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Increased brassinolide accumulation and increased growth in low-light-grown transgenic tobacco

R. GREBENOK[*,+], T. UGINE[**], M. GALANTE[*], N. GREBENOK[*], H. IQBAL[*], N. OLSZEWSKI[*], X. KRULL[*], T. LINGENFELTER[*], R. SEDANO[*], E. CORIGLIANO[*], and A. GREBENOK[*]

Department of Biology, Canisius University, Buffalo, 14208 New York, U.S.A[*]
Department of Biology, Cornell University, Ithaca, 14853 New York, U.S.A[**]

Abstract

Transgenic tobacco, demonstrating bacterial cholesterol oxidase (CO) activity in the chloroplast, grown at PAR ~280 µmol(photon) $m^{-2}s^{-1}$ (low light), contained thylakoid membranes that include a greater variety of steroids and had reduced contents of sterol and sterol-esters per milligram chlorophyll in comparison to controls. The mature transgenic plants, grown at low light, demonstrate a ~3× larger root dry mass, ~3× larger stem dry mass, ~2× larger leaf dry mass, ~2× increased leaf number, and ~5× increased flower number than controls. Mature transgenic flowering plants, develop to first flower ~2× faster and grow ~30% taller than control flowering plants. The transgenic seedlings contain approximately 2× higher amounts of brassinolide (BR) per g fresh mass than controls. We propose that since the CO enzyme produces increased contents of oxidized steroids in the thylakoid membrane, this encourages enhanced photosynthesis, enhanced BR contents, and increased biomass accumulation.

Keywords: brassinolide; cholesterol oxidase; oxidized sterols; photosynthesis; productivity.

Introduction

The light reactions of photosynthesis are well characterized as a membrane-associated process (Andersson *et al.* 2001, Leister 2019). The light reactions are responsible for the initial capture of exogenous light energy and when the energy is used metabolically, results in the production of NADPH, ATP, starch, and glyceraldehyde-3-phosphate, among other components. These compounds are subsequently used to produce reduced forms of plant materials and refractory plant biomass. Several *in vivo* strategies have demonstrated the increased activities of several components of the light reactions of photosynthesis, with a demonstrated increase in plant biomass. Several of these studies focused on the overexpression of proteins that maintain some known importance to the photosynthetic process, while others are speculative. The results of these studies demonstrate that the process of photosynthesis is amenable to modification and that increased biomass in transgenic plants is achievable through manipulation

Highlights

- Tobacco expressing cholesterol oxidase demonstrates increased growth and mass accumulation
- Tobacco expressing cholesterol oxidase demonstrates more rapid development than control plants
- Characteristics of the transgenic plants correlate with increased brassinolide accumulation

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[+]Corresponding author
e-mail: grebenor@Canisius.edu

Abbreviations: AD – cholesterol; AE – campesterol; AF – β-sitosterol; AG – stigmasterol; BD – cholest-4-en-3-one; BG – stigmast-4, 22-dien-3-one; BF – sitost-4-en-3-one; BR – brassinolide; CD – cholestan-3-one; CE – campestan-3-one; CF – sitostan-3-one; CO – cholesterol oxidase; ER – endoplasmic reticulum.

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of the components of the light reactions of photosynthesis (Heyer *et al*. 2004, Simkin *et al*. 2017, Głowacka *et al*. 2018, Zhou *et al*. 2018, Leister 2019).

Chloroplasts produce many components, including chlorophyll, carotenoids, lipids, and many other molecules that support their function. However, this production does not extend to sterols, as sterol production is typically associated with the endoplasmic reticulum in cells of higher plants (Hartmann and Benveniste 1987, Lichtenthaler *et al*. 1997, Andersson *et al*. 2001, Laule *et al*. 2003). However, sterols have been reported to exist in the membranes of higher plant chloroplasts (Hartmann and Benveniste 1987). Polar lipids have been demonstrated to move from the endoplasmic reticulum membrane into the outer chloroplast membrane and the existence of specialized structured membrane contact sites connecting the endoplasmic reticulum membrane to the outer chloroplast membrane have been demonstrated (Kwok and Hanson 2004, Andersson *et al*. 2007, Xu *et al*. 2008, Wang *et al*. 2012, Mehrshahi *et al*. 2013, Pérez-Sancho *et al*. 2016). The physical connection between the outer chloroplast membrane and the membrane of the endoplasmic reticulum allows for molecule exchange between the two sites, suggesting that the sterols found in the chloroplast membranes of a higher plant are derived from the membrane of the endoplasmic reticulum (Hartmann and Benveniste 1987, Andersson *et al*. 2001, Laule *et al*. 2003, Mehrshahi *et al*. 2013, Pérez-Sancho *et al*. 2016). As the light reactions of photosynthesis are a thylakoid-membrane-localized process, sterol's impact on the characteristics of the thylakoid membrane has been recently examined. It has been shown that sterol-mediated characteristics of the thylakoid membrane are of great importance to the function of the light reactions of photosynthesis in both plant-derived membranes and artificial membrane assemblages (Yamamoto *et al*. 1981, Ford and Barber 1983, Busheva *et al*. 1998, Siegenthaler and Trémolières 1998, Heyer *et al*. 2004, Dekker and Boekema 2005, Popova *et al*. 2007, Wang *et al*. 2016).

Cholesterol oxidase (3-hydroxysteroid oxidase, EC 1.1.3.6, CO) is a flavin adenine dinucleotide-dependent bifunctional enzyme that catalyzes the oxidation and isomerization of cholesterol, 5-cholesten-3β-ol, to yield 4-cholesten-3-one with the concomitant production of hydrogen peroxide (Smith and Brooks 1977, MacLachlan *et al*. 2000). While CO acts on a relatively wide range of sterol substrates, the presence of a C3 β-hydroxyl group, typical of the common phytosterols of tobacco [cholesterol (AD), campesterol (AE), stigmasterol (AG), and β-sitosterol (AF)], is required for enzyme activity (Fig. 1) (Smith and Brooks 1977, MacLachlan *et al*. 2000). The gene encoding the CO enzyme has been cloned from several bacterial sources. The enzyme has been expressed in higher plants on several occasions. When CO is expressed in plants, increased contents of the steroids containing the 4-en-3-one, the 3-one, and stanol chemistries (Fig. 1), further referred to as "oxidized steroids" for our purposes, are reported (Corbin *et al*. 2001, Venkatramesh *et al*. 2003, Heyer *et al*. 2004). The oxidized steroids, which are produced by the action of CO, are readily identified as precursors or intermediates in the brassinosteroid pathway (Fujioka *et al*. 1997, Noguchi *et al*. 1999, Bajguz *et al*. 2020).

