Photosystem II Subunit S overexpression increases the efficiency of water use in a field-grown crop

Katarzyna Glowacka
University of Nebraska - Lincoln, kglowacka2@unl.edu

Johannes Kromdijk
University of Illinois at Urbana-Champaign

Katherine Kucera
University of Illinois at Urbana-Champaign

Jiayang Xie
University of Illinois at Urbana-Champaign

Amanda P. Cavanagh
University of Illinois at Urbana-Champaign

See next page for additional authors

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Authors
Katarzyna Glowacka, Johannes Kromdijk, Katherine Kucera, Jiayang Xie, Amanda P. Cavanagh, Lauriebeth Leonelli, Andrew D. B. Leakey, Donald R. Ort, Krishna K. Niyogi, and Stephen P. Long
Photosystem II Subunit S overexpression increases the efficiency of water use in a field-grown crop

Katarzyna Głowacka¹,², Johannes Kromdijk¹, Katherine Kucera¹, Jiayang Xie¹,³, Amanda P. Cavanagh¹, Lauriebeth Leonelli⁴, Andrew D.B. Leakey¹,³,⁵, Donald R. Ort¹,⁶, Krishna K. Niyogi⁴,⁷ & Stephen P. Long¹,⁸

Insufficient water availability for crop production is a mounting barrier to achieving the 70% increase in food production that will be needed by 2050. One solution is to develop crops that require less water per unit mass of production. Water vapor transpires from leaves through stomata, which also facilitate the influx of CO₂ during photosynthetic assimilation. Here, we hypothesize that Photosystem II Subunit S (PsbS) expression affects a chloroplast-derived signal for stomatal opening in response to light, which can be used to improve water-use efficiency. Transgenic tobacco plants with a range of PsbS expression, from undetectable to 3.7 times wild-type are generated. Plants with increased PsbS expression show less stomatal opening in response to light, resulting in a 25% reduction in water loss per CO₂ assimilated under field conditions. Since the role of PsbS is universal across higher plants, this manipulation should be effective across all crops.
Demand for primary foodstuffs, that is, grains and seeds of our major crops, may increase by 70–100% by 2050. One major barrier to meeting this large demand will be availability of water for crop production. Crop productivity strongly depends on having a sufficient supply of freshwater, and agriculture consumes 90% of total global freshwater. A large proportion of global food crops depend on irrigation, which is depleting global groundwater, and putting the sustainability of global food production at risk. To capture atmospheric CO₂ during photosynthesis, stomatal pores need to stay open to allow CO₂ diffusion into the leaf. However, stomatal opening causes most of the water absorbed by plant roots to be lost via transpiration. Transpiration is proportional to the water vapor pressure deficit (VPD) from leaf to air, which represents the gradient between the humidity in leaf internal airspaces and drier air surrounding the leaf. With the global rise in air and surface temperatures, VPD has been increasing, thus increasing demand for irrigation.

Because stomatal opening controls both the CO₂ influx and the water vapor efflux, stomata have to respond to many different cues to balance the fluxes. Progress has been made in unraveling the molecular basis of the response of stomata to intercellular CO₂ concentration and blue lights, but much less is known about stomatal response to light quantity. Stomatal opening in response to light is typically much less pronounced in detached epidermal layers, but can be restored when the connection with mesophyll cells is restored. Therefore, although some control resides in the guard cells, stomatal responses to light intensity seem to rely strongly on a signal derived from the underlying mesophyll tissue. The rate of photosynthetic CO₂ assimilation at high light intensity is usually limited by the restriction of CO₂ influx imposed by stomata. Thus, control of stomatal opening by a signal derived directly from photosynthesis could provide a feedback loop to match the light energy processed by the photosynthetic light-dependent reactions with sufficient supply of CO₂. However, several mutants deficient in specific components of the photosynthetic light-dependent or carbon reactions typically show vast decreases in the rate of CO₂ assimilation without corresponding changes in stomatal conductance. For example, in tobacco plants containing reduced amounts of cytochrome b₅₅₃ ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), glyceraldehyde 3-phosphate dehydrogenase, or sedoheptulose-bisphosphatase net assimilation rate was substantially reduced, but stomatal conductance often remained relatively unaltered compared to the wild-type (WT). These results show clearly that the stomatal opening signal does not scale directly with the rate of CO₂ uptake. However, the interpretation of these results is severely complicated by the strong decrease of net CO₂ assimilation rate associated with these transgenic alterations, which greatly increases the CO₂ concentration in the intercellular airspaces within the leaf (Cᵢ), providing a potent signal for stomatal closing.

