Planta (2017) 245:1009–1020
DOI 10.1007/s00425-017-2659-y

ORIGINAL ARTICLE

Photosynthetic antenna engineering to improve crop yields

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Received: 9 January 2017 / Accepted: 3 February 2017 / Published online: 10 February 2017

Abstract

Main conclusion **Evidence shows that decreasing the light-harvesting antenna size of the photosystems in tobacco helps to increase the photosynthetic productivity and plant canopy biomass accumulation under high-density cultivation conditions.**

Decreasing, or truncating, the chlorophyll antenna size of the photosystems can theoretically improve photosynthetic solar energy conversion efficiency and productivity in mass cultures of algae or plants by up to threefold. A Truncated Light-harvesting chlorophyll Antenna size (TLA), in all classes of photosynthetic organisms, would help to alleviate excess absorption of sunlight and the ensuing wasteful non-photochemical dissipation of excitation energy. Thus, solar-to-biomass energy conversion efficiency and photosynthetic productivity in high-density cultures can be increased. Applicability of the TLA concept was previously shown in green microalgae and cyanobacteria, but it has not yet been demonstrated in crop plants. In this work, the TLA concept was applied in high-density tobacco canopies. The work showed a 25% improvement in stem and leaf biomass accumulation for the TLA tobacco canopies over that measured with their wild-type counterparts grown under the same ambient conditions. Distinct canopy appearance differences are described between the TLA and wild type tobacco plants. Findings are discussed in terms of concept application to crop plants, leading to significant improvements in agronomy, agricultural productivity, and application of photosynthesis for the generation of commodity products in crop leaves.

Keywords Chlorophyll-deficient mutant · Canopy density · Light-harvesting antenna size · *Nicotiana tabacum* · Productivity · TLA technology

Abbreviations

Car	Carotenoids
PS	Photosystem
TLA	Truncated light-harvesting antenna

Introduction

Photosynthetic organisms, including bacteria, algae, and plants, have evolved extensive arrays of light-harvesting pigments, comprising chlorophylls (Chl), carotenoids (Car), and bilins that absorb sunlight and transfer the excitation energy to photochemical reaction centers. The latter convert the absorbed irradiance to chemical energy via the photochemical charge separation reaction, which is viewed as the beginning step of photosynthesis. The evolution of sizable arrays of light-harvesting antennae in all photosynthetic systems confers a selective survival advantage for the organism in nature, where sunlight intensity is often the growth-limiting parameter. Successful competition in nature requires capturing more sunlight for self, even if wasted, and preventing light capture by competing neighbors (Melis 2009). Consequently, top layers of

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plant canopies and upper layers of microalgae in high-density liquid cultures absorb sunlight far in excess of what is needed to saturate photosynthesis (Nakajima and Ueda 1997; Melis et al. 1999; Polle et al. 2003; Ort et al. 2011). Excess absorbed irradiance is dissipated in an orderly manner by the photosystems via non-photochemical quenching (NPQ) mechanisms, which evolved to protect the photosynthetic apparatus and prevent photosensitized bleaching (Müller et al. 2001; Ruban 2016).

In organisms of oxygenic photosynthesis, large arrays of light-harvesting pigment-protein complexes are assembled as peripheral components of photosystem I (PSI) and photosystem II (PSII) (Masuda et al. 2002). Minimizing, or truncating, the chlorophyll (Chl) antenna size of the photosystems would limit excess-absorption of sunlight and can, therefore, improve photosynthetic solar energy conversion efficiency and productivity in high density foliage or microalgal cultures (Melis 2009). The rationale in plants is that individual chloroplasts and photosystems with a smaller chlorophyll antenna size in the upper canopy leaves would have a diminished probability of absorbing sunlight, thereby permitting greater penetration and a more uniform distribution and utilization of irradiance throughout the foliage of the high-density crop plant. Such altered optical properties are expected to alleviate over-absorption and wasteful dissipation of sunlight by the upper canopy and enhance photosynthetic productivity of the foliage as a whole. The **T**runcated **L**ight-harvesting **A**ntenna (TLA) concept, referring to a smaller than wild type chlorophyll antenna size of the photosystems, has found application and noteworthy success in the case of high-density cultivation of microalgae (Nakajima and Ueda 1997, 1999; Melis et al. 1999; Polle et al. 2003; Nakajima et al. 2001; Mussgnug et al. 2007) and cyanobacteria (Kirst et al. 2014), but has not yet been applied to or demonstrated with crop plant canopies.

Nicotiana tabacum (tobacco) as a platform for foliar production of commodity and biopharmaceutical products offers advantages including a large leaf surface area, and a high leaf-to-stem ratio. It can be coppiced to generate multiple harvests per year (Andrianov et al. 2010). Large-scale agricultural infrastructure for planting, growing, harvesting and handling tobacco leaves is in place, and application of this crop avoids the potential conflict of food versus fuel. Moreover, tobacco farmers would benefit by using tobacco as a commodity crop plant. However, important for the above considerations is the foliar biomass productivity, improvement of which is the focal point of this investigation.

In the present work, the TLA concept was applied to high-density tobacco canopies. We show significant biomass accumulation improvements in TLA tobacco canopies over that measured in wild-type counterparts grown under the same agronomic and ambient conditions. Distinct canopy and plant anatomical appearance differences are also described between the TLA and wild-type tobacco plants.

Materials and methods

Plant material

Nicotiana tabacum, cv John William's Broadleaf, and the yellow-green mutant *Su/su* (Homann and Schmid 1967; Okabe et al. 1977) were grown in the greenhouse under ambient sunlight conditions. Seeds were kindly provided by Dr. Georg H. Schmid, University of Bielefeld, Germany. Prior analysis has shown these yellow-green *Su/su* mutant to have a substantially smaller than wild type light-harvesting antenna size (Melis and Thielen 1980; Thielen and van Gorkom 1981). Hence, the yellow-green *Su/su* mutant is referred to as TLA tobacco in this work.

