

Letters

Targeting of flavodoxin to chloroplasts of mesophyll but not bundle sheath maize cells confers increased drought tolerance

Abiotic stresses, especially drought, represent the main factor limiting agricultural productivity world-wide. Maize, one of the top crops in terms of food, feed and biofuel production, is a C_4 summer monocotyledonous species grown as a single-cross hybrid displaying high heterosis (conducive to improved grain yield), and high sensitivity to drought stress at different growth stages (Sheoran *et al.*, 2022). Photosynthesis is an early target of most environmental hardships including drought, which inhibits CO_2 assimilation associated with stomatal closing. Under such conditions, reducing power generated by the photosynthetic electron transport chain (PETC) cannot be used in the regenerative step of the Calvin cycle, leading to the accumulation of NADPH, over-reduction of the PETC and downregulation of ferredoxin levels (Tognetti *et al.*, 2006). The excess of excitation energy and reducing equivalents are delivered to adventitious acceptors, mostly O_2 , with the generation of partially reduced and/or activated energy-rich reactive oxygen species (ROS) such as the superoxide anion radical, hydrogen peroxide and singlet oxygen, which act both as toxic compounds and signaling intermediates (Foyer *et al.*, 2017; Dvořák *et al.*, 2021). Oxidative stress is thus an almost universal outcome of environmental adversities.

Plants have evolved multiple protective mechanisms to control ROS formation, scavenge them once formed, and repair the damage they cause (Lodeyro *et al.*, 2021). Alternative electron pathways and acceptors such as the Mehler–Asada cycle, the plastid terminal oxidase and the flavin–diiron proteins can relieve the over-reduction of the PETC by delivering the surplus of reducing equivalents to water, thus limiting ROS propagation (Lodeyro *et al.*, 2021). Cyanobacteria and oceanic algae have an additional electron shuttle, flavodoxin, a flavin mononucleotide-harboring soluble carrier isofunctional with ferredoxin (Pierella Karlusich *et al.*, 2014). Expression of this flavoprotein in photosynthetic microorganisms is induced in response to environmental stresses and iron starvation, which lead to downregulation of ferredoxin levels and inhibition of photosynthesis, allowing growth and reproduction under adverse conditions (Pierella Karlusich *et al.*, 2014). It is worth noting that unlike purely dissipative electron acceptors, flavodoxin can deliver reducing equivalents to many productive routes associated with photosynthesis, including nitrite and thioredoxin reduction (Tognetti *et al.*, 2006). Flavodoxin-encoding genes disappeared from the plant genome during the evolution of the *Viridiplantae*

lineage (Pierella Karlusich *et al.*, 2015), but the flavoprotein retained its capacity to functionally interact with plant photosynthetic components (Nogués *et al.*, 2004). Indeed, expression of a chloroplast-targeted cyanobacterial flavodoxin in tobacco plants led to increased tolerance to multiple sources of abiotic stress (Tognetti *et al.*, 2006), as in microorganisms. Moreover, the introduction of this trait enhanced stress tolerance and harvest index in several C_3 crops, including the pasture *Agrostis* (Li *et al.*, 2017), tomato (Mayta *et al.*, 2019) and potato (Pierella Karlusich *et al.*, 2020), highlighting its potential for crop improvement.

Despite their negative impact on growth and yield, the effects of environmental hardships on C_4 photosynthesis are not fully understood (Zhang *et al.*, 2018). Moreover, application of the flavodoxin technology to maize and other C_4 species presents some conceptual challenges. As indicated, flavodoxin protective functions depend on its functional interaction with the PETC in chloroplasts. However, as a C_4 plant, maize has dimorphic chloroplasts in the differentiated mesophyll (Ms) and bundle sheath (BS) cells (Rogowski *et al.*, 2019; Schlüter & Weber, 2020). Mesophyll chloroplasts can perform linear electron transport (LET) from water to $NADP^+$, similar to that of C_3 plants, and do not assimilate CO_2 , whereas BS chloroplasts have little grana, lower levels of photosystem (PS) II and perform cyclic electron transport (CET) around PSI. Carbon assimilation takes place in these plastids, using NADPH generated by malic enzyme (Rogowski *et al.*, 2019). Attempts to determine which of the two types of chloroplasts and photosynthetic pathways is more sensitive to abiotic stresses and oxidants led to conflicting results (Kingston-Smith & Foyer, 2000; Omoto *et al.*, 2013; Liu *et al.*, 2022), likely due to the different conditions and assays employed. Further research is thus required to identify which type of intervention would be more effective to improve stress tolerance in maize using the flavodoxin approach.