A recent analysis suggested the redox state of chloroplastic quinone A (Qₐ) as an early signal for stomatal opening in response to light. with a more reduced Qₐ pool corresponding to increased stomatal opening. Qₐ is the primary electron acceptor downstream of photosystem II and its oxidation state reflects the balance between excitation energy at photosystem II and the rate of the Calvin–Bentz cycle. This predicts that decreasing the excitation pressure at photosystem II should directly affect stomatal opening in response to light by keeping Qₐ more oxidized. This prediction is tested here by altering expression of Photosystem II Subunit S (PsbS). PsbS expression directly affects the rate at which excitation energy absorbed by the antenna complex of photosystem II is used to reduce Qₐ, because of its role in non-photochemical quenching (NPQ). NPQ protects the photosynthetic machinery under excessive light conditions via controlled dissipation of absorbed light energy as heat. PsbS expression strongly stimulates NPQ and promotes photoprotection under high light or rapidly fluctuating conditions, but typically does not affect steady-state rates of net CO₂ assimilation, thus keeping control of stomatal movements via Cₐ relatively unaltered. Manipulation of PsbS expression thus provides an ideal test case to verify if Qₐ redox state is indeed an early signal for light-induced stomatal opening. Since PsbS stimulates the thermal dissipation of excitation energy, we predicted that increased expression of this protein would keep the redox state of Qₐ more oxidized, decrease stomatal opening in response to light, and decrease water loss at the leaf level. To test this hypothesis, Nicotiana tabacum lines with both increased and decreased PsbS expression were generated and analyzed under controlled and field conditions. N. tabacum cv “Petite Havana” was transformed with the coding sequence of N. benthamiana PsbS fused to the cauliflower mosaic virus 35S promoter for constitutive strong expression (Supplementary Fig. 1). Four independent, single-copy transformation events with increased NPQ amplitude (PSBS-28, PSBS-34, PSBS-43, and PSBS-46) were selected and selfed to obtain progeny homozygous for the transgene. Additionally, two events exhibiting spontaneous partial silencing of PsbS expression (psbs-4 and psbs-50) were selected for further analysis. Our results show that the light response of stomatal conductance is clearly affected by PsbS expression. Plants overexpressing PsbS show an average 25% reduction in water loss per CO₂ assimilated under field conditions.

Results

PsbS expression under controlled conditions. PSBS-28, PSBS-43, psbs-4, and WT N. tabacum plants were grown in a controlled-environment cabinet and PsbS transcript and protein levels were measured in samples from the youngest fully expanded leaves. PSBS-28 and PSBS-43 samples showed 4.2-fold and 3.5-fold increases in total (transgenic and native) PsbS transcript relative to WT (Fig. 1a), whereas transcript levels in psbs-4 were 10-fold less than WT. PsbS protein expression, normalized to the large subunit of the oxygen-evolving complex (PsbO) as a relative measure for the abundance of photosystem II, was 2.7-fold higher in PSBS-43 and 3.5-fold higher in PSBS-28 relative to WT while virtually absent in psbs-4 (Fig. 1b, c).

PsbS expression affects intrinsic water-use efficiency. Net CO₂ uptake (Aₜ) increased in response to light intensity until approximately 800 μmol m⁻² s⁻¹ and was not significantly affected by PsbS expression (P = 0.6, analysis of variance (ANOVA); Fig. 1d). The maximum capacity for carboxylation of ribulose-bisphosphate (Vₐₕₐₛₖₚ) and the maximum rate of whole-chain electron transport (Jₐₘₜₐₓ) showed weak positive trends with PsbS content (Supplementary Fig. 2a–d). Rubisco content was similar between lines (Supplementary Fig. 2e), but Rubisco activation state was slightly lower in psbs-4 (85%), compared to WT (95%), whereas the overexpressing lines were similar to wild-type (PSBS-28, 93%) or slightly higher (PSBS-43, 102%; Supplementary Fig. 2f). Stomatal limitation to net CO₂ assimilation also significantly differed with PsbS content (Supplementary Fig. 2g); however, the aforementioned changes in photosynthetic capacity and Rubisco biochemistry counteracted these differences, leaving Aₜ unchanged between all lines.

As expected, differences in PsbS protein expression led to pronounced differences in NPQ (Fig. 1e). At high light, NPQ was significantly higher in PSBS-28 and PSBS-43 relative to WT (P ≤ 0.05, Dunnett’s two-way test) and significantly lower in psbs-4 (P ≤ 0.02, Dunnett’s two-way test). In concert with these
differences, the redox state of QA was significantly more oxidized in PSBS-28 and PSBS-43 relative to WT (P ≤ 0.02, Dunnett’s two-way test; Fig. 1f) and more reduced in psbs-4 (P ≤ 0.002, Dunnett’s two-way test; Fig. 1f). These differences in QA redox state at high light were reflected in differences in stomatal conductance (gₛ) (Fig. 1g). The change in gₛ was consistent with altered regulation of stomatal opening rather than any changes in stomatal or epidermal anatomy, that is, pore dimensions or stomatal density. Stomatal density was 21% (abaxial) to 23% (adaxial) lower in psbs-4 and 18% (abaxial) lower in PSBS-43, relative to WT (P = 0.006 for abaxial and P = 0.02 for adaxial, ANOVA; Supplementary Fig. 3a), but unchanged in PSBS-28.