Pigment determination and leaf protein analysis

The chlorophyll and carotenoid concentration of leaves was determined spectrophotometrically in 80% acetone (Arnon 1949) or 100% methanol extracts (Lichtenthaler 1987) of tobacco leaves. Protein analyses were conducted with solubilized leaf extracts resolved in precast SDS-PAGE gels (BIO-RAD). Loading of samples was based on chlorophyll content.

Chloroplast and thylakoid membrane isolation

For the analyses reported in this work, three leaves were sampled from the mid-lower to the mid-upper point of the plant. Three independent measurements were conducted with these samples. This was repeated at different times with samples from three different plants. Leaves were homogenized in ice-cold chloroplast isolation buffer containing 0.4 M sucrose, 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone 40, 1% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 µM phenylmethylsulfonyl fluoride (PMSF). The suspension was filtered to separate unbroken leaf pieces from the cell lysate. Chloroplasts were pelleted by centrifugation at 5000*g* for 10 min and washed twice with chilled chloroplast isolation buffer. For thylakoid membrane isolation, chloroplasts were lysed by re-suspension in a glass homogenizer in hypotonic buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone 40, 1% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 µM PMSF. Thylakoid membranes were then

pelleted by centrifugation at 75,000*g* for 45 min at 4 °C. Membranes were resuspended in 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ for spectrophotometric analyses.

Spectrophotometric and kinetic analyses

The light-*minus*-dark absorbance difference signal at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII (Melis and Brown 1980; Melis 1989) were measured with a laboratory constructed sensitive absorbance difference spectrophotometer. Photosystem quantification estimates were then based on the measurements of P700 and Q_A. The functional light-harvesting chlorophyll antenna size of PSI and PSII was estimated from the rate constant of P700 photo-oxidation and Q_A photoreduction, measured upon weak green actinic illumination of isolated and DCMU-poisoned thylakoid membranes (Melis 1989).

Measurement of photosynthesis

Photosynthetic gas exchange measurements were made using a portable open gas analysis device (LI-6400, Li-Cor Inc., Lincoln, NE, USA). Light response curves were measured on the youngest and second youngest fully expanded and attached leaves of at least 2 different plants from the same canopy. Leaf temperature and CO_2 concentration in the leaf chamber were 25 °C and 400 µmol mol^{-1} (ambient CO_2 concentration), respectively. The vapor pressure deficit was maintained below 1 kPa. The adaxial side of the leaf was illuminated by the light source (10% blue, 90% red). The starting light intensity was 1500 µmol photons m^{-2} s^{-1}, then lowered to 1200, 1000, 800, 600, 400, 200, 100, 50, and 0. Measurements at various light intensities were recorded after the rate of photosynthesis reached steady state (after about 10 min).

Canopy density biomass accumulation experiments

Heterozygous TLA (*Su*/*su*) *N. tabacum* seeds were sprinkled on soil in seed pots in the greenhouse nursery for germination. Wild type (dark green), TLA (light-green), and white/lethal phenotypes emanated from the heterozygous seeds. Viable seedlings were transferred to 4 × 4 inch peat pots (soil source: Sunshine Mix#1, Sun Gro Horticulture, McClellan Park, CA 95652, USA) for primary growth as individual plants (2–3 weeks). Development of the TLA plants upon seed germination (seedling stage) in the peat-pots was slower than that of the wild type due to the limiting light-intensity conditions prevailing in the nursery. Regardless, wild type and TLA plants were transferred, at the same time, into soil in 2-gallon pots having a 9-inch diameter. Experiments were conducted in 5 × 5 canopy pairs, with a wild type and TLA canopy growing during the same period of time, in the same greenhouse and under identical ambient conditions. Twenty-five (25) such wild type or TLA pots were placed adjacent to each other in a 5 × 5 configuration for growth and biomass production measurements, comprising a 1.3 m^2 (2025 $inch^2$) canopy. Such high-density canopies with plants separated from one another by 9-inches were allowed to develop until about half of the plants showed signs of bolting. Growth time in the canopy varied between 6 and 8 weeks, depending on the time of the year (canopy experiments were conducted from May through October) and the prevailing luminosity at the time of growth (duration of day time and sunlight intensity). The biomass of the entire canopy (all 25 plants) was then harvested, comprising the entire plant matter above the surface of the soil, and the fresh weight was recorded. Dry biomass weight (DW) was estimated by assuming a 12% wet-to-dry conversion factor.

Results

Pigmentation characteristics of wild type (WT) and the TLA tobacco

Leaves of the TLA tobacco displayed a distinct light-green coloration compared to the dark green wild type strain (Fig. 1, upper panel). Leaf cross section observations showed a higher density of cells in the wild type (Fig. 1, lower panel, WT) compared to that in the TLA mutant (Fig. 1, lower panel, TLA). Importantly, the latter showed more extensive intercellular air spaces in the spongy tissue, with a concomitantly lower density of spongy cells. Analysis of the pigment content showed significantly lower total chlorophyll per leaf area in the TLA leaves compared to the wild type. The total chlorophyll (Chl) content of wild type leaves was about 24.9 mg cm^{-2}, whereas the TLA strain contained only about 12.1 mg cm^{-2} (Table 1). The Chl *b* content of the TLA leaves was disproportionately lower relative to the lowering in Chl *a*. Measurements showed that Chl *a* content in the TLA leaves was lowered to 57% of the wild type, whereas Chl *b* content was lowered to about 23% of wild type. In consequence, the Chl *a*/Chl *b* ratio of the TLA was elevated to a ratio of 8:1, relative to the wild type that displayed a Chl *a*/Chl *b* ratio of about 3:1. The latter is typical for the fully developed photosynthetic apparatus in the leaves of green plants (Anderson 1986; Masuda et al. 2002), whereas a substantially greater Chl *a*/Chl *b* ratio of the TLA leaves indicates a truncated light-harvesting Chl antenna size (Melis 1991). The carotenoid (Car) content also differed between wild type and TLA tobacco. Total carotenoid content in the

Fig. 1 *Nicotiana tabacum* wild type and TLA leaves. *Upper panel* visual appearance and coloration of *N. tabacum* wild type and TLA leaves. Note the dark green coloration of the wild type (WT) and the light green coloration of the TLA tobacco leaves. *Lower panel* microscopic leaf cross section observations showing a higher density of cells in the wild type (WT) compared to that in the TLA mutant (TLA). The latter showed more extensive intercellular air spaces in the spongy tissue, with a concomitantly lower density of spongy cells

TLA strain was slightly lower, about 85% of that in the wild type (Table 1), resulting in a Car/Chl ratio of 0.16 in the wild type, but 0.29 in the TLA mutant (Table 1). These results are consistent with the notion of a truncated light-harvesting Chl antenna size in the tobacco mutant plants.