Brassinolide (BR) is a member of a class of steroid hormones whose synthesis and function in higher plants have been well-studied (Clouse *et al*. 1992, Zurek *et al*. 1994, Fujioka *et al*. 1997, Noguchi *et al*. 1999, Yang *et al*. 2018, Bajguz *et al*. 2020, Albertos *et al*. 2022). Many publications represent BR as highly effective, presenting a pronounced phenotypic effect, chiefly growth-associated, in many plants (Clouse *et al*. 1992, Zurek *et al*. 1994, Oh and Clouse 1998, Kim *et al*. 2007, Yang *et al*. 2018, Bajguz *et al*. 2020, Sheng *et al*. 2022). In physiological systems designed to examine the BR effect, it was shown that BR resulted in many modifications to a plant's phenotype including enhanced stem elongation, modified root growth, and enhanced flowering, among other physiological and developmental phenotypes (Clouse *et al*. 1992, Zurek *et al*. 1994, Kim *et al*. 2007, Yang *et al*. 2018, Bajguz *et al*. 2020, Sheng *et al*. 2022). Recently, foliar BR application has been examined concerning photosynthesis, demonstrating that increased plant growth, increased synthesis of photosynthetic pigments, and increased photosynthetic efficiency occur in *L. chinensis* in comparison to controls when plants were grown in low light (Yang *et al*. 2018).

Plant growth and development have been extensively studied. BR, other hormones, light intensity, and many other abiotic factors play very important roles in the process (*see* Clouse *et al*. 1992, Zurek *et al*. 1994, Oh and Clouse 1998, Kim *et al*. 2007, Yang *et al*. 2018, Bajguz *et al*. 2020, Shafiq *et al*. 2021, Albertos *et al*. 2022, Sheng *et al*. 2022). Therefore, we are interested in determining how low light intensity may impact the photosynthetic biomass accumulation and development in our transgenic tobacco model that demonstrates cholesterol oxidase activity in the chloroplast, increased oxidized sterol contents in chloroplast membranes, and enhanced rates of whole chain electron transport in comparison to control plants (Heyer *et al*. 2004). A recent meta-analysis of 57 studies revealed current food security issues and a predicted 35 to 56% increase in food demand by the global population by the year 2050 (van Dijk *et al*. 2021). In many global climate change scenarios, it is suggested that modified environmental conditions and limited plant productivity will be a reality in the future and are likely to limit food productivity associated with photosynthesis (Tobey *et al*. 1992, Olmstead and Rhode 2011, Shafiq *et al*. 2021). Specifically, we are interested in seeing if the expression of the CO enzyme in the chloroplast affects the ability of our transgenic tobacco model to produce more photosynthetically dependent products than control plants.

Materials and methods

Steroid definition, identification, and analysis: Steroids are a large class of organic compounds with a characteristic molecular structure containing four rings of carbon atoms (three six-membered and one five) (Fig. 1). They include

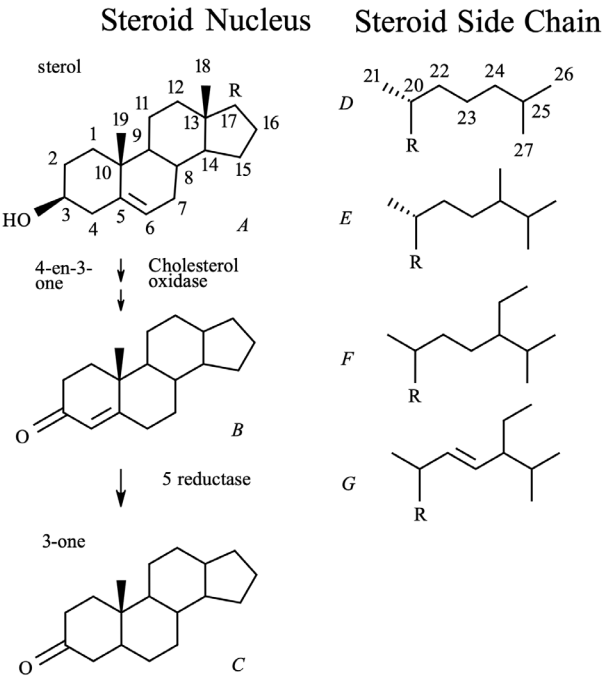

Fig. 1. Proposed pathway to produce oxidized steroid in transgenic tobacco plants expressing a chloroplast targeted cholesterol oxidase enzyme. The cholesterol oxidase enzyme catalyzes the initial oxidation and isomerization of C5 of the sterol, followed by 5α reductase-like activity. 3-ketosteroids and 4-en-3-one steroids with structures corresponding to cholesterol (AD), cholest-4-en-3-one (BD), cholestan-3-one (CD), campesterol (AE), campestan-3-one (CE), sitosterol (AF), sitostan-3-one (CF), stigmasterol (AG), and stigmasta-4, 22-dien-3-one (BG) were observed in transgenic tobacco system. Each steroid identified and quantified in this system contains a steroid nucleus (*A*, *B*, *C*) and a sidechain (*D*, *E*, *F*, *G*).

many hormones, alkaloids, and vitamins. Sterol can be defined as any member of a group of naturally occurring unsaturated steroid alcohol, typically a waxy solid. Further, sterol can be defined as an organic compound, whose molecule is derived from that of gonane by replacement of a hydrogen atom in position C3 by a hydroxyl group (Fig. 1). Therefore, the authors used the term sterol throughout the text where they are referring to a 4-ringed molecule containing a C3 hydroxyl group (Fig. 1*A*), while the authors referred to a molecule as a steroid when they are referring to a 4-ringed molecule maintaining a C3 ketone group (Fig. 1*B*).