Stomatal pore dimensions on both abaxial and adaxial leaf surfaces were very similar between all lines (Supplementary Fig. 3b–d). In addition, all measurements of QA redox state and gₛ in all lines could be described by a single highly significant positive correlation (P < 0.0001, ANOVA; Fig. 1h), consistent with a role for QA redox state as an early determinant for stomatal opening in response to light intensity. Furthermore, the differences in gₛ associated with PsbS expression in combination with unchanged Aₐ resulted in a strong correlation between intrinsic water-use efficiency (Aₐ/gₛ, WUEi) and PsbS expression (R² = 0.92, P = 0.03, ANOVA; Fig. 1i).
WUE and productivity under field conditions. The differences in WUEi observed under controlled conditions were subsequently tested under field conditions. A field experiment was conducted with transformants with both increased (PSBS-28, PSBS-34, PSBS-43, and PSBS-46) and decreased PsbS expression (psbs-4 and psbs-50) in an incomplete block design (Supplementary Fig. 4). Western blotting confirmed differences in PsbS expression in the youngest fully expanded leaf for each genotype at 34, 37, 41, and 45 days after emergence (DAE) (Fig. 2a–h). As predicted, transformants showed a broad range of PsbS expression from almost none to 3.7-fold higher than WT, which were directly reflected in levels of NPQ measured on leaf discs (Fig. 2i). Critically, as under controlled conditions, net CO2 assimilation rate did not differ among the transformants and WT (Fig. 3a, Supplementary Fig. 5a), but g, correlated negatively to PsbS expression (P = 0.0001, ANOVA; Fig. 3b and Supplementary Fig. 5b). The reduction in g, due to PsbS overexpression varied between 4 and 30%, whereas g, was increased by 46% due to decreased PsbS expression (Fig. 3b). Once again, WUEi was significantly affected by genotype (P = 0.007, ANOVA; Fig. 3c and Supplementary Fig. 5d) and correlated positively with PsbS expression (R^2 = 0.94, P = 0.004, ANOVA; Fig. 3d). Increased PsbS expression resulted in 25–33% increased WUEi, whereas decreased PsbS expression led to 14% reduction in WUEi. Final size and dry weight were determined at the end of the field experiment. All biomass productivity traits were significantly affected by PsbS expression (P ≤ 0.008, ANOVA; Fig. 3e–g). Decreased PsbS expression significantly reduced dry weight (22%, P ≤ 0.002, Dunnett’s two-way test; Fig. 3e, Supplementary Fig. 6), leaf area (15%, significant only in psbs-50, P = 0.03, Dunnett’s two-way test; Fig. 3f) and plant height (15%, P < 0.0001, Dunnett’s two-way test; Fig. 3g). The productivity measures in transformants with increased PsbS expression did not show a consistent response. PSBS-28 showed significant decreases in dry weight (−18%, P = 0.008, Dunnett’s two-way test; Fig. 3e), leaf area (−19%, P = 0.005, Dunnett’s two-way test; Fig. 3f), and plant height was also significantly smaller in PSBS-28 and PSBS-34 (−9 and −8%, P ≤ 0.01, Dunnett’s two-way test; Fig. 3g), whereas the same productivity measures were not significantly affected in PSBS-43 and PSBS-46, relative to WT.

Discussion

Our results provide direct proof, through genetic manipulation, that increasing PsbS expression suppresses stomatal opening with little effect on CO2 uptake and so increases WUE. We showed a strong dependence of g, on PsbS expression (Figs. 1g, 3b) and that overexpression of PsbS significantly improved WUEi (Fig. 3c), representing a strong decrease (averaging 25%) in the amount of water used for each molecule of CO2 assimilated at leaf level by an irrigated field crop. Novel bioengineering strategies to improve crop WUE such as exemplified here are urgently needed, especially considering the long timelines for developing new crop varieties. Although this test of concept was performed on tobacco, the role of PsbS in NPQ is universal across higher plants, so this manipulation can be expected to be effective across all crops.