Photochemical apparatus organization in wild type and TLA tobacco

The concentration of the photosystems in isolated tobacco thylakoid membranes was measured using the sensitive absorbance difference spectrophotometric method (Melis and Brown 1980; Melis 1989) from the amplitude of the light *minus* dark absorbance difference signal at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII. Isolated thylakoid membranes from the wild-type tobacco showed an overall Chl/Q_A ratio of 383:1, whereas this ratio dropped to 129:1 for the TLA mutant (Table 2). Moreover, the overall Chl/700 ratio was 412:1 for the wild type, whereas this ratio dropped to 378:1 for the TLA mutant. These quantifications translated into a photosystem (PSII/PSI) molar ratio of 1.08:1 for the wild type and 2.93:1 for the TLA mutant. Enhancement of the PSII/PSI molar ratio in the TLA tobacco is a compensation response of the chloroplasts to the disproportionately smaller antenna size of PSII, resulting from the disproportionate lowering of Chl *b* over that for Chl *a* in these mutants (Greene et al. 1988).

Functional light-harvesting Chl antenna size of the photosystems

Chlorophyll *b* pigments are exclusively present in the peripheral light-harvesting antenna proteins but not in the core photosystem or reaction center complexes. The higher Chl *a*/*b* ratio in the TLA strain compared to the wild type suggested a lower amount of Chl *a*–*b* light harvesting antenna proteins in the TLA chloroplasts. The functional light-harvesting Chl antenna size of PSI and PSII was measured from the kinetics of P700 photooxidation and Q_A photoreduction kinetics, respectively (Melis 1989). Results from these measurements are summarized in Table 2. In the wild type, due to the biphasic kinetics of Q_A photoreduction, two rate constants of PSII photochemistry were discerned, K_{II} α [=8.4 s^{-1}] and K_{II} β [=5.1 s^{-1}], reflecting the well-known PSII heterogeneity of PSII-α and PSII-β centers in chloroplasts (Melis and Homann 1976). Under the same weak actinic illumination conditions, the rate constant K_I of PSI photochemistry was measured to be equal to 6.8 s^{-1}. These rate constants are a measure of the rate of light absorption and utilization by the photosystems in the wild type, and are directly proportional to the specific photosystem functional light-harvesting Chl ($a + b$) antenna size. The precise number of the functional PSII chlorophylls was derived (Melis 1989) as 248 Chl molecules for PSII-α and 151 Chl for PSII-β. The average functional PSII chlorophyll antenna size in the wild type was estimated to be 196 molecules. Similarly, the precise number of the functional PSI chlorophylls was estimated to be 202 in the wild type (Table 2). In the TLA mutant, all

Table 1 Chlorophyll (Chl) and carotenoid (Car) content per leaf area and pigment ratios of *Nicotiana tabacum* (tobacco) wild type and TLA plants

	WT	TLA	% Change to
Total Chl, mg cm^{-2} of leaf area	24.9 ± 2.1	12.1 ± 1.3	49 ± 6
Chl *a*, mg cm^{-2} of leaf area	18.8 ± 1.6	10.7 ± 1.0	57 ± 7
Chl *b*, mg cm^{-2} of leaf area	6.1 ± 0.6	1.4 ± 0.4	23 ± 11
Chl *a*/Chl *b* ratio, mol:mol	3.1 ± 0.1	8.1 ± 1.7	–
Car, mg cm^{-2} of leaf area	4.1 ± 0.4	3.5 ± 0.6	85 ± 21
Car/Chl ratio (w:w)	0.16 ± 0.02	0.29 ± 0.05	–
Average intensity of sunlight passing through an expanded leaf of a young plant	140 ± 13 µmol photons m^{-2} s^{-1} (6.4%)	615 ± 22 µmol photons m^{-2} s^{-1} (28.0%)	–
Average intensity of sunlight passing through an expanded leaf of a mature plant	105 ± 15 µmol photons m^{-2} s^{-1} (4.8%)	265 ± 57 µmol photons m^{-2} s^{-1} (12.0%)	–

Also shown are the transmittance of bright sunlight (2200 µmol photons m^{-2} s^{-1}) through the wild type and TLA leaves from young (2–3 weeks) and mature (5–6 weeks) plants (n = 3–5; means ± SD)

Table 2 Photochemical apparatus characteristics of *Nicotiana tabacum* wild type (WT) and TLA plants grown under ambient sunlight in high canopy density conditions

	WT	TLA
Chl/Q_A (mol:mol)	383 ± 8	129 ± 5
Chl/P700 (mol:mol)	412 ± 13	378 ± 29
PSII/PSI (mol:mol)	1.08 ± 0.04	2.93 ± 0.25
K_{II} α (s^{-1})	8.4 ± 0.1	3.4 ± 0.1
K_{II} β (s^{-1})	5.1 ± 0.2	2.4 ± 0.1
K_I (s^{-1})	6.8 ± 0.7	3.6 ± 0.6
Proportion PSII-α (%)	46 ± 2	23 ± 3
Proportion PSII-β (%)	54 ± 4	77 ± 9
N_{II} α (number of Chl molecules specifically in PSII-α)	248 ± 2	112 ± 3
N_{II} β (number of Chl molecules specifically in PSII-β)	151 ± 4	80 ± 2
N_{II} average	196 ± 3	88 ± 2
N_I (number of Chl specifically in PSI)	202 ± 10	120 ± 14

Photosystem Chl antenna size and reaction center concentrations were measured spectrophotometrically (Melis 1989) ($n \geq 3$; mean ± SD)

photosystem light-harvesting Chl ($a + b$) antenna sizes were smaller with 112 Chl for PSII-α and 80 Chl for PSII-β (average PSII Chl antenna size of 88 molecules) and a PSI average functional Chl antenna size of 120 molecules (Table 2). These results clearly show the effect of Chl deficiency on the functional antenna size of the photosystems, and are consistent with measurements conducted earlier (Melis and Thielen 1980; Thielen and van Gorkom 1981).