Sterols and steroids were quantified by GC/MS using previously produced standard curves for all common and commercially available molecules. Fresh plant material or thylakoid membrane sample was extracted with chloroform and methanol (1:1, v/v) by previously published methods (Grebenok and Adler 1991). In short, the extracted fraction is evaporated to dryness, and resuspended in 70% methanol:water, and the sterols/steroids were extracted from the methanol:water with water equilibrated hexane. The sterol/steroid (hexane) fraction was evaporated to dryness under nitrogen and subsequently resuspended in a minimal volume of hexane for conjugation with N, O-bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA-tmcs/99:1) (*Sigma Chemical*, St. Louis). The conjugated hexane sample was subsequently washed with water and analyzed by GC/MS (*Agilent*, *Inc*., Palo Alto, CA, USA). Throughout the paper free sterols are those identifiable in the tissue without further processing, while sterol-ester pools are analyzed following base saponification of the initial hexane fraction. Base saponification includes the treatment of extracted sterols/steroids (hexane fraction) with a 5% methanolic KOH solution at 70°C for 2 h in a shaking water bath. Subsequently, the freed sterols are extracted from the methanolic KOH with hexane and the hexane fraction is washed to neutrality, conjugated, characterized, and quantified by GC/MS. Sterols and steroids were analyzed by GC/MS using the following conditions: inlet temperature of 280°C, transfer line temperature of 290°C, and column oven temperature programmed from 80 to 300°C with the initial 80°C temperature maintained for 1 min and the final temperature for 20 min and a ramp rate of 25°C min^{-1}. The column used was a glass capillary *RS-5* (30 m) *Restek* (*Restek*, *Inc*., Bellefonte, PA, USA) with a film thickness of 0.25 µm. Helium at a flow rate of 1.30 ml min^{-1} served as carrier gas. The tabletop *Agilent 5973* mass selective detector maintained an ion source temperature of 250°C and a quadrupole temperature of 180°C. Standard protocols include the determination of the ionization rates of the common sterols: cholesterol (AD), campesterol (AE), stigmasterol (AG), β-sitosterol (AF), and the common steroids: campestan-3-one (CE), cholestan-3-one (CD), sitostan-3-one (CF), stigmasta-4, 22-dien-3-one (BG), cholest-4-en-3-one (BD), and sitost-4-en-3-one (BF) (Fig. 1). All molecules were identified through co-chromatography with authentic standards and by mass spectrum analysis (Heyer *et al*. 2004). The identification of the sitost-4-en-3-one (BF) and cholest-4-en-3-one (BD) was facilitated by the purchase of purified standards from *Steraloids Inc*. (Newport, RI, USA). The prominent mass ions for cholest-4-en-3-one (BD) were as follows: m/z 384 [40%], 229 [50%], and 124 [100%]. Sitost-4-en-3-one (BF) maintains prominent mass ions of: m/z 412 [40%], 229 [50%], and 124 [100%]. We presume that the cholest-4-en-3-one (BD) and sitost-4-en-3-one (BF) are structures directly derived from the respective corresponding parent sterol through the enzymatic activity of the CO enzyme (Smith and Brooks 1977, MacLachlan *et al*. 2000, Motteran *et al*. 2001, Moreau *et al*. 2002, Heyer *et al*. 2004) (Fig. 2).

Plants and plant growth: Tobacco (*Nicotiana tabacum*) lines are as previously described (Corbin *et al*. 2001). Steroid reference chemicals were purchased from *Steraloids Inc*., fine chemicals were purchased from *Sigma Chemical* (St. Louis, MO, USA), and GC/MS supplies were purchased from *Agilent*, *Inc*., Palo Alto, CA, USA.

Tobacco plants expressing a CO enzyme that had been transformed with a vector carrying the chloroplast-targeted CO gene, *pMON33814*, were morphologically indistinguishable from control plants that were transformed with a control vector, except for the growth differences

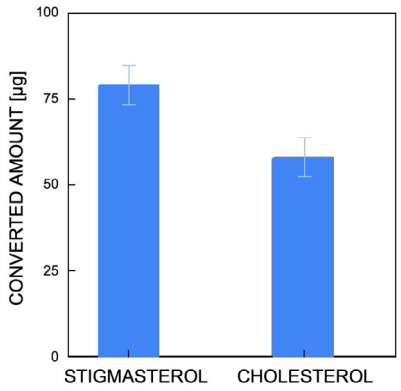

Fig. 2. *In vitro* cholesterol oxidase activity in transgenic chloroplasts. Average conversion of sterol to steroid-4-en-3-one attained following equilibration of listed sterol into thylakoid membrane obtained from transgenic tobacco line 500103. The products of 11 and 12 equilibrations of 200 micrograms starting sterol, respectively were averaged for stigmasterol and cholesterol conversions. Each bar represents an average (mean) amount converted with error bars representing standard errors of the mean. Equilibration of the identical sterols into control thylakoid membrane under comparable laboratory conditions provided no detectable steroid-4-en-3-one product identified by GC/MS (data not shown).

reported here (Corbin *et al.* 2001). The authors define a cohort of plants as a group of plants generated from a unique planting of seeds. Sterol accumulation was examined in cohorts of plants representing three independent R2 lines, 50087, 50095, and 500103. Control and transgenic plants were soil-grown under artificial lighting conditions on a 20:4 light-to-dark regime at ~25°C in a room environment. The light intensity approximated PAR of 280 µmol(photon) $m^{-2}s^{-1}$ as measured by the light sensor on the *LI-COR 6400 XT* device (low light) (*LI-COR, Inc*., Lincoln, NE, USA). Plastic eight-inch pots and plastic trays (*Garden Supply Inc*.) were used for application of water and fertilizer. A standard dilution of *Miracle Grow* (*Scotts Miracle Gro Company* and *Evergreen Garden Care Ltd.*) was used as fertilizer (used as instructed on the packaging). Transgenic and control plants were fertilized equally. Water was given to all plants sufficient to maintain a damp soil.

Seeds obtained from plants identified as transgenic or control by GC/MS were used to generate the plants that composed the cohorts. Transgenic lines and control lines were maintained based on the demonstration of steroid characteristics, with independent self-pollination of transgenic and control lines occurring. Plants were randomly selected from the cohort of plants grown and were sub-cultivated into pots according to experimental design. It was typical to have plants from a cohort not used in the design, preparation, or execution of the experiment. Some plants used for the various experiments died (for unknown reasons) during the execution of the experiments and data from these plants was not included in the final data sets reported. The determination of chlorophyll concentration of isolated chloroplast samples was according to MacKinney (1941). In short, 50 µl of the isolated chloroplasts were extracted in a 3 ml volume of 80% acetone:water and the acetone solution was examined *via* a *Shimadzu 1900i* spectrophotometer at 645 and 663 nm. The absorbances were used to calculate the concentration of chlorophyll *a* and *b*.