Here a large improvement of leaf level WUEi is shown (Figs. 1i, 3c), which can be expected to conserve soil moisture and may result in increased productivity if the crop becomes water-limited. However, many feedbacks could lessen this improvement at the whole crop level. Open-air elevation of CO2 has provided a direct test of the significance of such feedbacks. When stomatal conductance in a mature soybean canopy was reduced by 10% due to elevated CO2, this resulted in increased productivity if the crop becomes water-limited. However, many feedbacks could lessen this improvement at the whole crop level. Although this test of concept was performed on tobacco, the role of PsbS in NPQ is universal across higher plants, so this manipulation can be expected to be effective across all crops.
overexpression was not advantageous for biomass productivity, but instead led to slight decreases in final plant size and dry weight (Fig. 3e–g and Supplementary Fig. 6). We hypothesize that the increase in PsbS and associated increased levels of NPQ may have adversely affected the light-use efficiency of CO₂ assimilation under fluctuating light as previously shown in rice [32]. We have previously demonstrated that this efficiency is an important determinant of biomass productivity of tobacco under similar field conditions [31] and therefore may explain these findings.

Since gₛ directly affects the supply of CO₂ to photosynthesis, decreases in gₛ often result in decreased Aₛ [32]. Interestingly, the effects of PsbS on stomatal conductance did not translate into differences in Aₛ, even though CO₂ supply to photosynthesis was slightly affected (Supplementary Fig. 2g). Instead, maximum RuBP carboxylation capacity (Vₘₐₓ) and the maximal rate of linear electron transport (Iₘₐₓ) showed weak positive relationships with the amount of PsbS (Supplementary Fig. 2c, d), consistent with previous findings in rice with altered PsbS levels [30] and Rubisco activation state also showed a weak positive trend with PsbS content (Supplementary Fig. 2f). These results indicate that plants may be able to compensate for the effects of a decrease in stomatal conductance on CO₂ uptake by increasing photosynthetic capacity, thereby limiting the negative feedback on biomass productivity.

**Methods**

**Plant material.** WT N. tabacum cv. “Petit Havana” seeds carrying TMV resistance (NN) were a gift from Professor Spencer Whitney. Lines exhibiting increased or reduced expression of PsbS were generated within this study as described below.

**Recombinant DNA and transformation.** The N. benthamiana PsbS gene coding sequence (www.uniprot.org, Q2LAHO_NICBE) was cloned in between the cauliflower mosaic virus 35S and octopine synthase terminator in the pEARLYGATE binary vector. The resulting binary vector pEG100-NpPsbS conferred microbrial resistance to kanamycin and bialaphos resistance in plants (Supplementary Fig. 1). Nicotiana tabacum cv. “Petite Havana” was transformed with pEG100-NpPsbS using the Agrobacterium tumefaciens-mediated protocol [33]. Copy number and homozogosity were assessed using digital droplet PCR [34]. Results shown are for homozygous offspring unless otherwise described.

**Transcription and protein expression.** Five leaf discs (total 2.9 cm²) were from the youngest fully expanded leaf of five plants per genotype (controlled conditions) or four plants per genotype (field). Samples were taken 2 h after the start of the photoperiod. Protein and mRNA were extracted from the same leaf sample (NucleoSpin RNA/Protein kit, REFl704933, Macherey-Nagel GmbH & Co., Düren, Germany). Extracted mRNA was treated by Dnase (Turbo DNA-free kit; AM1907, Life Technologies, Carlsbad, CA, USA). Quantitative reverse transcription PCR was used to quantify PsbS transcripts (5′-GGCACAGCTGTAATCTTGAAAC-3’ and 5′-CAGGGACAGGGTCATCAATAAA-3′) relative to Actin (5′-CTTCTCCCTTTAATC-3′ and 5′-ACACGCTGTAATGGGCGATAC-3′) and to determine PsbS expression levels relative to wild-type tobacco. Plant material was transformed with pEG100-NbPsbS mediated protocol [33]. Copy number and homozogosity were assessed using digital droplet PCR [34]. Results shown are for homozygous offspring unless otherwise described.