Protein analysis

Isolated (intact) tobacco chloroplasts were solubilized and subjected to SDS-PAGE and Western-blot analysis with specific polyclonal antibodies cross-reacting with the PsaA-PsaB PSI-RC, the D2 (PsbD) PSII-RC protein, the LHCB1 light-harvesting protein, or the LHCB2 protein. Loaded on a per Chl basis (0.5–2 µg per lane), the SDS-PAGE Coomassie stain showed substantially lower levels of the LHC-II proteins in the TLA mutant than in the wild type (Fig. 2). At the same time, there was more Rubisco (RBCL) in the TLA mutant than in the wild type. Western-blot analysis showed that the TLA chloroplasts contain lower levels of the PsaA-PsaB PSI-RC proteins (Fig. 3), relative to the wild type, consistent with the greater PSII/PSI ratio measured spectrophotometrically (Table 2). Levels of the D2 34 kD (PsbD) PSII reaction center protein were about the same, whereas levels of the LHCB1 were somewhat lower and levels of the LHCB2 proteins appreciably lower in the TLA than wild type (Fig. 3). These relative protein abundance measurements are consistent with the smaller (truncated) light-harvesting Chl antenna

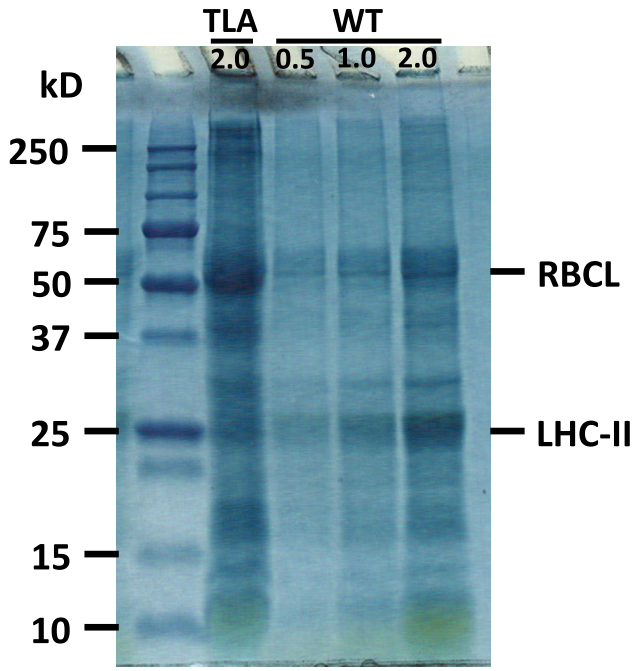


Fig. 2 Coomassie stain of total chloroplast protein extracts from *Nicotiana tabacum* wild type (WT) and TLA chloroplasts resolved by SDS-PAGE. Lanes were loaded with 2 µg chlorophyll (**a**, **b**) for the TLA analysis and with 0.5, 1.0, or 2.0 µg chlorophyll (**a**, **b**) for the wild type (WT) analysis. On a chlorophyll basis, the TLA sample contained more RBCL, the large subunit of RubisCO, than the wild type. However, the TLA sample contained substantially lower levels of LHC-II, apoproteins of the major light-harvesting complex of PSII, than the wild type (note: the rough resolution of the protein bands is due to the presence of excess chlorogenic acid, contained within the tobacco leaves and extracts, which interferes with the SDS-PAGE process)

size of the TLA mutant and corroborate the results of the spectrophotometric and kinetic analysis.

Light-saturation curves of photosynthesis

To investigate the functional properties of the photosynthetic apparatus in TLA tobacco relative to that in wild type, the light-saturation curve of photosynthesis was measured under in vivo conditions (Fig. 4). Results were normalized to the same light-saturated rate P_{max} to better illustrate differences in half-saturation intensity between the two samples. On a per leaf surface area basis, the absolute dark respiration rate of wild type and TLA tobacco was about -2 µmol CO_2 m^{-2} s^{-1}, whereas the absolute light-saturated rate P_{max} varied in different leaves between 20 and 35 µmol CO_2 m^{-2} s^{-1}, depending on plant age, leaf development stage and probably other physiological conditions. It should be pointed out that, on occasion, P_{max} of the TLA tobacco exceeded that of the wild type, when measured under ambient (400 ppm) CO_2 conditions. The reasons for this variation were not investigated further in this work.

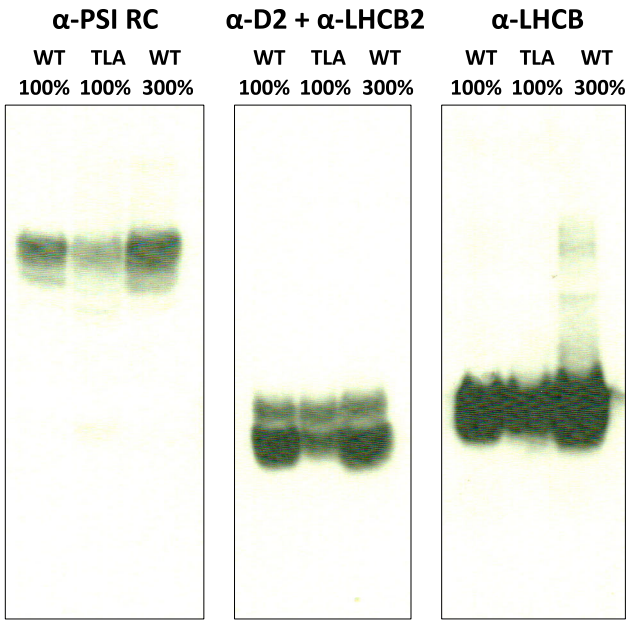


Fig. 3 Western-blot analysis of total chloroplast protein extracts from *N. tabacum* wild type (WT) and TLA leaves. Protein extracts were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with specific polyclonal antibodies raised against the PsaA/PsaB photosystem-I reaction center proteins (α-PSI RC), the PsbD photosystem-II reaction center protein (α-D2), or the light-harvesting chlorophyll-proteins LHCB1 and LHCB2 of PSII (α-LHCB). Note the approximately even levels of D2 protein (relative measure of PSII) in the WT and TLA lanes, and the substantially lower PSI RC and LHCB protein levels in the TLA tobacco relative to the wild type