All biomasses reported are dry masses of materials. For the generation of dry mass, plant parts were dried using an air flow-through system connected in series to our research building vacuum. The plant material was held for 48 h in a pan in a drying oven (*National Appliance Company – Model 5851*) set to ~80°C. Room air was passed through the drying oven and pulled into a 500-ml flask ¾ submerged in a cooling/circulating polyethylene glycol bath, whose temperature was set to ~10°C (*Model 1162*, *VWR Scientific Inc*.). The submerged flask was filled with glass wool to facilitate water condensation, and the flask was emptied daily. The air from the submerged flask was diverted to a second 500-ml flask that was ~½ full of *Drierite* ($CaSO_4$) (*Hammond Drierite Company*), which was changed as needed (indicated by a change from blue to pink). The destination for the air was the research building vacuum system. Plant material was cut into small pieces to facilitate its drying and all plant material was determined to be dry according to assessment of the *Drierite* characteristics.

Isolation of class C (leaky) chloroplasts and purified thylakoid membranes: Plant leaves used to isolate chloroplasts were harvested from specific locations on the plant with at least 14 fully expanded leaves. Leaves were identified according to the width of the apical leaves, with the apical leaf measuring at least 7 cm across at the widest point being defined as leaf #1, which was typically the fifth leaf below the apical tip of the plant. Leaves were then numbered moving basipetally on the plant beginning from leaf #1. Odd-numbered leaves were then pooled and used for chloroplast isolation. Typically batches of 50 to 100 g of leaves were used to isolate intact chloroplasts and thylakoid membranes as described by Melo *et al.* (1995). All steps were carried out at 4°C and in dim room light. In short, freshly harvested, deveined leaves were placed in an ice-cold homogenization buffer described by Melo *et al.* (1995) and homogenized in a Waring blender for 15 s on the puree setting. The crude homogenate was filtered through 8 layers of cheesecloth and centrifuged at 1,000 rpm for 1 min. The supernatant was transferred to a fresh tube and centrifuged at 4,000 rpm. The final pellet was resuspended in 1 ml of SHM assay buffer, containing 0.35 M sucrose, 1 mM HEPES, 25 mM KCl, and 25 mM ascorbic acid. The final pellet was resuspended in a volume of assay buffer at approximately 1 mg ml^{-1} of suspension. The sample was divided into aliquots used for enzyme assays or steroid analysis. Sample aliquots used for steroid analysis were frozen at –80°C, while those used for enzyme assays were not frozen, but kept on ice and in the dark, until use.

The isolation of further purified thylakoid membranes was according to Andersson *et al.* (2001) in which isolated chloroplasts were lysed and centrifuged at

16,000× *g* for 5 min. The pellet was resuspended in 20 ml of homogenization buffer (Andersson *et al*. 2001) and 10 ml of an 8% *Percoll* (*Sigma*) cushion, in the same medium, was layered beneath the resuspended membrane pellet. The thylakoids were pelleted through the *Percoll* layer by centrifugation at 20,000× *g* for 15 min. The pelleted thylakoid membranes were suspended in 5 ml of homogenization buffer, as included in Melo *et al*. (1995) and aliquoted for equilibration studies. Throughout the process, the samples were maintained in the dark and at 4°C.

CO *in vitro* assay – steroid equilibrations: The CO activity was measured in thylakoid membrane preparations and purified thylakoid preparations. In short, the purified thylakoid membranes were obtained as described above (Melo *et al*. 1995, Andersson *et al*. 2001) and aliquots were subjected to the equilibration of sterol in a 200-µl volume of hexane. The sample was kept on ice and in the dark unless otherwise noted. A thylakoid membrane sample containing at least 0.25 mg of chlorophyll was suspended in 500 µl of homogenization medium (Melo *et al*. 1995). Experimental samples were over-lain with 200 µl of hexane containing a sterol at a concentration of 1 µg sterol per µl of hexane, while control samples were over-lain with 200 µl of hexane without sterol. The control and experimental samples were treated similarly throughout the protocol. The hexane was evaporated under a stream of nitrogen and subsequently, each sample was completely enclosed in aluminum foil and vortexed for 1 min to prompt sterol equilibration into the thylakoid membranes. The equilibrated samples were then incubated at room temperature for 10 min. The reaction was stopped by adding an equal volume of ethanol to the reaction mix. The samples were immediately transferred to a fresh centrifuge tube and centrifuged at 4,000× *g* for 2 min to pellet the membrane. The supernatant was drawn off and kept for sterol analysis. The equilibrated thylakoid pellet was washed 5× with osmotic shock buffer by vortexing for 1 min, followed each time by centrifugation at 4,000× *g* for 2 min to remove any non-equilibrated sterol. The supernatants produced from each centrifugation were combined for sterol analysis. The equilibrated thylakoid membrane pellet generated following the fifth spin was resuspended in 5 ml of 70% methanol:water and washed 5× with equal volumes of hexane to remove equilibrated sterol and sterol products of the CO enzyme from the thylakoid membrane preparation, which were identified by GC/MS.

BR identification and analysis: BR was quantified by GC/MS using previously produced standard curves for BR (*Sigma Chemical*, *B1439*). Fresh plant material was extracted with 200 ml of methanol overnight, preceded by a 20-second maceration in a waring blender, set at puree. The isolation and purification of BR followed previously published methods with variations delineated (Pachthong *et al*. 2006). In short, the extracted fraction was subjected to multiple liquid partitions between water, methanol, n-hexane, and chloroform, and finally the sample was evaporated to dryness and resuspended in a minimal volume of chloroform (1 ml). The chloroform fraction was subject to column chromatography using 12 g of *Silica Gel G* in a gravity flow column. The starting material mass was ~20 g and the extracted material weighed ~1 g, so a 12-g column was used to ensure sufficient theoretical plates for separation and purification. The material placed on the column was subjected sequentially to 0, 3, 5, 10, 15, 20, 25, 30, 40, and 50% methanol in chloroform (v/v). The volumes of each eluent were 31.25 ml, equaling approximately 3 column volumes of solvent per fraction. The BR was eluted in the 10 and 15% methanol fractions. The BR fractions were subjected to boronation according to Pachthong *et al*. (2006) analyzed and quantified by GC/MS. The additional clean-up procedures utilized by Pachthong *et al*. (2006) were not used in this procedure. The separation and GC-MS characteristics of the sample BR was comparable to the separation and GC/MS characteristics of the standard BR (*Sigma Chemical*, *B1439*).