**Total protein concentration was quantified using a protein quantification assay (ref. 74067.50, Macherey-Nagel GmbH & Co., Düren, Germany).** Samples containing 1 µg total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis electrophoresis, blotted to membrane (Immobilon-P, IPVH00100, Millipore, Billerica, MA, USA) and semi-dry blotting (Trans-Blot SD, Bio-Rad, Hercules, CA, USA), and sequentially immuno-labeled with primary antibodies raised against AtPsbO (1:2,000 dilution, AT0S533, Agrisera, Vännäs, Sweden) and AtPsbO (1:20,000 dilution, AT0S6142-33, Agrisera, Vännäs, Sweden) followed by incubation with secondary antibodies (1:2,500 dilution; W401B, Promega, Madison, WI, USA). The sequential use of the two primary antibodies was verified empirically against blots where only one antibody was used and dilution series were used to establish the quantifiable range. Chemiluminescence was detected using a scanner (ImageQuant LAS-4010, GE Healthcare Life Sciences, Pittsburgh, PA, USA). A protein ladder (Precision Plus Protein Standards, #1610375, Bio-Rad, Hercules, Healthcare Life Sciences, Pittsburgh, PA, USA) (Data Set 2 and 11) in a gel was used to determine the size of each band. Protein bands were quantified using densitometry with ImageQuant TL software (version 7.6, GE Healthcare Life Sciences, Pittsburgh, PA, USA, Data Repository: https://data.mendeley.com/datasets/nbipw9kkgdraft/?view=10508d31-685a-4a62-89fe-cb591c569e97). PsbS expression was normalized based on PsbO bands.
Photosynthetic gas exchange under controlled conditions. Seedlings of psbs-4, PSBS-28, and WT were germinated on growing medium (LC1 Sunshine mix, Sun Gro Horticulture, Agawam, MA, USA) in a controlled-environment walk-in growing chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA) with photoperiod set to 12 h and temperature controlled at 23/18 °C (day/night). Five days after germination, psbs-4 seedlings with low NPQ were identified through chlorophyll fluorescence imaging and together with PSBS-28, PSBS-43, and WT seedlings transplanted to 3.8-L pots and randomly positioned in a controlled-environment chamber (PGC20, Conviron, Winnipeg, MB, Canada) with photoperiod set to 16 h and air temperature controlled at 20/25 °C (night/day). Light intensity at leaf-level was controlled at 500 µmol m⁻² s⁻¹. Plants were watered and plant positions were repositioned at random every 2 days until the fifth leaf was fully expanded. Gas exchange measurements were performed using an open gas exchange system (LI6400XT, LI-COR, Lincoln, NE, USA) equipped with a 2-cm² leaf chamber and integrated modulated fluorometer. All chlorophyll fluorescence measurements were performed using the multiphase flash routine36. To determine the light response of AN, whole-chain photosynthetic electron transport, gas exchange and pulse amplitude-modulated chlorophyll fluorescence were measured at a range of light intensities. Block temperature was controlled at 25 °C, [CO₂] inside the cuvette was maintained at 380 µmol mol⁻¹ and leaf-to-air water VPD was controlled to 1.1–1.4 kPa. Leaves were clamped in the leaf cuvette and dark-adapted for 1 h, after which minimal (Fᵣ) and maximal fluorescence (Fₘ) were measured to determine maximal efficiency of whole-chain electron transport36 (F/Fₘ, Eq 1).

\[
Fᵣ/Fₘ = (Fₘ - Fᵣ)/Fₘ.
\]  

Subsequently, light intensity (100% red LEDs, λₑₘₙₙ = 630 nm) was slowly increased from 0 to 50, 80, 110, 140, 170, 200, 300, 400, 500, 600, 800, 1,000, 1,500, and 2,000 µmol m⁻² s⁻¹. When steady state was reached, AN, gₛ, and Cᵣ were logged, and F and Fₘ were measured to estimate the operating efficiency of whole-chain electron transport36 (F/Fₘ, Eq 2). Since stomatal movements can include very long-term diurnal components37,38, our routine was aimed at measuring only relatively short-term stomatal responses to changes in light intensity, and steady-state waiting times were kept between 10 and 20 min per step. NPQ of chlorophyll fluorescence was determined according to the variable NPQ (Fₘ/Fₘ'), and leaf-to-air water VPD was corrected to measurements. Chlorophyll fluorescence parameter qL (Eq. 4) was used to estimate the fraction of NPQ in its oxidized state (and correspondingly, Qₐ redox state as 1−qL). The derivation of this parameter is assuming a “lake” model for photosynthetic antenna complexes (i.e., antennae are shared between reaction centers)39.

\[
qL = (Fₘ/Fₘ') - 1.
\]

To evaluate the CO₂ response of AN, leaves were allowed to reach steady state at a light intensity of 2,000 µmol m⁻² s⁻¹ (100% red LEDs, λₑₘₙₙ = 630 nm), with block temperature controlled at 25 °C and [CO₂] in the airstream set to 400 µmol mol⁻¹. Subsequently, [CO₂] was varied from 400 to 300, 200, 100, 75, 400, 400, 500, 600, 700, 800, 1,000, 1,200, and 1,500 µmol mol⁻¹. When steady state was attained, AN, gₛ, and Cᵣ were logged. Vₘₚₙₖₚₐₜₜₚₐₜₜ was determined from the response of AN to chloroplastic CO₂ concentration (Cₐ) by fitting a biochemical model41 with temperature corrections42 to measurements. Cᵣ required an estimate of mesophyll conductance to CO₂ transfer (gₚₐₜₜₚₐₜₜ), which was estimated independently for each point in the CO₂ response curve from parallel chlorophyll fluorescence measurements according to the variable f method41. Fₘₚₙₖₚₐₜₜₚₐₜₜ was determined by fitting a non-rectangular hyperbola to light response curves of linear electron transport estimated from chlorophyll fluorescence44. Stomatal limitation of AN was computed...
using measurements at ambient CO$_2$ ($C_a = 380$ μmol mol$^{-1}$) and saturating light intensity, and predicted values of $A_m$ when stomata are not limiting (i.e., $C_i$ would equal $C_a$) [43].