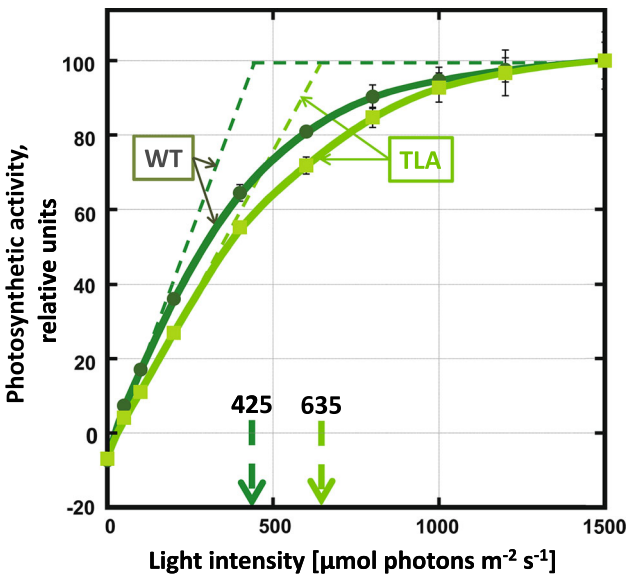


Fig. 4 Light-saturation curves of photosynthesis in *Nicotiana tabacum* wild type (*dark green*) and TLA leaves (light green) at ambient CO_2 concentration (400 µmol mol^{-1}). Saturation of photosynthesis in the wild type was estimated to occur at 425 µmol photons m^{-2} s^{-1}, whereas in the TLA mutant saturation occurred at 635 µmol photons m^{-2} s^{-1}

Interestingly, in all cases examined, and irrespective of the P_{max} value attained, the photosynthesis-saturation intensity, measured from the intercept of the initially linear increase of the light-saturation curve with the P_{max} asymptotic line (dashed lines in Fig. 4) indicated a saturation intensity of 425 µmol photons m^{-2} s^{-1} for the wild type and 635 µmol photons m^{-2} s^{-1} for the TLA mutant. This difference suggested that wild type has on the average a 1.5-fold greater light-harvesting antenna size of the photosystems than the TLA tobacco. However, the spectrophotometric and kinetic analysis showed that wild type has on the average a twofold greater light-harvesting antenna size of the photosystems than the TLA tobacco (Table 2). The discrepancy could be attributed to different specifics for the two measurements. In the spectrophotometric and kinetic analysis, Chl *a* and Chl *b* molecules were sensitized by weak green actinic illumination, which is absorbed about equally by the two pigments. Hence, all pigments are counted about equally. In the light-saturation curves of photosynthesis, the higher density packaging of chlorophyll molecules in the wild type than in the TLA mutant causes greater attenuation of light absorption, thus underestimation of the real antenna size of the wild type. Moreover, the 90% red and 10% blue actinic light, used for the saturation curves of photosynthesis, sensitizes both Chl and Car molecules in the light-harvesting antenna. TLA chloroplasts have a greater Car/Chl ratio than the wild type (Table 1), and Car excitation in the blue region of the spectrum may further attenuate the difference in light harvesting between the two samples. In addition, increased penetration of light into the leaves of the TLA mutant (Table 1) might result in higher rates of photosynthesis because of improved light distribution within the leaf.

Biomass accumulation under high canopy and foliage density conditions

Three different canopy sets comparing the growth and biomass accumulation of wild-type and TLA tobacco plants were investigated in canopy density experiments. Comparative canopy biomass accumulation measurements were conducted in the greenhouse during the natural tobacco growth season spanning the period from May to October. The layout of the canopy entailed individual tobacco plants growing in 2-gallon pots, having a rim diameter of 9-inches. Pots were placed against one-another in a 5 × 5 canopy configuration, comprising 25 plants separated by a 9-inch distance from each other. Relative to the corresponding wild type, TLA canopies reached the harvesting stage with a lag of 1–2 weeks, due to their slower development (1–2 weeks) earlier at the seedling stage in the greenhouse nursery (please see "Materials and methods" section). There were distinct visual differences in mature canopy appearance between the wild type and TLA tobacco. Wild-type plants in the canopy tended to develop longer internode distances with lower density of leaves in their upper canopy than the TLA plants (Fig. 5). There was an overall higher foliage density in the TLA canopy, compared to the wild type. Importantly, there were differences in the total biomass that was harvested from the two types of canopies. Wild-type biomass averaged 736 ± 67 g DW m^{-2} of canopy surface area, whereas TLA biomass yield averaged 928 ± 47 g DW m^{-2} (Fig. 6a). There was variation among the absolute yields of

Fig. 5 Visual appearance of a pair of *Nicotiana tabacum* wild type and TLA canopies, shown at the end of their respective growth period and immediately before harvesting of the plants. The wild type tobacco leaves had a *dark green* coloration (*upper panel*) and the TLA tobacco leaves a light coloration (*lower panel*). The wild type plants also had longer internode distances with fewer leaves in their upper canopy, as compared with that of the TLA plants. The overall foliage density was greater in the TLA canopy, compared to the wild type. Note the 5-day difference in the harvesting of the two canopies, introduced to account for the slower start of the TLA plants in the nursery