Steroid and sterol thin-layer chromatography and column chromatography: For the initial identification of each steroid, selected samples were subjected to thin-layer chromatography and column chromatography before GC/MS analysis. The hexane fraction of selected samples (isolated as described above and before conjugation) was evaporated to dryness and resuspended in a minimal volume of hexane (100 µl). The fraction was subject to column chromatography using a 500 mg *Discovery SPE DSC-Silica* pre-made gravity flow column (*Supelco*, *Inc*., Bellefonte, PA, USA). The material placed on the column was subjected sequentially to 2 ml of hexane (100%), hexane–toluene (50:50) (v/v), toluene (100%), toluene–ether (50:50) (v/v), ether (100%), ether–methanol (50:50) (v/v), methanol (100%) (v/v). The steroid-3-ones eluted in the hexane-toluene (50:50) and the toluene (100%) fractions. The steroid-4-en-3-ones and the sterols eluted in the toluene–ether (50:50) and the ether (100%) fractions. The separation and GC/MS characteristics of the sample steroid and sterol were comparable to the separation and GC/MS characteristics of the standard steroid-3-one, steroid-4-en-3-one, and sterol.

Thin-layer chromatography was performed on *Silica G*, 20 cm, 500-micron glass-backed plates developed in 100 ml of toluene:ethyl acetate (90:10) (v/v) in a standard glass thin-layer chromatography tank (*Analtech*, *Inc*., Newark, DE, USA). Completed thin-layer plates were developed using gaseous iodine. The separation and GC/MS characteristics of the sample steroid and sterol were comparable to the separation and GC/MS characteristics of the standard steroid-3-one, steroid-4-en-3-one, and sterol.

Statistical methods: All analyses were performed with *JMP PRO 16* (*SAS Institute*, *Inc*., Cary, NC, USA). For each dataset, we first square root-transformed the data to meet the assumptions of normality. Next, we performed a mixed-model analysis of variance and included the fixed effect "treatment" (transgenic *vs*. control) and a random "cohort" effect. For the analysis of the BR per g of plant

tissue we transformed the data using the natural log, as the square root transformation did not result in normally distributed data.

Results

Cholesterol oxidase (CO) is a bacterial enzyme that catalyzes the oxidation and isomerization of cholesterol, 5-cholesten-3β-ol, to yield 4-cholesten-3-one with the concomitant production of hydrogen peroxide (Smith and Brooks 1977, MacLachlan *et al*. 2000) (Fig. 1). The activity of a CO enzyme, present in a chloroplast preparation, obtained from mature transgenic tobacco grown in low light, is demonstrated. Conversion of stigmasterol (AG) and cholesterol (AD) to the respective 4-en-3-one products, sitost-4-en-3-one (BF), and cholest-4-en-3-one (BD) was observed in association with the chloroplasts obtained from the transgenic tobacco (Fig. 2), while no conversion of sterol to the 4-en-3-one steroid was observed in chloroplasts obtained from control plants (data not shown).

Sterols with structures typical of CO substrates, cholesterol (AD), campesterol (AE), stigmasterol (AG), and β-sitosterol (AF), are present in the thylakoid membrane fractions isolated from both the mature transgenic and control tobacco (Table 1). The enzymatic conversion of the sterols in the transgenic thylakoid membrane fraction to those steroids containing 4-en-3-one chemistry was apparent and expected. In contrast, those same chemistries were absent from the control thylakoid membranes (Smith and Brooks 1977, Hartmann and Benveniste 1987, Morré *et al*. 1991, MacLachlan *et al*. 2000, Andersson *et al*. 2001). The transgenic thylakoid membranes demonstrated sterol plus sterol-ester contents that decreased by ~10×. Additionally, we identified five species of atypical oxidized steroids in the transgenic thylakoid membranes: cholest-4-en-3-one (BD), cholestan-3-one (CD), campestan-3-one (CE), sitostan-3-one (CF), and stigmasta-4, 22-dien-3-one (BG), that were not found in control membranes (Fig. 1, Table 1). Other forms of steroids occur in the transgenic thylakoid membranes, and it is apparent that additional enzymes, such as reductases and glycosyltransferases may act on steroids that reside in the chloroplast. While the activities of these enzymes were not the focus of this report, their actions may be responsible for the generation of the 3-ketosteroids and the sterol esters observed (Fig. 1, Table 1).

Four cohorts of plants were analyzed for the growth and BR measures unless otherwise noted. Stems from transgenic plants exhibited a greater than 3× increase in mass compared to controls. Transgenic stems had an average dry mass of 3.9 ± 0.2 g per stem, while control plants had an average stem dry mass of 1.1 ± 0.9 g per stem (Fig. 3*A*). The transgenic plants had an average leaf dry mass of 4.7 ± 0.3 g per leaf. Control plants had an average leaf dry mass of 2.1 ± 0.1 g per leaf (Fig. 3*A*). Thus, leaves from the transgenic plants had more than a two-fold increase in dry mass in comparison to control plants. Roots of transgenic plants also exhibited a greater than 3× increase in dry root mass compared to control plants. The transgenic plant roots had an average dry mass of 0.7 ± 0.05 g per plant, while control plants had an average root dry mass of 0.24 ± 0.03 g per plant (Fig. 3*B*).

We also observed an increase in number of leaves in mature transgenic plants in comparison to mature control plants (Fig. 4*A*). Control plants had an average of 28 ± 7 leaves per plant, while the transgenic plants had an average of 45 ± 13 leaves per plant, about a 1.6× increase. Transgenic plants had an average height of 122 ± 7 mm per plant at 9 weeks, and control plants had an average height of 86 ± 6 mm per plant at 9 weeks (Fig. 4*B*). One contribution to increased dry mass in stems in transgenic plants *vs*. control plants was the ~1.4× increase in plant height for the transgenic plants. Transgenic plants had ~ 5× more flowers than control plants. The transgenic plants had an average of 10 ± 0.9 flowers per plant, while control plants had an average of 2.2 ± 0.1 flowers per plant (Fig. 4*C*).

Table 1. Percentage and concentration of thylakoid membrane sterols/steroids[a] obtained from control and transgenic *Nicotiana tabacum* grown at low light. [a] Percentage of free and esterified sterols/steroids in the thylakoid membrane. Sterol/steroid represents the average of three analyses with a variation of 8%. [b] Sterols/steroids were quantified by GC-Fid and structurally identified by GC/MS. Percentage of sterols/steroids identified were represented. [c]Sterols/steroids not detected (nd) – less than 0.5 ng mg^{-1} chlorophyll. [d] Total micrograms of sterol/steroid per milligram chlorophyll.

