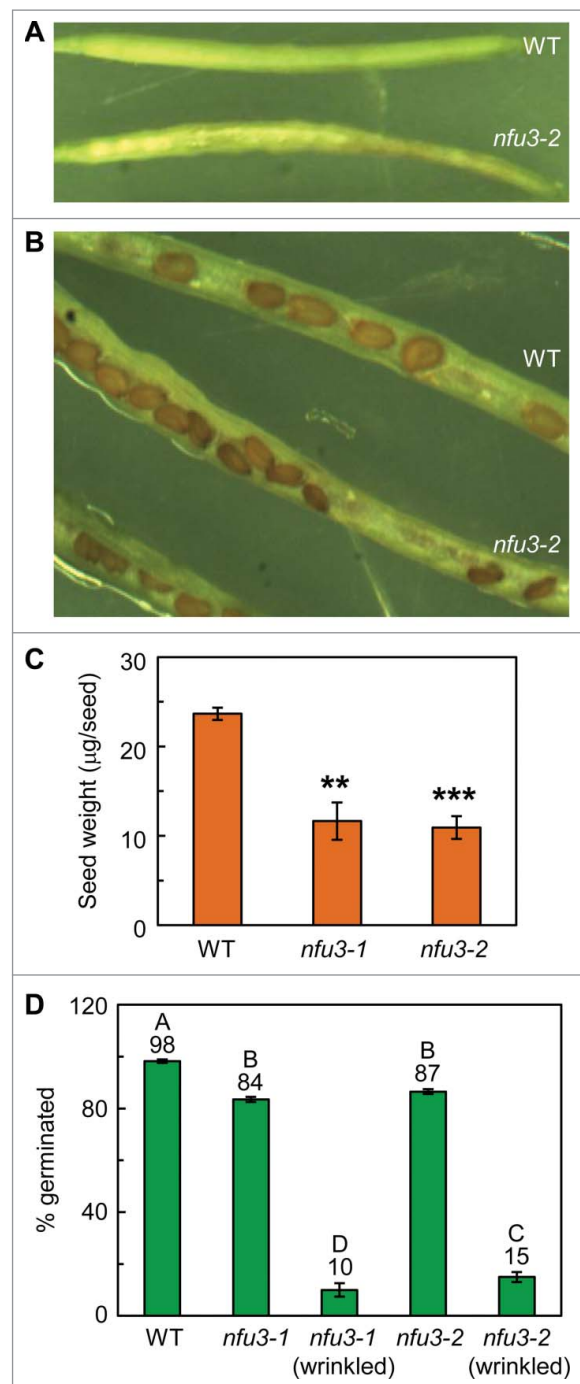
Figure 3. Analyses of seeds from wild-type and *nfu3* plants.(A) Intact desiccating siliques from wild-type and *nfu3–2* plants. (B) Opened desiccating siliques from wild-type and *nfu3–2* plants. (C) Average weight of a seed from wild-type and *nfu3* plants. Data are presented as mean ± SE (n = 5–7). The asterisk indicates a significant difference between the mutant and the wild type (WT) (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (D) Germination of seeds from wild-type and *nfu3* plants. Data are presented as mean ± SE (n = 4). Values not connected by the same letter are significantly different (Student's t test, $p < 0.05$). Plants used for seed analyses were grown on a 12-h light/12-h dark photoperiod with an irradiance of 150 μmol photons m^{-2} s^{-1} during the light period.

3Fe-4S clusters are required in various vital biologic processes, many of which directly affect plant growth and development.

For example, PSI core subunits PsaA, PsaB, and PsaC require 4Fe-4S clusters to be functional.[1-4] Loss-of-function mutations in NFU3 resulted in substantial reductions in the abundances of PsaA, PsaB, and PsaC, a near-complete loss of

PSI activity, and a secondary reduction in PSII activity.[4] PSI and PSII are essential to photosynthesis and the products of photosynthesis are required for plant growth and development. Therefore, the growth and developmental defects in the *nfu3* mutants could be secondary effects of the near-complete loss of PSI activity due to limited availability of the 4Fe-4S cluster to PSI. Indeed, other mutants with reduced photosynthetic efficiency also displayed reduced growth and development.[14,19-21] NFU2 is involved in assembly and transfer of 4Fe-4S and classic 2Fe-2S clusters and the *nfu2* mutants displayed similar trends as the *nfu3* mutants.[14] The *nfu2* mutants had a ∼30% reduction in PSI activity (calculated from Fig. 3 in reference[14]) and this is accompanied by delayed growth, flowering, and seeding.

The NAD(P)H dehydrogenase (NDH) complex in the chloroplast is another example of 4Fe-4S-containing protein complexes.[1,3,22] The NdhI and NdhK subunits of this complex bind one and 2 4Fe-4S clusters, respectively.[1,3,22] Although the abundances of NdhI and NdhK proteins were not determined, the levels of 2 other chloroplastic NDH subunits (NdhB and NdhH) were substantially reduced in the *nfu3* mutants.[4] This suggests that the activity of chloroplastic NDH might be compromised in the *nfu3* mutants. In addition to NAD(P)H dehydrogenation, the chloroplastic NDH complex also acts as ferrodoxin-dependent plastoquinone reductase, mediating a minor pathway of PSI cyclic electron transport.[22,23] PSI cyclic electron transport is essential for balancing ATP/NADPH production, which is required in various growth and developmental processes.[24-26] The NDH-dependent PSI cyclic electron transport is specifically important for stress tolerance and photoprotection.[24]