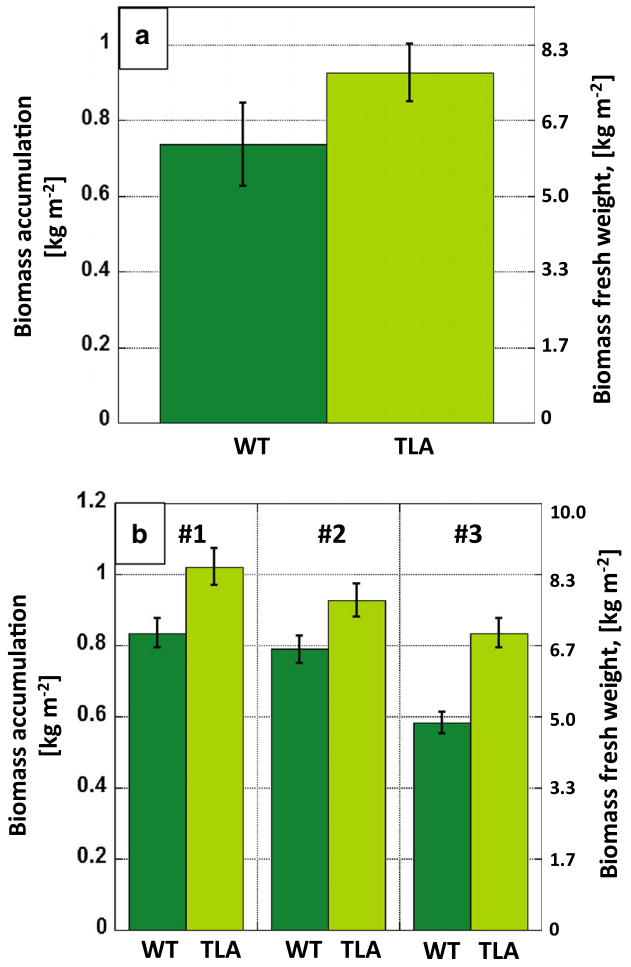


Fig. 6 Above ground total biomass harvested from wild type and TLA canopies. **a** Average values of biomass harvested from three different pairs of wild type and TLA canopies grown at different periods of time during the growth season in the greenhouse. TLA canopies produced about 25% more biomass than the corresponding wild-type. **b** Biomass accumulation by individual wild type–TLA pair of canopies, showing variation among the absolute yields in each of the three separate pairs, depending on time of the year and greenhouse location of the plants. Note that in all cases the TLA canopy produced more biomass than the corresponding wild type

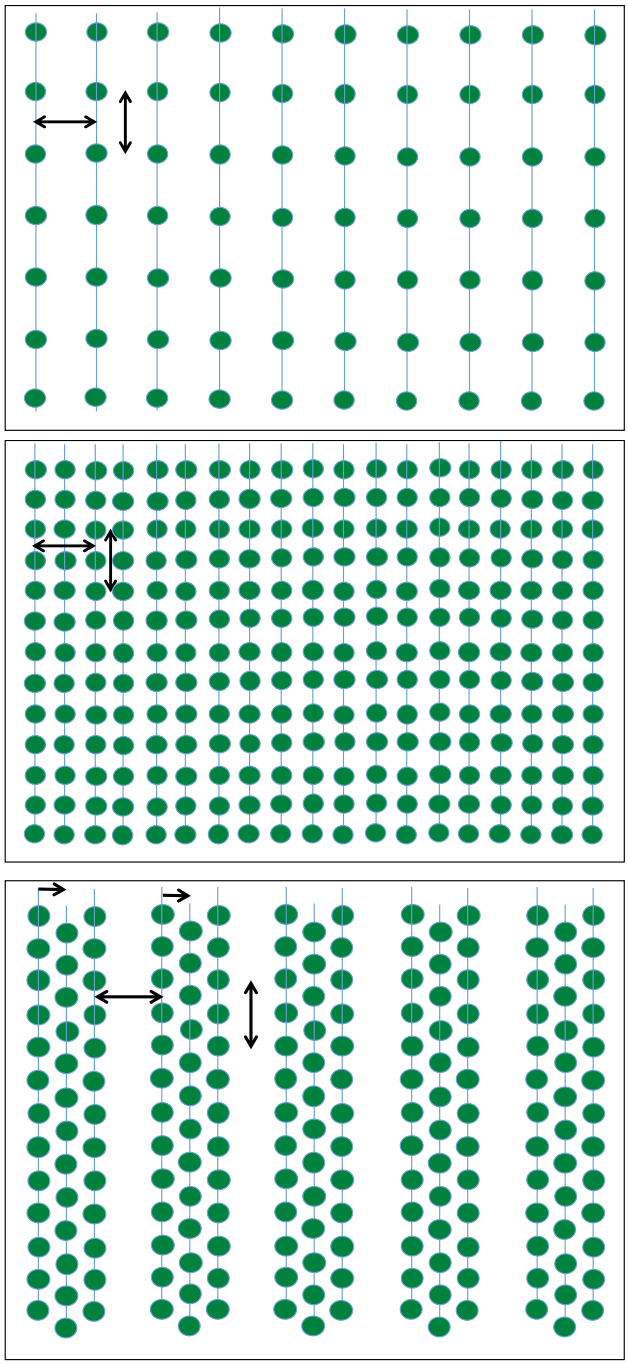
the three separate experimental canopy pairs (Fig. 6b), depending on time of the year and greenhouse location, but in all cases the TLA canopy produced more biomass than the corresponding wild type. In a one-tailed, paired statistical analysis of the biomass accumulation data, the calculated P value was 0.014, i.e., substantially lower than $P = 0.05$, considered to be the threshold of statistically significant results. Thus, there was a notable 25% improvement in biomass accumulation, attributed to the better sunlight distribution through the TLA canopy, as compared to that of the wild type.