	Control		Transgenic	
Sterols/steroids[b] [%]	free	ester	free	ester
Cholesterol (AD)	12	55	nd	2
Cholest-4-en-3-one (BD)	nd[c]	nd	6	5
Cholestan-3-one (CD)	nd	nd	6	3
Campesterol (AE)	15	8	nd	nd
Campestan-3-one (BE)	nd	nd	13	12
Sitosterol (AF)	17	5	nd	12
Sitostan-3-one (CF)	nd	nd	23	7
Stigmasterol (AG)	54	32	8	25
Stigmast-4,22-dien-3-one (BG)	nd	nd	44	34
Sterol/steroid concentration[d]	9.9	56	3.6	3.9

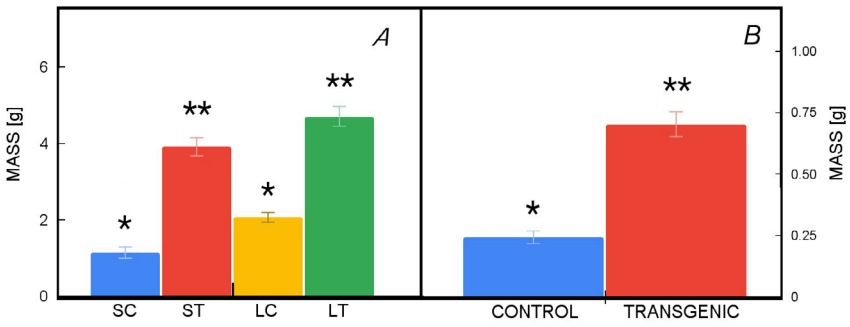

Fig. 3. The assessment of stem dry mass for transgenic plants (ST) and control plants (SC) and leaf dry mass for transgenic plants (LT) and control plants (LC) (panel *A*) and root dry mass (panel *B*) in control and transgenic plants grown at low light. The bars represent the average (mean) of the data set, while the error bars represent the standard error of the mean. Stem dry mass ($F_{(1,\ 91.4)}$ = 135.9, P<0.0001) and leaf dry mass (panel *A*) ($F_{(1,\ 82.5)}$ = 94.1, P<0.0001) and root dry mass (panel *B*) ($F_{(1,\ 90.8)}$ = 52.1, P<0.0001) in control and transgenic plants grown at low light were assessed examining 55 transgenic and 39 control plants. Bars with different numbers of asterisks above them are significantly different from each other (P<0.05).

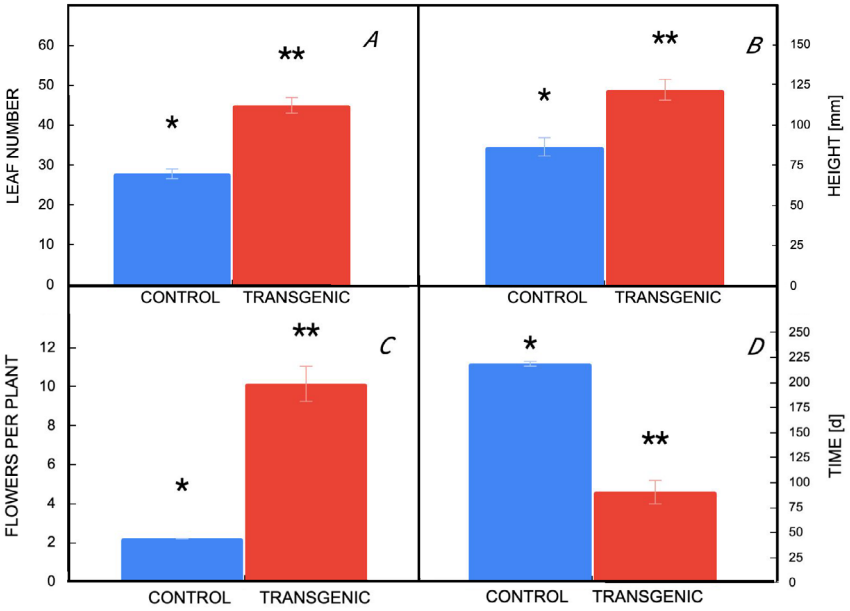

Fig. 4. The number of leaves (panel *A*), height of plant at week 9 (panel *B*), number of flowers (panel *C*), and development to first flower (panel *D*) in control and transgenic plants grown at low light were assessed. The bars represent the average (mean) of the data set, while the error bars represent the standard error of the mean for each data set. For the number of leaves (panel *A*) ($F_{(1,\ 90.3)}$ = 62.9, P<0.0001), 55 transgenic and 39 control plants were assessed. For the height of plants at week 9 (panel *B*) ($F_{(1,\ 76)}$ = 16.4, P=0.0001), 38 transgenic and 43 control plants were assessed. For the flower number per plant (panel *C*) ($F_{(1,\ 58.8)}$ = 42.1, P<0.0001) and development to first flower (panel *D*) ($F_{(1,\ 57.3)}$ = 491.0, P<0.0001), 50 transgenic and 12 control plants for each measure were assessed. Bars with different numbers of asterisks above them are significantly different from each other (P<0.05).

Transgenic plants grew faster over time ($F_{(6,\ 71)}$ = 3.0), several control plants took approximately 250 d to flower, while the transgenic plants produced flowers on average of 90 d following germination. The assessment of the rate at which the developmental state of flower generation was reached was made by counting the days post germination that it took a plant to reach the stage of first flower formation. The transgenic plants reached the first flower stage in an average of 91 ± 2 d, with some transgenic plants reaching the first flower stage as early as 71 d after germination. Control plants reached the first flower stage in 219 ± 12 d (Fig. 4*D*). The time to first flower was assessed for approximately 20 d following control plant flowering (or 275 d post germination) to ensure all plants were allowed to flower.