Rubisco activation state and content. Plants were grown under controlled conditions as described above. Youngest fully expanded leaves were clamped in the cuvette of an open gas exchange system (LI6400XT with 2 × 3 LED light source), with light intensity set to 1800 μmol m$^{-2}$ s$^{-1}$, CO$_2$ concentration set to 400 μmol mol$^{-1}$, and block temperature set to 25 °C. After steady-state gas exchange was reached, leaves were rapidly removed and a disc of 0.55 cm$^2$ from the center of the portion of the leaf that had been enclosed in the cuvette was snap frozen in liquid N. Rubisco activity was determined by the incorporation of 14CO$_2$ into acid-stable products at 25 °C following an existing protocol [45]. Samples were ground in ten-fold glass homogenizers with ~2 ml cm$^{-2}$ CO$_2$-free extraction buffer containing 100 mM NaClO$_4$, 15 mM Na$_2$CO$_3$, 2 mM Na$_2$EDTA, 20 mM MgCl$_2$, 5 mM dithiothreitol (DTT), 5 mg ml$^{-1}$ polyvinyl pyrrolidone, 15 mM amino-$n$-caproic acid and 3.5 mM benzamidine, and 5% v/v protease inhibitor cocktail (P9599, Sigma, St. Louis, MO, USA). Within 30 s of extraction, samples were assayed for initial Rubisco activity in a buffer containing 100 mM Bicine-NaOH (pH 9.5), 2 mM Na$_2$EDTA, 20 mM MgCl$_2$, 1 μmol ribulose-1,5-bisphosphate, and 12.8 mM NaH$_4$CO$_3$ (15 Bq nmol$^{-1}$, Vitrax, Placentia, CA, USA). Assays were run for 30 s and with the addition of 300 μL 5 N formic acid. The radioactivity of acid-stable products was determined by liquid scintillation counting (Packard Tri-Carb 1900 TR, Canberra Packard Instrument Co., Downers Grove, IL, USA). After determining initial activity, the extract was incubated with 10 mM NaHCO$_3$ and 20 mM MgCl$_2$ for 20 min at room temperature, and the total activity of the extract was assayed as above. Unless stated otherwise, all other reagents were purchased from Sigma (St. Louis, MO, USA).

Purified RubBP was used in both initial and total activity assays to avoid underestimation of the activity of Rubisco [46]. The activation state of Rubisco is determined by the ratio of initial to total activity. Rubisco content was determined from carbylated samples extracted as above using a [14C]carboxy-arabinitol bisphosphate-binding assay [47] with a specific activity of 583 Bq nmol$^{-1}$ [48], Rubisco, assuming eight binding sites per Rubisco [5].

Stomatal density and stomatal complex dimensions. Plants were grown under controlled conditions as described above. Fresh leaf samples were taken from the youngest fully expanded leaf and mounted onto a microscope slide using double-sided tape. The adaxial and abaxial leaf surfaces were measured using a leaf scanner (Topcon, Tokyo, Japan) using an objective of ×40 and a ×100 magnification. Measurement set was included as a random effect in analysis of variance was applied to transcription levels, protein expression, gas exchange data, Rubisco content and activation state, stomatal density, and dimension data. All statistical analysis was performed with SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). Data were tested with the Brown-Forsythe test for homogeneity of variance and the Shapiro–Wilk test for normality. One-way analysis of variance was applied to transcription levels, protein expression, gas exchange data, Rubisco content and activation state, stomatal density, and dimension data. Measurement set was included as a random effect in analysis of the