A third example is 3Fe-4S-containing Fd-GOGAT1 and Fd-GOGAT2[1-4], which are expressed in leaf chloroplasts and root plastids, respectively.[27] Fd-GOGATs have 2 functions: nitrogen assimilation and re-assimilation of photorespiratory ammonia.[27] Under ambient CO_2 conditions, loss-of-function mutants of Fd-GOGAT1 had a smaller plant size with small and pale green leaves,[27] similar to the *nfu3* mutants (Fig. 2A). Interestingly, the abundance of Fd-GOGAT1 was 45% lower in the *nfu3* leaves than in wild-type leaves.[4]

In addition to PSI, NDH, and Fd-GOGATs, other 4Fe-4S- (*e.g.*, nitrite reductase and sulfite reductase) and 3Fe-4S-(*e.g.,* NADH-GOGAT) containing proteins and complexes exist in the chloroplast.[1-3] Although not determined, the abundances of these proteins and complexes might also be reduced in the *nfu3* mutants, due to limited availability of 4Fe-4S and 3Fe-4S clusters. Taken together, NFU3 is required for the assembly and transfer of 4Fe-4S and 3Fe-4S clusters in the chloroplast. NFU3 influences plant growth and development by modulating the availability of 4Fe-4S and 3Fe-4S clusters to 4Fe-4S- and 3Fe-4S-requiring proteins and complexes involved in various growth and developmental processes. Therefore, NFU3 is essential to overall plant fitness.

Materials and methods

Plant materials and growth conditions

The 2 Arabidopsis (*Arabidopsis thaliana*) T-DNA insertion lines (*nfu3–1* [GABI_381H10] and *nfu3–2* [GABI_791C01],

both in Columbia ecotype) used in this study were obtained from the Arabidopsis Biological Resource Center. Plants were grown in a growth chamber on a 12-h-light/12-h-dark photoperiod, as described previously.[28] The light intensity was 150 μmol photons m^{-2} s^{-1}, the temperature was 20°C, and the relative humidity was 50%.

Transient Expression of NFU3-CFP in Nicotiana benthamiana

Transient Expression of the NFU3-CFP fusion protein in *N. benthamiana* was performed as described previously.[7] The full-length NFU3 coding region without the stop codon (NFU3$^{1–708 \ bp}$, corresponding to NFU3$^{1–236 \ AA}$) was amplified using the mRNA:cDNA hybrid, Phusion High-Fidelity DNA Polymerase (New England Biolabs), a forward primer NFU3_F (5′-CACCATGGGTTCTG TTTCGGGTCAA-3′), and a reverse primer NFU3_R (5′-CTCTAGAAGCTGGACTGCAC-3′). The resulting PCR products were gateway-cloned into the pENTR^{TM}/D-TOPO® vector (Thermo Fisher) and sequenced to confirm the absence of errors. The confirmed NFU3$^{1–711 \ bp}$ fragment was sub-cloned into the pPH5ADEST-CFP vector (unpublished; provided by Jian Yao, Department of Biological Sciences, Western Michigan University, Kalamazoo, MI), using the Gateway® LR Clonase® II enzyme mix (Thermo Fisher). The resulting pPH5ADEST-NFU3-CFP construct, which is under the control of cauliflower mosaic virus 35S RNA promoter, was introduced into *Agrobacterium tumefaciens* strain C58C1. Transformed *A. tumefaciens* was cultured over-night at 30°C in Luria-Bertani medium containing appropriate antibiotics, harvested by centrifugation at 3,500 rpm at room temperature for 10 min, washed once in infiltration buffer (10 mM MES pH 5.8; 10 mM $MgCl_2$; 0.2% sucrose), and resuspended to OD600 = 0.2 in infiltration buffer containing 300 μM acetosyringone. The *A. tumefaciens* cultures were inoculated into mature leaves of *N. benthamiana* and transient expression of the NFU3-CFP fusion protein was analyzed by confocal microscopy at 36–48 hours after inoculation.

Chlorophyll fluorescence analysis

Minimal fluorescence (F_o) and maximal fluorescence (F_m) were measured on dark-adapted plants at room temperature with the MAXI version of the IMAGING-PAM M-Series chlorophyll fluorescence system (Heinz Walz GmbH), as described previously.[29,30] Maximum photochemical efficiency of PSII (F_v/F_m) is calculated using the following equation: $F_v/F_m = (F_m – F_o)/F_m$, where F_v is variable fluorescence of dark-adapted leaves.[4]

Seed morphology

Desiccating siliques were harvested and imaged under a Nikon SMZ-U dissecting scope and a QIMAGING RETIGA EXi Fast 1394 color camera. After the intact silique was imaged, one valve was removed to expose and image seeds in the silique. The average seed weight was determined by measuring the precise weight of ∼2.0 mg seeds and subsequent counting of the seed number under a dissecting microscope.

Seed germination

Harvested seeds were mixed well and aliquots of ∼100 seeds were imbibed in 0.01% Triton for 30 min, surface-sterilized with 95% ethanol for 5 min and 10% bleach for 5 min, and rinsed with sterile water 5 times. Sterilized seeds were sown in 25×150 mm Petri dishes containing 25-mL of solid germination media (1X Murashige and Skoog Basal Salt Mixture, 1X Gamborg's Vitamin Solution, 10 g/L sucrose, 0.25 g/L MES, 7 g/L agar, pH 5.65–5.8). Sown seeds were stratified at 4°C for 2–3 d and placed in a growth chamber on a 12-h-light/12-h-dark photoperiod for 7 d. The germination rate was determined by counting the numbers of germinated seedlings and un-germinated seeds.

ORCID

Yan Lu 🆔 http://orcid.org/0000-0002-3374-7376

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