To address these unresolved issues, we generated two types of maize transgenic lines (*pfld*-Ms and *pfld*-BS, for plastid-targeted flavodoxin), in which flavodoxin expression and targeting were restricted to either Ms or BS chloroplasts, respectively, by using appropriate promoters and transit peptides (Fig. 1a, Supporting Information Fig. S1; Table S1; see also Methods S1). The flavodoxin gene was introduced in maize immature zygotic embryos from the HiII(AxB) hybrid cultivar by *Agrobacterium*-mediated transformation (Raineri *et al.*, 2019). After bialaphos selection and plant regeneration (Methods S1), independent T0 plants containing plasmids with the two constructs were crossed with the B73 inbred line (Fig. S2; Raineri *et al.*, 2019), resulting in flavodoxin-expressing and nonexpressing (null-segregating) plants in the progeny (Figs 1b, S3a). Flavodoxin expressed from both constructs accumulated as a processed mature-sized protein (mFld), although *pfld*-BS leaf extracts contained substantial amounts of the precursor (preFld, Fig. 1b). This might be caused

by lower efficiency of the C_3 targeting sequence employed, or to less import capacity in BS chloroplasts compared with *Ms* plastids. The BS-specific promoter from *Flaveria trinervia* (a C_4 dicot, see Methods S1) yielded weak expression when used in C_3 monocot rice (Ermakova *et al.*, 2021), but expression levels from the same

promoter appeared higher in the C_4 monocot *Setaria viridis* (Ermakova *et al.*, 2023). Accordingly, levels of mFld accumulation in BS cells of the C_4 monocot maize were comparable to those of *Ms* lines when equivalent samples from the two tissues were resolved in the same gel (Fig. S3b). Correct tissue-specific flavodoxin sorting