Field experimental design. Seedlings were transplanted to an experimental field site at the University of Illinois Energy Farm (40.11’N, 88.21’W) on June 6, 2016. The field was prepared 2 weeks prior to transplant by rototilling, disk sampling, and harrowing. At this time, chlorpyrifos (1.5 g m$^{-2}$ Loridan 15 G Insecticide, Dow AgroSciences Canada Inc., Calgary, AB, Canada) was worked into the soil to suppress cutworm damage, sulferzone (29 μl m$^{-2}$ Spartan 4F preemergence herbicide, FMC Agricultural Solutions, Philadelphia, PA, USA) was broadcast to reduce the emergence of weeds and slow-release fertilizer (30 g m$^{-2}$ ESN Smart Nitrogen, Agrium US Inc., Denver, CO, USA) was put down. After transplant, all seedlings were sprayed with thiometamol (7 mg/plant Platinum 75 MG insecticide, Syngenta Crop Protection LLC, Greensboro, NC, USA) to prevent damage from insect herbivory, and 12 days after the field transplant, all plants were sprayed with fermentation solids and solubles from Bacillus thuringiensis, subsp. kurstaki, strain ABTS-351 (2.6 M L$^{-1}$ DiPel Pro dry flowable biological insecticide, Valent Biosciences Corp.) to suppress tobacco hornworm. The field experiment was set up as an incomplete randomized block design with 12 blocks of 6 × 6 plants spaced 30 cm apart (Supplementary Fig. 4). Each block contained four replicates of four plants per genotype in north–south (N–S) or impaction, surrounded by one border row of WT. WT was present in all blocks (n = 12), whereas the four PSBs overexpression and two psb knock-down lines were randomly assigned to six blocks (n = 6). The blocks were positioned in a 3 (N–S) × 4 (E–W) rectangle with 75 cm spacing between blocks. The entire experiment was surrounded by two border rows of WT.

Light intensity (LI-190QR quantum sensor, LI-COR, Lincoln, NE, USA) and air temperature (Model 109 temperature probe, CampbellScientific, USA) were measured nearby on the same field site and half-hourly averages were logged using a datalogger (CR1000, Campbell Scientific, USA). Precipitation was measured at two sites close to the field using precipitation gauges (NOAA IV Precipitation Gauge, ETI Instrument Systems Inc., Fort Collins, CO, USA) (Supplementary Fig. 7). Watering to restore field capacity was provided daily when needed through parallel drip irrigation lines with emitters every 30 cm (17 mm PC Drip Line #DL777, The Drop Store, Vista, CA, USA) spanning the whole experiment in E–W direction and spaced 30 cm apart. After watering and precipitation events, two trenches with a depth of approximately 10 cm were dug in N–S direction between the blocks and connected on the south side of the experiment to a 15 cm deep E–W trench. Photosynthesis measurements were performed on the youngest fully expanded leaf 22 days after transplanting. Plants were harvested on July 7, 2016. After final harvest, stem length and the number of leaves were determined, and leaf area was measured with a conveyor-belt scanner (LI-3100C Area meter, LI-COR, Lincoln NE, USA). Leaf, stem, and root fractions were dried to constant weight at 60 °C in a custom-built drying oven equipped with condenser to further dry the recirculated air, after which the dry weights were determined.

Non-photochemical quenching in field-grown plants. Leaf discs were sampled pre-dawn from field-grown plants of pbs-4, pbs-5, PSBS-28, PSBS-34, PSBS-43, PSBS-46, and WT control and stored in darkness in glass vials for up to 4 h until measurement. Humidity in the vials was maintained fully saturated by placing a piece of wet filter paper in each vial. Dark-adapted leaf discs were positioned on a piece of wet filter paper in a chlorophyll fluorescence imager (CIFinder, Techmetrics, Cambridge, UK) to optimize maximal fluorescence. After dark adaptation, leaf discs were exposed to 15 min of 1000 μmol m$^{-2}$ s$^{-1}$, after which maximal fluorescence without dark adaptation was determined ($F_o$). NPQ was then determined according to Eq. 3.

Photosynthetic gas exchange in field. The response of photosynthetic gas exchange to light intensity was measured on the youngest fully expanded leaf of four plants of pbs-4, PSBS-28, PSBS-34, PSBS-43, and WT control in the N–S direction between the blocks and connected on the south side of the experiment to a 15 cm deep E–W trench. Photosynthesis measurements were performed on the youngest fully expanded leaf 22 days after transplanting. Leaves were logged. After gas exchange measurements were performed, leaf absorbance was determined using an integrating sphere (LI1800, LI-COR, Lincoln, NE, USA) connected to a spectrometer (USB-2000, Ocean Optics Inc., Dunedin, FL, USA).

Statistical analysis. All statistical analysis was performed with SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). Data were tested with the Brown-Forsythe test for homogeneity of variance and the Shapiro–Wilk test for normality. One-way analysis of variance was applied to transcription levels, protein expression, gas exchange data, Rubisco content and activation state, stomatal density, and dimension data. Measurement set was included as a random effect in analysis of the
field gas exchange data to account for variation caused by N−S plant position and time of day. Biomass, leaf area, and plant height data were analyzed with a linear mixed model accounting for block and genotype effects with Welch–Satterthwaite adjustment of degrees of freedom to account for the different replication rate of WT (PROC MIXED). Significant genotype effects in ANOVA (α = 0.05) were followed by testing of genotype means against WT control (α = 0.05), using Dunnett’s multiple comparison correction. Correlations between Qa, redox state with k, and protein levels and with ΔA2/ΔP were evaluated using Pearson’s correlation coefficient.