Discussion

A challenge for plant scientists on a global scale is to generate enough food and feed for human and farm-animal nutrition, so as to meet the needs of an expanding world population (Alexandratos and Bruinsma 2012; Godfray et al. 2010; Ort et al. 2015; Kromdijk et al. 2016). Several analyses of the photosynthetic solar-to-biomass energy conversion efficiency identified truncation of the light-harvesting antenna of the photosystems as a potentially high-dividend strategy for increasing crop productivity (Nakajima and Ueda 1997; Melis et al. 1999; Polle et al. 2003; Melis 2009; Ort et al. 2011; Kirst and Melis 2014). While assembly of large arrays of light-harvesting antenna pigments is a critical evolutionary adaptation and a survival strategy for plants competing to grow in limited light in the wild, this is strongly counterproductive in crop monocultures, where growth takes place under direct and excess sunlight. The large arrays of light harvesting antenna complexes in crop plants cause the surface of the canopies to over-absorb solar irradiance, far in excess of what is needed to saturate photosynthesis, forcing them to engage in wasteful dissipation mechanisms to deal with the excess energy. This property further causes shading of the inner and lower canopy leaves preventing them from attaining their maximum photosynthetic potential. The net effect of this large antenna configuration in crop plants is to lower the overall solar-to-biomass energy conversion efficiency of photosynthesis from a theoretically possible 8–10% to less than 1% (Melis 2009).

Work reported in this paper showed that TLA technology could be applied to a crop monoculture, resulting in measurable improvement in biomass yield. This improved productivity is attributed to the smaller Chl antenna size of the photosystems, a property that alleviated excessive absorption of sunlight and the ensuing wasteful dissipation of the energy by leaves in the surface of canopies, thus permitting a greater penetration of irradiance deeper into the canopy to enhance overall canopy performance. Such improvement would not materialize, or it would be very substantially attenuated in an alternative leaf organization with fewer chloroplasts, or fewer electron transport chains per chloroplast, but with photosystems retaining the full antenna size of the wild type. In the latter cases, there would be no difference in the light-saturation curves of photosynthesis from leaves of a wild type and a strain with lower Chl content per leaf area.

For the purposes of this work, plants were grown in the greenhouse in separate 2-gallon pots that had a 9 inch-diameter. The plants did not have to compete with each other for water or other soil nutrients, so that soil, watering, and fertilizer issues were removed from consideration,



◀**Fig. 7** Schematic presentation of wild type and TLA plant agronomy. *Upper panel* schematic of traditional plant (tobacco) agronomy shows the typical 18-inch distance between adjacent plants, translating into an average of 4.35 plants cultivated per m^2. This planting density prevents individual plants from unduly shading one-another. *Middle panel* schematic of modified tobacco agronomy shows a narrow 9-inch distance between adjacent plants, translating into a canopy plant density of 14.9 plants per m^2. This substantially greater planting density takes advantage of the TLA property of the photosystems, and of the ensuing lower chlorophyll content per leaf area, properties that afford a greater transmittance of sunlight through the canopy foliage, thereby enhancing productivity. *Lower panel* schematic of alternative plant (tobacco) agronomy shows planting in bunches of three rows with an 18-inch distance between the bunches of three. Plants within each bunch of rows are separated by a 9-inch distance from each other. This configuration translates into 12.7 plants per m^2. Such planting density also benefits from the TLA size of the photosystems, and of the ensuing lower chlorophyll content per leaf area, properties that afford a greater transmittance of sunlight through the canopy foliage, thereby enhancing productivity. Long arrows indicate a distance of 18 inches; the short unidirectional arrows indicate 7.8-inch distance

leaving the sunlight utilization efficiency by the canopy as the only variable of interest. It is likely that such high-density growth experiments performed in the field with a greater number of plants in each canopy would probably yield a significantly stronger effect.

TLA-supported agronomic improvements

Application of the TLA concept to agriculture would afford higher density planting, thereby minimizing the surface area that is needed for the generation of a given amount of biomass. For example, traditional tobacco and grapevine cultivation entails planting in rows with individual plants separated from each other by about 18 inches (Fig. 7, upper). This practice translates into a density of 4.35 plants cultivated per m^2. Application of the TLA concept to tobacco and grapevine, as applied in this work, would permit cultivation in rows with individual plants separated from each other by as little as about 9 inches (Fig. 7, middle). This improvement would increase canopy density to 14.9 plants per m^2 with obvious benefits to yield per hectare.

Practical considerations often dictate spacing for access to the plants in the field. A schematic of alternative tobacco agronomy with three rows bunched together and separated by an 18-inch distance from the next bunch of three is shown in Fig. 7, lower. In this configuration, the middle row in the bunch of three is offset such that that a distance of 9-inch still separates plants from each other within the bunch, while a 7.8-inch distance separates the three rows (Fig. 7, lower, short unidirectional arrows). Such configuration with the 18-inch gap between the bunch of rows translates into 12.7 plants per m^2. This planting density would also benefit from the TLA property of the photosystems, and of the ensuing lower chlorophyll content per leaf area, properties that afford a greater transmittance of sunlight through the foliage of the plants, thereby enhancing canopy productivity. Alternative planting configurations are also possible with two, four, or five bunch of rows, with the bunch of rows separated by a space of 15, 18, or 23 inches to enable access to the plants throughout the field.

Table 3 Chlorophyll composition and antenna size of the photosystems in selected TLA crop plants

Plant species	Chl *a*/Chl *b* ratio (mol:mol)	Average PSII antenna size (Chl molecules)	PSI antenna size* (Chl molecules)	References
Hordeum vulgare (barley) chlorina *f2*	Chl *b*-less	50	150	Ghirardi et al. (1986)
Glycine max (soybean) y_9y_9	5.0	135	160	Ghirardi and Melis (1988)
Glycine max (soybean) $Y_{11}y_{11}$	5.5	112	170	Ghirardi and Melis (1988)
Zea mays (maize) OY-YG	5.6	140	150	Greene et al. (1988)
Beta vulgaris (sugarbeet) PBI line LMG	5.2	120	120	Abadia et al. (1985)