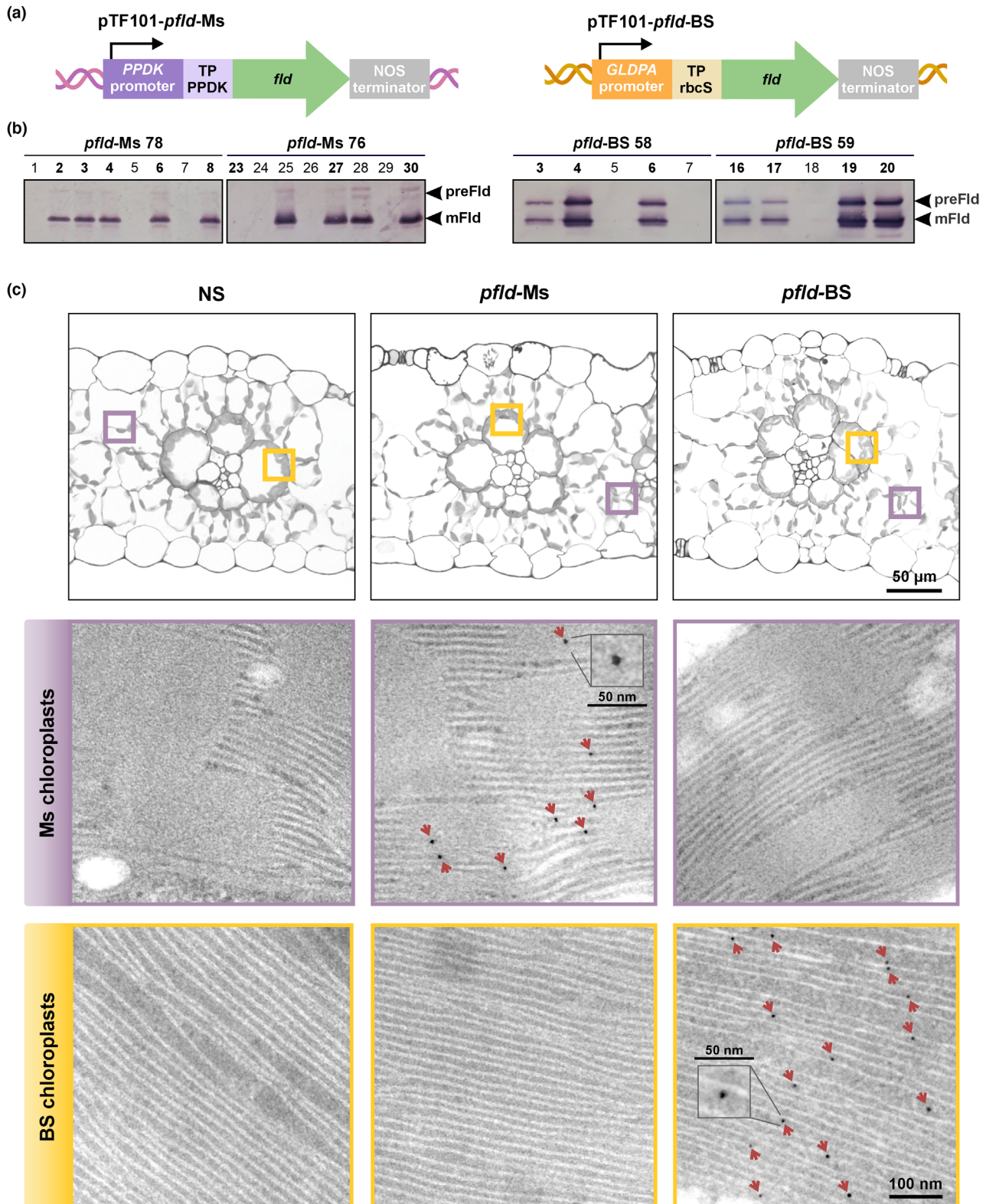


Fig. 1 Expression of a chloroplast-targeted flavodoxin in maize mesophyll and bundle sheath cells. (a) Schematic diagram of the constructs used to transform *Zea mays* cv Hill(AxB) showing tissue-specific promoters and transit peptides (TP) that allow flavodoxin expression in mesophyll (Ms) and bundle sheath (BS) cells. Plasmids employed for transformation are further described in Supporting Information Fig. S1. GLDPA, glycine decarboxylase; NOS, nopaline synthase; PPKK, pyruvate-orthophosphate dikinase; *rbcS*, RubisCO small subunit. (b) Flavodoxin expressed from both constructs accumulated in leaves from T1 maize plants obtained after introgression of *pflid* primary transformants into the B73 background (Fig. S2; see also Methods S1). Cleared leaf extracts corresponding to 5 mg of fresh weight were resolved by 15% SDS-PAGE and assayed by immunoblotting with flavodoxin-specific antisera. Typical results obtained with different independent lines are shown. Arrowheads indicate the electrophoretic mobilities of the flavodoxin precursor (preFId) and mature protein (mFId). Lanes without flavodoxin detection represent null-segregating (NS) lines. The complete blot is shown in Fig. S3(a). (c) Histological and ultrastructural analysis of leaf cross-sections of NS, *pflid*-Ms and *pflid*-BS plants using optical and transmission electron microscopy (TEM). Upper panel, light microscopic images showing typical Kranz anatomy. Lower panel, immunolocalization of flavodoxin in the mesophyll and bundle sheath chloroplasts for NS, *pflid*-Ms and *pflid*-BS genotypes. Arrowheads in the TEM images indicate immunogold labeling, and magnified regions are shown in the insets.

