Overexpression of citrus UDP-GLUCOSYL TRANSFERASE gene enhances anthocyanin and proanthocyanidins contents and confers high light tolerance

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Abstract

Background Citrus fruits are consumed freshly or as juice to directly provide various dietary flavonoids to humans. Diverse metabolites at different levels are present among Citrus genera and many flavonoids biosynthetic genes were induced after abiotic stresses. To better understand the underlying mechanism, we designed experiments to overexpress a UDP-GLUCOSYL TRANSFERASE gene from Citrus sinensis (sweet orange) to evaluate its possible function in metabolism and response to stress.

Results Our results demonstrated that overexpression of Cs-UGT78D3 resulted in high accumulation of proanthocyanidins in the seed coat and a dark brown color to transgenic Arabidopsis seeds. In addition, the total contents of flavonoid and anthocyanin were significantly enhanced in the leaves of overexpressed lines. Gene expression analyses indicated that many flavonoid (flavonol) and anthocyanin genes were up-regulated by 4-15 folds in transgenic Arabidopsis. Moreover, after 14 days of high light stress treatment, the transgenic Arabidopsis lines showed strong antioxidant activity and higher total contents of anthocyanins and flavonoids in leaves compared with the wild type.

Conclusion Our study concluded that the citrus Cs-UGT78D3 gene is involved in proanthocyanidins accumulation in seed coats and confers tolerance to high light stress by accumulating the total anthocyanin and flavonoid contents with better antioxidant potential (due to photoprotective activity of anthocyanin) in the transgenic Arabidopsis.

Background

Citrus is mostly cultivated fruit crops worldwide and its fruits are consumed freshly or as juice (oranges and mandarins). The fruit provide abundant amount of vitamins, carotenoids, folate, dietary fibers, flavonoids [1], proanthocyanidins (PAs) and anthocyanins [2] and is becomes a direct source of dietary metabolites. Flavonoids are further divided into various subclasses such as flavanones, isoflavones, flavonols, flavones, flavanols and anthocyanins [3] which play important role in the protection and defense of plants against biotic and abiotic factors [4, 5]; in addition flavonoids also convenes health promoting effects and reduce the risk of many chronic diseases such as cancer in humans [6]. Although citrus is one of the most widely cultivated fruit crops in the world, but little is known about the genetic mechanism and the genes involved in the biosynthesis pathways and variation of metabolites [7].

Proanthocyanidins (condensed tannins) are oligomeric or polymeric flavonoid compounds [8]. PAs are widely distributed in whole plant kingdom but mostly they are accumulated in seeds and fruits [8]. PAs functionally protects plants from abiotic (UV radiation) and biotic (fungus and insect pests) factors [9] and also beneficial for humans (anticancer, anti-inflammatory activity and protect skin from sun damage) [8, 10]. Anthocyanins are also subclass of flavonoids which is involved in pollen dispersal, fruits and flower colors, plant defense, and development [11]. The chemical structures of anthocyanins and flavonols are supposed to have strong antioxidant properties which are vital for scavenging of reactive oxygen species (ROS) [11]. These compounds protect plants from various environmental factors such as

intense light, cold, UV damage, high temperature and biotic stresses [12]. Recently, it was reported that several flavonoid pathway genes (o-methyl transferase, flavonol synthase, flavonoid 3'-monooxygenase, leucoanthocyanidin dioxygenase etc.) that belongs to flavone, flavonol and anthocyanin biosynthesis pathways were significantly up-regulated in citrus after Huanglongbing invasion [13–16]. Flavonoid compounds are famous due to antimicrobial, photoprotective and have resilient antioxidant properties to scavenging the ROS abiotic [17, 18] and biotic stress [19]. Whenever citrus plants are exposed to different abiotic and biotic stresses then they stimulates its secondary defense machinery (such as anthocyanins and flavonoids) to protect itself from challenging environmental conditions.

In citrus, many studies have been reported at transcriptional [20] and anatomical level to understand the tolerance and defense mechanism [21] but little information is available on citrus secondary metabolic pathway genes and their role in defense by enhancing protective metabolites [22]. Many researches have been reported that citrus plant accumulates secondary metabolites (flavonoids, anthocyanin etc.) after abiotic [23] and biotic stress [19]. However, the molecular mechanisms controlling the biosynthesis, alteration and distribution of anthocyanin and flavonoids are mostly unknown [24] and no study has been published to validate the function of metabolic pathway genes to understand the citrus leaves secondary metabolites role and their potential in triggering the tolerance against unfavorable conditions.