Photosystem Chl antenna size and reaction center concentrations were measured spectrophotometrically (Melis 1989) ($n = 3$; mean ± SD.) All corresponding wild type strains showed a Chl *a*/Chl *b* ratio of about 3.0 ± 0.5. Wild type possessed an average PSII antenna size in the range of 215–230 chlorophyll (*a* and *b*) and a PSI antenna size in the range of 180–210 chlorophyll (*a* and *b*) molecules. Some of the mutated genes conferring a small or truncated antenna size (TLA) to green plants and algae are known and include TLA1 (variant of the plain MOV34/MPN domain (Tetali et al. 2007; Mitra et al. 2012), TLA2 (CpFTSY; Kirst et al. 2012a), TLA3 (CpSRP43; Kirst et al. 2012b), CAO (chlorophyllide *a* oxygenase; Ghirardi et al. 1986; Polle et al. 2000), Mg-chelatase subunit-I (CHL-I; Hansson et al. 1999; Fitzmaurice et al. 1999)

Preliminary evidence of such agronomic improvements was exemplified in this work with tobacco. However, the TLA principle can apply to other agricultural plants, as the TLA property per se has been demonstrated in barley, soybean, corn, sugar beet, and possibly additional crop species (Table 3). For example, favorable corn planting densities for high yield are in the range of 65,000–75,000 plants per ha (~7 plants per m^2). On the basis of the considerations described in this work, this planting density could double to 140,000 plants per ha (14 plants per m^2), substantially increasing crop yield. Similarly, currently optimal sugar beet planting density is about ~100,000 plants per ha (~10 plants per m^2), a density that could double to 200,000 plants per ha (20 plants per m^2) upon application of the TLA-concept to this crop.

The question of whether viable TLA plants can be generated in crop species and algae has been addressed in the literature, as plants and algae with a mutation resulting in smaller antenna size are known. For example, mutations that result in small or truncated antenna size can occur in genes such as *TLA1*, a variant of the plain MOV34/MPN domain (Tetali et al. 2007; Mitra et al. 2012), *TLA2* (CpFTSY; Kirst et al. 2012a), *TLA3* (CpSRP43; Kirst et al. 2012b), TLA4 (CpSRP54; Jeong et al. 2017), *CAO* (chlorophyllide *a* oxygenase; Ghirardi et al. 1986; Polle et al. 2000), Mg-chelatase subunit-I (*CHL-I*; Hansson et al. 1999; Fitzmaurice et al. 1999). In some of the above examples, a TLA plant, i.e., a plant having a smaller chlorophyll antenna size of the photosystems, has a mutation in a known gene as indicated in the references cited above. In other examples, a verified TLA plant has a genetic modification resulting in decreased chlorophyll antenna size, where the mutation has not been identified. Examples of crop plant lines having a smaller antenna size of the photosystems are provided in Table 3.

TLA technology benefits to agriculture

The model plant *N. tabacum* (tobacco) was used in this demonstration. However, the TLA principle could apply to other crop plants, promising to increase yields, while minimizing the space needed for cultivation. Higher density planting of grapevine, soybean or corn, among other crops, offers ancillary benefits, as these crops would achieve canopy closure more quickly and thus (1) minimize losses of soil moisture, (2) lower the amount of fertilizer needed, as a smaller plot size with higher density of plants would minimize unwanted fertilizer runoff, and (3) alleviate the need to use herbicides, as quick and unbroken canopy closure will create the shading needed to prevent, or minimize, the growth of weeds.

Prior work applied and tested the TLA concept in microalgae and cyanobacteria. Nagajima and co-workers (Nakajima and Ueda 1997, 1999; Nakajima and Itayama 2003) provided early evidence of improvement in cyanobacterial productivity at high irradiances upon reducing the content of the light harvesting pigments in the cells. Similarly, evidence of improvement in culture productivity was offered with green microalgae possessing a truncated light-harvesting antenna size (Nakajima et al. 2001; Polle et al. 2003; Mussgnug et al. 2007). Polle et al. (2003) reported that *TLA1* mutants of *Chlamydomonas reinhardtii* accumulated 1.56-fold more biomass than the corresponding wild type, when grown in the

greenhouse under ambient sunlight conditions. Kirst et al. (2014) reported that phycocyanin-less mutants of *Synechocystis* PCC 6803 accumulated 1.57-times more biomass than the corresponding wild type under simulated sunlight conditions in the laboratory. Shin et al. (2016) worked with a *Chlorella vulgaris* TLA mutant exhibiting a 56.5 and 75.8% decrease in chlorophyll *a* and *b* contents, respectively, and a 1.45-fold greater biomass productivity under high light, as compared to the corresponding wild type. Furthermore, Mussgnug et al. (2007) showed that in a mixed culture of wild type and TLA microalgae, under sub-saturating light growth conditions, the wild type dominated and quickly took-over the culture at the expense of the TLA mutants. The latter were outcompeted, providing experimental evidence in support of the selective survival advantage conferred by the evolution of sizable arrays of light-harvesting antennae. Cazzaniga et al. (2014) noted that domestication of the green alga *Chlorella sorokiniana*, entailing a reduction of the Chl antenna size, improved light-use efficiency in a photobioreactor.

However, not all TLA transformants among microalgae and cyanobacteria proved to perform better than the corresponding wild type (de Mooij et al. 2015). This shortcoming was attributed to adverse effect(s) of the transformation process on cell fitness, suggesting a requirement for the development of transformation protocols that avoid the off-target mutation of other essential or secondary genes.

Author contribution statement HK and STG conducted most of the experimental work. KKN provided equipment for the light-saturation curves of photosynthesis, read and edited the manuscript. PGL and AM conducted the canopy-density experiments, and read and edited the manuscript. AM conceived the idea and planned the work.

Acknowledgements We thank Hannah Clifton and Christina Wistrom for the greenhouse support they provided during the canopy-density experiments. Thanks are also due to Dr. Denise Schichnes for help with the microscopic leaf cross-section preparation and observations. The work was conducted as part of FOLIUM, a DOE ARPA-E PETRO project, Grant # AR0000204. K.K.N. is an investigator of the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation (through Grant GBMF3070). PGL is supported by the U.S. Department of Agriculture Cooperative Extension Service through the University of California.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human participants and/or animals Research did not involve Human and/or Animal Subjects. Experimental protocols in this work were approved by the UC Berkeley Committee on Laboratory and Environmental BioSafety (CLEB).

Informed consent All authors have read and approved submission of this work.

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