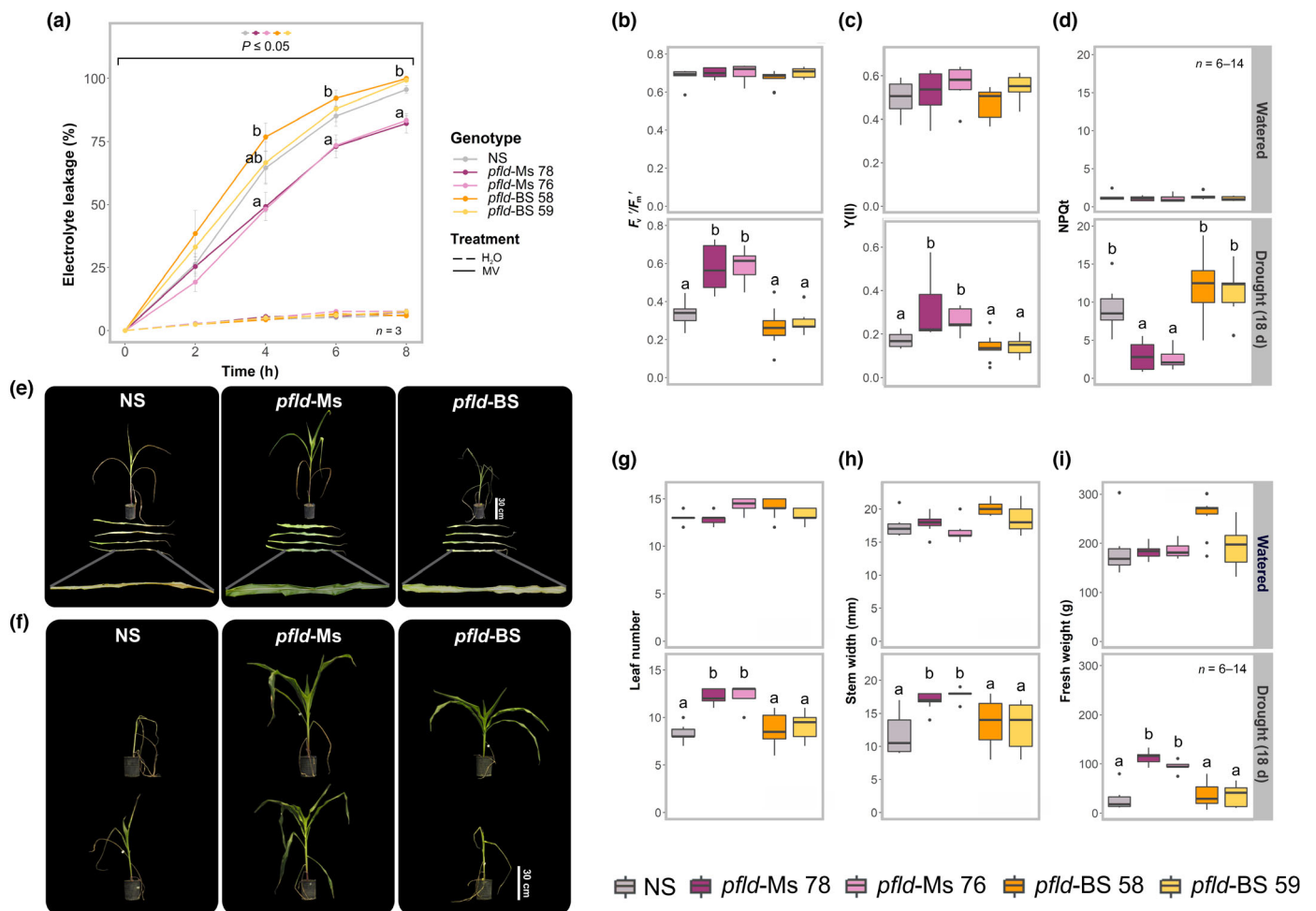


Fig. 2 Expression of chloroplast-targeted flavodoxin in maize mesophyll cells increased tolerance to oxidative and drought stress. (a) Leaf disks from null-segregant (NS), *pflid*-mesophyll (Ms) and *pflid*-bundle sheath (BS) plants were exposed to 25 μ M methyl viologen (MV) or kept in water, and illuminated for the times indicated. The conductance of the solution was determined and referred to the total ion contents measured after autoclaving the disks in the same solutions. Means \pm SE of eight independent plants per line are reported. (b–f) To assay drought stress, 4-wk-old maize T1 plants were watered regularly or water-deprived for up to 18 d. Photosynthetic parameters F_v/F_m (b), $Y(II)$ (c), and NPQ (d) were determined by chlorophyll fluorescence measurements in a MultispeQ V 2.0 system. Means \pm SE of 6–14 independent plants per line are reported. Time courses are shown in Supporting Information Fig. S6–S8. (e) Typical leaves from NS, *pflid*-Ms, and *pflid*-BS plants at the end of the drought treatment (18 d). (f) Typical NS, *pflid*-Ms, and *pflid*-BS plants at the end of the rehydration period (11 d). (g–i) *pflid*-Ms plants displayed better growth parameters after rehydration, as reflected by the number of leaves (g), stem width (h), and total fresh weight (i). Data shown are means \pm SE of n replicates, as indicated in the panels. Boxplots exhibit the median as a horizontal line and the whiskers both above and below depict the data range. Outliers are represented by the points outside the whiskers. Statistically significant differences were determined by one-way ANOVA and Tukey's test ($P \leq 0.05$) for comparison between lines. Additionally, Dunnett's test ($P \leq 0.05$) was employed to identify significant differences within each line over time relative to the initial stage.

and chloroplast localization was confirmed by immunogold labeling in cross sections of young leaves (Fig. 1c; see also Methods S1 and Table S2). Null-segregating (NS) plants were used as controls, after confirming the absence of the flavodoxin gene and protein by PCR and western assays, respectively. Independent lines *pflid*-Ms 76, *pflid*-Ms 78, *pflid*-BS 58 and *pflid*-BS 59, shown in Fig. 1 (b), were used for further experiments.