The Citrus genera consist of about 162 species [25]; out of these oranges, mandarins, pummelos, lemons and grapefruits are the famous cultivated Citrus species worldwide. Varied degrees of anthocyanin contents are present among Citrus genera; some citrus species possess high level of anthocyanin and some lower [2]. However, UDP glycosyl-transferase genes are directly involved anthocyanin and flavonol glycoside biosynthesis [26] in *Arabidopsis thaliana.* But in citrus, no one knows about the molecular mechanism controlling the biosynthesis of these metabolites. So, we have chosen one gene encoding *UDP-GLUCOSYL TRANSFERASE* from *Citrus sinensis (*sweet orange) to overexpress in *Arabidopsis thaliana* to evaluate its function. This study will helps to understand the metabolic biosynthesis, regulation of genes and possible tolerance role of *UDP-GLUCOSYL TRANSFERASE* gene towards the stress; in addition, it also facilitates pharmacological industry and assists metabolic engineering to breed a cultivar with increased protective metabolites (phytochemicals) in citrus.

3. Results

3.1 Selection and overexpression UDP glycosyl-transferase gene

Citrus species contain varied degree of metabolites. Based on published transcriptome data [27], we have selected UDP glycosyl-transferase genes and confirmed that the UDP glycosyl-transferase genes were differentially expressed among various citrus species by quantitative real time PCR (qRT-PCR) (data not shown). One UDP glycosyl-transferase gene was selected to overexpress in *Arabidopsis thaliana* to evaluate its function.

Transgenic Arabidopsis plants were selected at T_1 generation, on MS+ kanamycin medium for positive transgenic plant screening, and for further confirmation of transformed plants we did PCR by CaMV35S forward and reverse Cs5g24820 gene-specific primer. At T_2 generation, three independent transgenic lines (OX–1, OX–5 and OX–7) were selected based on gene expression and PCR results. Furthermore for light stress experiment, T_4 generation was produced via cross checking and selfing. According to qRT-PCR results, the *Cs-UGT78D3* gene was more than 25 folds up-regulated in all transgenic lines as compared to wild type (Figure 1A).

3.2 Overexpression of *UDP-GLUCOSYL TRANSFERASE* gene promotes proanthocyanidins accumulation in seed coats

The T_2 generation the transgenic Arabidopsis seed coats color becomes brownish (Figure 1B) and was significantly differentiated from the wild type seed coats color which was pale yellow (Figure 1C). However, at T_3 and T_4 generation, the seed coat color was stabilized i.e. dark brown (Figure 1B). Generally, high accumulation of PAs in the Arabidopsis seed testa gives dark brown color to seed coats [28].

Vanillin reacts with leucoanthocyanidins monomers, terminal subunits of PAs, catechins and gives red color [29, 30]. Vanillin assay was conducted on T_3 stage transgenic seeds. Interestingly, after vanillin treatment, the dark brown color of transgenic seed testa, showed distinct red color (Figure 1D) as compared with wild type seeds (where only seed coat margins showed red color) (Figure 1E). So, it is confirmed that the brown color of transgenic seed coats is due to PAs accumulation in seed testa (Figure 1B, D) than wild type (Figure 1E).

High concentration of proanthocyanidins contents (PAC) were observed in seeds of all transgenic lines than wild type (Figure 3A). Additionally, some gene related to proanthocyanidins biosynthesis pathway such as AT5G42800 (DFR or TT3), AT2G37260 (TTG2), AT4G09820 (TT8) and AT1G61720 (BAN) were significantly up-regulated (more than 4 folds) in transgenic seeds than wild type seeds (Figure 2). Up-regulation of TTG2, TT8 and BAN during seeds formation will facilitates high accumulation of proanthocyanidins in the seeds coats of *Arabidopsis thaliana* [31, 32].

3.3 High level of phenolic, flavonoid and anthocyanins in transgenic plants

Higher concentration of total phenolic (Figure 3D) and total flavonoid contents (Figure 3C) were observed in transgenic lines as compared to wild type. According to qRT-PCR expression data, the *Cs-UGT78D3* gene was more than 25 folds (Figure 1A) and some genes related to flavonols biosynthesis pathway such as TT3, AT3G55120 (TT5), AT3G51240 (TT6) and AT5G07990 (TT7) were induced 2–5 folds in all transgenic lines as compared with wild type. TT5 is involved in the flavonoid and anthocyanin biosynthesis pathway [33]. The high concentration of total phenolic contents (TPC) and total flavonoid

contents (TFC) in the leaves with significantly up-regulation of genes related to flavonoids pathways in all transgenic lines indicates that this gene is involved in increasing the total phenolic and flavonoid contents. Many plant phenolic and flavonoid compounds possess significant antioxidant properties that help plants to alleviate the free radical damage produced during light stress [17].