Because BR is involved in flowering, growth, and other development processes (Clouse *et al.* 1992, Zurek *et al.* 1994, Fujioka *et al.* 1997, Noguchi *et al.* 1999, Yang *et al.* 2018, Bajguz *et al.* 2020, Albertos *et al.* 2022), we undertook to determine if BR contents were altered in the transgenic plants. The sterol and steroid of transgenic thylakoid membranes in seedlings were likely similar to that of mature tissues. Therefore, the steroids shown in Table 1 are those that are expected to be in the chloroplast membranes of the seedlings assessed for BR synthesis. We have no reason to believe that the rates of sterol production, CO activity, or movement of steroids throughout the subcellular membrane locations differ developmentally, but we did not examine these aspects of our system. It has been shown that the oxidized steroids typical of those we identified in the transgenic chloroplast membranes serve as intermediates in the pathway for BR synthesis (Fujioka *et al.* 1997, Noguchi *et al.* 1999, Bajguz *et al.* 2020). We found that transgenic plants had an average of 37 ± 12 ng(BR) g^{-1}(plant fresh mass). Control plants had an average of 12 ± 5 ng(BR) g^{-1}(plant fresh mass) (Fig. 5*A*). The BR analysis involved paired experiments in which transgenic seedlings and control seedlings were gathered at the same time for examination. The rate of germination of the transgenic and the control seedlings differed, with the transgenic seeds germinating ~20% faster than the control plants but it was not examined further (data not shown). The transgenic plants demonstrated ~10× enhanced content of BR per seedling *vs.* control

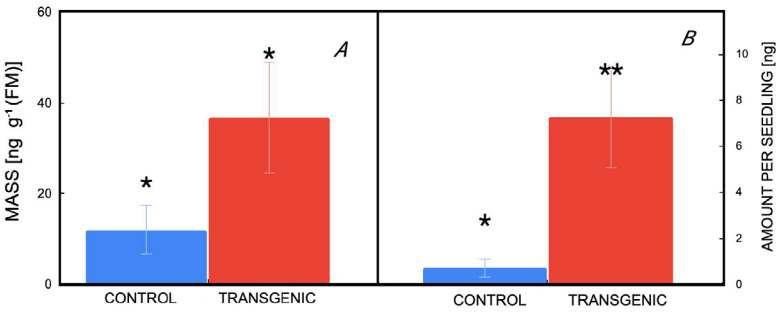

Fig. 5. For brassinolide amount per gram fresh mass (panel *A*) ($F_{(1, 3)}$ = 9.3, P=0.06) and brassinolide amount per seedling (panel *B*) ($F_{(1, 3)}$ = 46.1, P=0.007), 4 independent plantings (cohorts) of transgenic and control plants were grown for 4 weeks post germination, with the total plants analyzed numbering 280 transgenic and 1,130 control. Each bar represents an average of brassinolide amount with error bars representing standard error of the mean. Bars with different numbers of asterisks above them are significantly different from each other (P<0.05).

seedlings (Fig. 5*B*). Visual observation of early growth in the BR analysis showed that the transgenic seedlings maintained ~4-fold increased size in above-ground tissue, and an ~2-fold increased root size in comparison to control seedlings. The enhanced and faster growth and development demonstrated by the transgenic seedlings may be a result of the enhanced root size, enhanced shoot size, the enhanced photosynthetic activity as reported by Heyer *et al.* (2004), the enhanced accumulation of BR, or most likely, a combination of the four phenomena.

We report that the activity of a bacterial CO in the chloroplasts of transgenic tobacco, grown at PAR of ~280 μmol(photon) $m^{-2}s^{-1}$, created elevated contents of oxidized sterols in the thylakoid membranes of these transgenic plants. This correlates with a ~10× increase in the accumulation of BR per seedling in comparison to control seedlings. The mature transgenic tobacco plants demonstrated increases ranging from 1.4 to 5× enhancements in stem growth, root growth, leaf growth, flower number, number of leaves, and a 100% faster development to the first flower, in comparison to controls.

Discussion

The *in vitro* activity of a cholesterol oxidase (CO) enzyme was demonstrated in a chloroplast preparation obtained from mature, low-light-grown, transgenic tobacco (Figs. 1, 2; Table 1). The chloroplast resident enzyme converted cholesterol (AD) and stigmasterol (AG) to the respective 4-en-3-one steroid products. The maximal amount of conversion of sterol substrate to the 4-en-3-one steroid product was ~40% of the amount exposed and is associated with substrates, which maintain a β-hydroxyl group at the C3 position and a double bond at the C5 position, chemistries typical of the plant sterols of tobacco, cholesterol (AD), campesterol (AE), stigmasterol (AG) and β-sitosterol (AF) (Figs. 1, 2; Table 1). Steroids bearing the 4-en-3-one chemistry do exist in the transgenic thylakoid, used for equilibrations. However, the contents of the 4-en-3-one steroid were low and uniform (Table 1). In contrast, the 4-en-3-one product of the CO enzyme was elevated and variable by comparison to unequilibrated transgenic thylakoid membrane (Fig. 2). Thus, the initial contents of 4-en-3-one steroid in the transgenic thylakoid membrane were considered, but not included, to demonstrate CO activity. The existence of 3-one-containing steroids in the thylakoid membrane indicates that the enzymes capable of reducing the C4 double bond, within the steroid structure, exist in the chloroplast of tobacco (Fig. 1, Table 1).

In the current transgenic system, we observed elevated contents of oxidized sterol in all tissues examined, including the root, stem, and leaves (data not shown), suggesting that sterol and oxidized steroids are moving out of the chloroplast and throughout the plant. The amount of oxidized sterol found in the transgenic plant leaf did not predict the extent of growth and developmental characteristics that were observed; however, the transgenic system did demonstrate an array of growth and developmental enhancements compared to control (Figs. 3, 4). The evidence suggests either of two possibilities, one, that once a threshold of steroid products of the cholesterol oxidase enzyme was reached in the thylakoid membrane, plant growth would be enhanced compared to controls or two, some other product of the cholesterol oxidase enzyme is responsible for the growth phenotypes observed in the transgenic plants (Figs. 3, 4). Thus, random populations of transgenic plants, and not individual plant lines, exhibiting a wide range of oxidized steroid phenotypes were compared with control plants. We have no evidence to believe that the presence of oxidized sterol or the reduction in sterol-ester contents in the thylakoid membranes play roles in the increased photosynthetic phenotype observed in the transgenic system but increases in oxidized steroid and reduction in sterol-ester contents in the thylakoid membrane correlated positively with the enhanced photosynthetic phenotypes observed in these transgenic plants. We did not examine the roles played by individual sterols/steroids or sterol-esters in the various organs or various membranes of the transgenic system and suggest that examination of sterol/steroid and sterol ester composition of the different plant tissue membranes, including the chloroplast membrane, in correlation with the photosynthetic activity of the plant, should be examined to fully explain the observations and the role played by the cholesterol oxidase enzyme.