Tognetti *et al.* (2006) reported that flavodoxin targeting to tobacco chloroplasts increased tolerance to oxidative stress driven by the superoxide propagator methyl viologen (MV). To probe the response of maize *pflid*-Ms and *pflid*-BS lines to this oxidant, leaf disks excised from 4-wk-old plants were exposed to 25 μ M MV, and membrane lysis was monitored by electrolyte leakage. Lines expressing flavodoxin in BS cells were strongly affected by treatment with the herbicide, comparable with wild-type (WT) siblings, whereas *pflid*-Ms plants suffered less oxidative damage (Figs 2a, S4; Dataset S1), suggesting that flavodoxin presence in Ms chloroplasts conferred a significant degree of tolerance.

The drought tolerance of the various maize lines was assayed in 4-wk-old T1 plants by suppressing watering for 18 d (Fig. S5). Photosynthetic parameters such as F'_v/F'_m (reflecting the integrity of PSII) and Y(II) (measuring electron flow through PSII) declined steadily from the seventh day of water deprivation in all lines, but *pflid*-Ms plants exhibited significant tolerance compared with the other genotypes (Figs 2b,c, S6–S9; Datasets S2, S3). The nonphotochemical quenching (NPQt) parameter, which estimates the extent of energy dissipation by heat (and hence the excess of excitation energy on the PETC), displayed the opposite behavior, with only marginal increases in drought-exposed *pflid*-Ms plants (Figs 2d, S8, S9; Datasets S2, S3). Drought symptoms were visible in leaves of all genotypes at the end of the treatment, but wilting was significantly more severe in WT and *pflid*-BS lines (Fig. 2e).

Upon rehydration (Fig. S5), all *pflid*-Ms plants recovered leaf turgor and resumed growth, while most WT and *pflid*-BS siblings remained arrested and wilted (Fig. 2f). After 13 d of rehydration, this differential response was reflected by an increased number of leaves (Figs 2g, S10a; Dataset S4), wider and longer stems (Figs 2h, S10b,c; Dataset S4), and as a consequence, higher fresh weight in *pflid*-Ms plants (Fig. 2i; Dataset S5).