Data availability. All relevant data and plant materials are available from the authors upon request. Raw data corresponding to the figures and results described in this manuscript have been deposited at: https://data.mendeley.com/datasets/nshpopkgk/draft/a10309d531-68b4-a662-809e-cb591c569e97.

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References
1. Long, S.P., Marshall-Colon, A. & Zhu, X.G. Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. Cell 161, 56–66 (2015).
2. Tilman, D., Balzer, C., Hill, J. & Befort, B. L. Global food demand and the sustainable intensification of agriculture. Proc. Natl. Acad. Sci. USA 108, 20260–20264 (2011).
3. Scanlon, B. R. et al. Groundwater depletion and sustainability of irrigation in the US High Plains and Central Valley. Proc. Natl. Acad. Sci. USA 109, 9320–9325 (2012).
4. Dalin, C., Wada, Y., Kastner, T. & Puma, M. J. Groundwater depletion embedded in international food trade. Nat. Commun. 543, 700–704 (2017).
5. Lobell, D. B. et al. Greater sensitivity to drought accompanies maize yield increase in the U.S. Midwest. Science 344, 516–519 (2014).
6. Ort, D. R. & Long, S. P. Limits on yields in the corn belt. Science 344, 484–485 (2014).
7. Lawson, T., Simkin, A. J., Kelly, G. & Granot, D. Mesophyll photosynthesis and guard cell metabolism impacts on stomatal behaviour. New Phytol. 203, 1064–1081 (2014).
8. Hu, H. et al. Carbonic anhydrases are upstream regulators in guard cells of CO2-controlled stomatal movements. Nat. Cell Biol. 12, 87–910 (2011).
9. Shimazaki, K.-I, Doi, M., Assmann, S. M. & Kinoshita, T. Light regulation of stomatal movement. Annu. Rev. Plant Biol. 58, 219–247 (2007).
10. Lee, J.-S. & Bowling, D. J. F. Influence of the mesophyll on stomatal opening. Aust. J. Plant Physiol. 22, 357–363 (1995).
11. Lawson, T. Guard cell photosynthesis and stomatal function. New Phytol. 181, 13–34 (2009).
12. Mott, E. A. Guard cell photosynthesis and stomatal function. Plant Cell Environ. 32, 1479–1486 (2009).
13. Baroli, I., Price, G.D., Badger, M.R. & von Caemmerer, S. The contribution of photosynthesis to the red light response of stomatal conductance. Plant Physiol. 146, 737–747 (2008).
14. von Caemmerer, S. et al. Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. J. Exp. Bot. 55, 1157–1166 (2004).
15. Price, G. D., Evans, J. R., von Caemmerer, S., Yu, J.-W. & Badger, M. R. Specific reduction of chloroplast glyceraldehyde-3-phosphate dehydrogenase activity by antisense RNA reduces CO2 assimilation via a reduction in ribulose bisphosphate regeneration in transgenic tobacco plants. Planta 195, 369–378 (1995).
16. Lawson, T., Lefebvre, S., Baker, N. R., Morison, J. I. L. & Raines, C. A. Reductions in mesophyll and guard cell photosynthesis impact on the control of stomatal responses to light and CO2. J. Exp. Bot. 59, 3609–3619 (2008).
17. Busch, F. A. Opinion: the red-light response of stomatal movement is sensed by the redox state of the photosynthetic electron transport chain. Photosynth. Res. 119, 131–140 (2014).
18. Müller, P., Li, X.-P. & Niyogi, K. K. Non-photochemical quenching. A response to excess light energy. Plant Physiol. 125, 1558–1566 (2001).
19. Ruban, A. V. Non-photochemical chlorophyll fluorescence quenching: mechanism and effectiveness in protecting plants from photodamage. Plant Physiol. 170, 1903–1916 (2016).
20. Li, X.-P., Müller-Moule, P., Gilmore, A. M. & Niyogi, K. K. Pbs-dependent enhancement of feedback de-excitation protects photosystem II from photoinhibition. Proc. Natl. Acad. Sci. USA 99, 15222–15227 (2002).
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Author contributions
K.G., J.K., K.K.N., and S.P.L designed experiments. L.L. prepared plasmid. K.G. and J.K. generated transgenic tobacco lines. K.G., K.K., and J.K. performed experiments. A.P.C. and D.R.O. measured Rubisco content and activation state. J.X. and A.D.B.L. measured stomatal density and dimensions of stomatal complex. K.G. and J.K. analyzed experiments. K.G., J.K., L.L., K.K.N., and S.P.L. wrote the manuscript.

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