However, the increased plant growth seen in the current transgenic system (Figs. 3, 4) and the enhanced photosynthetic electron transport demonstrated by Heyer *et al.* (2004), considering the modification of the sterol profile of the thylakoid membranes (Table 1) in transgenic plants is not surprising. Many have shown that modification of components of the thylakoid membrane is reflected in variations of the photosynthetic process (Yamamoto *et al.* 1981, Ford and Barber 1983, Siegenthaler and Trémolières 1998, Heyer *et al.* 2004, Popova *et al.* 2007, Simkin *et al.*

2017, Głowacka *et al*. 2018, Zhou *et al*. 2018, Leister 2019). Although it is not clear that membrane components impact the activity of transmembrane proteins, it is reasonable to suggest given the above evidence.

Corbin *et al*. (2001) demonstrated that the CO enzyme was localized to the chloroplast in the transgenic tobacco identical to the plants currently represented. Examination of the phenotype of transgenic tobacco expressing a CO enzyme in the cell cytosol in their 2001 study showed morphological stunting and sterility, phenotypes not observed in the current system (Corbin *et al*. 2001). The morphological stunting and sterility observed in plants expressing CO in the cytosol are likely due to the oxidation of a large portion of the cellular sterol pool, as sterols are biosynthesized by enzymes localized on the cytosolic surface of the ER membrane (Hartmann and Benveniste 1987, Lichtenthaler *et al*. 1997, Laule *et al*. 2003). The importance of maintaining a minimal amount of "normal" sterol [sterol with the double bond at the C5 position, such as cholesterol (AD)], in higher plants has been observed in an *Arabidopsis* system, where *Arabidopsis* with at least 30% of the "normal" sterol content displayed a normal organismal phenotype (Silvestro *et al*. 2013). The enzyme in the current transgenic system is likely physiologically sequestered away from the ER site of sterol biosynthesis. The enzyme likely resides in the chloroplast, as shown by Corbin *et al*. (2001), since no stunted morphology is observed, but many oxidized sterols were identified in the chloroplast membranes (Table 1) (Corbin *et al*. 2001, Heyer *et al*. 2004).

Interestingly, the production of oxidized steroids in the thylakoid membrane coincides with the increase in brassinolide (BR) accumulation in low-light-grown seedlings in our transgenic system. The transgenic seedlings grown in low light accumulate ~ 2× more BR than that observed in control seedlings (Fig. 5*A*). These transgenic seedlings also demonstrated an increased biomass phenotype. Enzymes involved in the BR synthesis pathway are localized to the cytosolic surface of the endoplasmic reticulum (Vukašinović and Russinova 2018). While several of the thylakoid membrane oxidized steroids in the transgenic system are proposed to serve as precursors to the production of BR, they would need to move into the proximity of the BR biosynthetic enzymes in the endoplasmic reticulum to be incorporated into BR (Vukašinović and Russinova 2018, Bajguz *et al*. 2020). The phenomenon of steroids moving out of the membranes of the chloroplast and into the endoplasmic reticulum membrane is logical based on the evidence presented. The hemifusion-based model proposed by Mehrshahi *et al*. (2013) in *Arabidopsis* demonstrates connections between the endoplasmic reticulum membrane and the chloroplast membrane demonstrates shared phospholipids, tocopherols, and enzymes between the two sites. However, considering our BR findings, growth phenotypes, and sterol/steroid membrane data, the evidence would support a bi-directional movement of membrane components (Hartmann and Benveniste 1987, Lichtenthaler *et al*. 1997, Corbin *et al*. 2001, Laule *et al*. 2003, Heyer *et al*. 2004).

An increased level of BR synthesis provides one potential explanation for the enhanced growth phenotypes observed in the current system. BR has been shown to participate in the regulation of several plant growth processes, including root and shoot growth, flowering, and plant response to environmental conditions (Zurek *et al*. 1994, Kim *et al*. 2007, Yang *et al*. 2018, Sheng *et al*. 2022). Increases or modifications in all these areas are typical of the phenotypes demonstrated by the transgenic tobacco expressing the CO enzyme (Figs. 3, 4) (Bajguz *et al*. 2020). While the data from the current study suggests that increased synthesis of BR is a possibility due to the increased contents of intermediates in the BR biosynthetic pathway, we have not shown increased BR synthesis in the current system. Our data reveal increased accumulated levels of BR (Fig. 5). Whether the increased growth observed occurred due to increased synthesis, decreased rates of destruction, or other means is yet to be determined. BR could be responsible for some, all, or none of the enhanced growth and biomass phenotypes observed, while the modification of light reactions of photosynthesis, as was reported by Heyer *et al*. (2004), may be responsible for some percentage and other possibilities that exist. However, examining the regulation of BR biosynthesis, BR receptor dynamics, and BR destruction associated with transgenic plants, would help clarify the role played by BR in this system (Goda *et al*. 2002, Schülter *et al*. 2002, Bajguz *et al*. 2020).

Regardless of the mechanism(s), the transgenic plants described in this system developed faster, grew taller, maintained larger roots, maintained larger stems, and produced more flowers and leaves than control plants. Further, the transgenic seedlings contained an elevated content of BR in comparison to controls. Although the mechanisms involved in this enhanced growth and faster development are not known conclusively, the authors propose that the presence of oxidized sterols in the chloroplast, due to the expression of cholesterol oxidase in the chloroplast, is ultimately responsible. It is reasonable to suggest that the global population will increase in the future, and it has been predicted that ~50% more food will need to be generated by 2050 (van Dijk *et al*. 2021). It is also reasonable to conclude that increased plant productivity, agricultural use of lands receiving lower light intensities, or other, yet-to-be-offered alternatives, will need to be used to meet the food demands (van Dijk *et al*. 2021). The data of the current report demonstrate that the presence of the CO enzyme in the chloroplast of transgenic tobacco grown at lower light intensities correlates with dramatic enhancements in plant growth and development. These enhancements include BR contents, increased flower generation, and increased plant biomass in comparison to controls. Whether the plant can use the given light more efficiently, as suggested by Yang *et al*. (2018), or the observed phenotypes are due to the production of elevated contents of steroid hormones is unclear. Regardless, this technology, placed in the correct context, may be of vital importance in meeting the agricultural needs of projected future populations in limiting environmental circumstances.

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