The use of tissue-specific transformation in maize has been sparse, especially for photosynthetic studies. Our results indicate that flavodoxin intervention provides better tolerance to oxidative and drought stress in maize, but only when targeted to Ms chloroplasts. These plastids perform LET, and the ability of flavodoxin to engage in this pathway as a ferredoxin analog is well-established in C_3 plants (Tognetti *et al.*, 2006). Flavodoxin accumulation in BS chloroplasts performing CET was instead inconsequential. It is possible that LET occurring in Ms plastids is the most stress-sensitive pathway of C_4 photosynthesis, as proposed by Liu *et al.* (2022), and thus the one that benefits more from the relief offered by flavodoxin to the excess of excitation energy. While these issues certainly deserve further investigation, our report identifies the type of intervention required to improve stress tolerance in maize plants using the flavodoxin approach, thus providing a more effective rationale to engineer this most important

crop for better growth and yield in suboptimal environments. Moreover, combination of tissue-specific expression with alternative electron shuttles such as flavodoxin provides a research tool to investigate the complex relationship between environmental hardships and C_4 photosynthesis.

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




Competing interests

None declared.

Author contributions

AFL, M-RH, MM, NC and RLC designed the research; MD, RCA, MC, JJPK and MM performed the experiments. All authors wrote and approved the final manuscript. MD and RCA contributed equally to this work.










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Data availability

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession nos S68006 for the flavodoxin gene, Z99767 for the *Flaveria trinervia* glycine decarboxylase gene and NM_001112268 for the maize

pyruvate-orthophosphate dikinase. The raw data that support the findings of this study are available in the [Supporting Information](#) of this article (Datasets S1–S5 include the raw data for Figs 1, 2, S1–S10).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Effect of methyl viologen on null-segregating and flavodoxin-expressing maize plants.

Dataset S2 Effect of drought on the photosynthetic activities of null-segregating and flavodoxin-expressing maize plants.

Dataset S3 Effect of water deprivation on the photosynthetic activities of null-segregating and flavodoxin-expressing maize plants.

Dataset S4 Effect of rehydration on growth of drought-stressed null-segregating and flavodoxin-expressing maize plants.

Dataset S5 Effect of rehydration on fresh weight increases of drought-stressed null-segregating and flavodoxin-expressing maize plants.

Fig. S1 Recombinant vectors used for expression of the flavodoxin gene in mesophyll or bundle sheath cells of maize T1 plants.

Fig. S2 Preparation of maize transgenic lines expressing a chloroplast-targeted flavodoxin in mesophyll (Ms) or bundle sheath cells.

Fig. S3 Expression of flavodoxin in leaves of maize *pfla*-mesophyll and *pfla*-bundle sheath transformants.

Fig. S4 Expression of chloroplast-directed flavodoxin in maize mesophyll cells, but not bundle sheath cells increases tolerance to methyl viologen (MV).

Fig. S5 Time line of the drought and rehydration treatments.

Fig. S6 Flavodoxin targeting to maize mesophyll chloroplasts protects photosystem II from drought stress.

Fig. S7 Flavodoxin targeting to maize mesophyll chloroplasts protects electron flow through photosystem II from drought stress.

Fig. S8 Flavodoxin targeting to maize mesophyll chloroplasts decreases nonphotochemical quenching under drought stress.

Fig. S9 Expression of a chloroplast-targeted flavodoxin in maize mesophyll cells protects photosynthesis from drought.

Fig. S10 Drought-stressed maize plants expressing a chloroplast-directed flavodoxin in mesophyll cells displayed better growth parameters after rehydration.

Methods S1 Detailed description of the experimental procedures used in this study.

Table S1 Primers used in this study.

Table S2 Protocol for microwave-assisted chemical fixation, dehydration and resin embedding of maize leaf cross sections.

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