Total anthocyanin contents (TAC) was significantly increased in all transgenic lines as compared with wild type (Figure 3B). The TT3, TT8, AT3G28430 (TT9) and BAN were significantly induced (more than 4 folds) in the seeds and leaves of all transgenic lines as compared to wild type (Figure 2, 6). Moreover, AT5G41315 (GL3) and AT5G24520 (TTG1) were also significantly induced more than 5 folds in all transgenic lines than wild type. However, 2 fold or less than 2 fold increments has been observed in AT4G22880 (ANS), AT1G17260 (AHA) and AT5G05600 genes expression in transgenic lines than wild type. The TT3 and TT5 genes are involved in anthocyanin biosynthesis [33]. The metabolic and qRT-PCR results revealed that this gene is involved in inducing the flavonoid and anthocyanins biosynthesis pathway to accumulate more metabolites in transgenic Arabidopsis.

3.4 Metabolic response of transgenic Arabidopsis against light stress

TAC, TPC, TFC were increased significantly under 14 days high light stress (HLS) treatment in all transgenic lines than wild type. In addition after HLS, all transgenic lines accumulated significantly high concentration of TAC (Figure 4E) than wild type. Moreover, all the transgenic lines showed dark purple color phenotype after HLS (Figure 5B) whereas the wild type leaves showed chlorosis and shrink phenotype (Figure 5B). Additionally, after 14 days of light stress, all transgenic lines showed high expression of many genes related to anthocyanin biosynthesis pathway such as TT8, TT9, BAN (Figure 6). Moreover, the TT3, TT5, TT8, TT9, BAN, TTG1 and GL3 genes were highly induced (2–15 folds) after high light stress in all transgenic lines and these genes are involved in proanthocyanidins and anthocyanins biosynthesis pathway (Figure 5C). Anthocyanins are water soluble color pigment which has strong photoprotective properties and probably that is the phenomena behind the HLS tolerance of transgenic lines. Moreover, it was also previously reported that the anthocyanin compounds processes strong antioxidant properties and scavenging the free radicals produced during HLS [34]. So, our phenotypic, metabolic and gene expression data supports that flavonoids and anthocyanin were significantly accumulated in all transgenic lines after HLS.

Chlorophyll degradation was slower and antioxidant activity was higher in transgenic Arabidopsis in response to high light stress. Chlorophyll a and b were decreased dramatically in wild type and empty vector lines after 14 days of HLS; but all the transgenic lines maintained high level of chlorophyll a and b than wild type (Figure 4A, B). Rapid degradation of chlorophyll, due to environmental stresses shows that the plants are more prone to oxidative stress with lower light harvesting capability [34, 35]. Our results showed rapid degradation of chlorophyll a and b in wild type plants while all transgenic lines possess high level of chlorophyll a and b after 14 days of HLS; which clearly showed that wild type plants were under high stress and more prone to free radical damage than transgenic Arabidopsis lines (Figure 4A).

The free radical scavenging potential during HLS has been examined in wild type and transgenic Arabidopsis lines. The antioxidant activity and capacity was extraordinarily higher in all transgenic lines after 14 days of HLS than wild type (Figure 4F, G). In addition, the antioxidant activity and capacity was non-significantly reduced after 14 days of high light stress in wild type and empty vector lines than control (without stress) wild type and empty vector lines respectively (Figure 4F, G). These results showed that the HLS transgenic Arabidopsis lines have high free radicals scavenging potential, as compared with wild type. Abiotic stress tolerance is associated with high antioxidant activity and capacity [35]. So, our results clearly indicates that the wild type plants were under high stress and more prone to free radical damage whereas the transgenic lines possesses high antioxidant potential after HLS and contributes to high light stress tolerance.

The Hydrogen peroxide contents were increased in HLS transgenic lines than control transgenic lines and similar trend was observed in wild type plants (Figure 4H). H_2O_2 is vital metabolite in plants that is involved in governing various defense responses against abnormal conditions such as cell signaling and sensing functions [36]. Recent researches have reported that H_2O_2 has been supposed to be involved in protecting plants during abiotic stresses by triggering the signal transduction pathways that facilities crops plant to acclimation in stressed environment [36, 37].

4. Discussion

In *Arabidopsis thaliana,* UDP glycosyl-transferase genes are positively correlated with anthocyanin biosynthesis and flavonol glycoside [26] accumulation. In Brassica species and *Arabidopsis thaliana* UDP glycosyl-transferase genes responded to stress and anthocyanin accumulation [38]. To the author's knowledge, no evidence has been obtained so far about citrus UDP glycosyl-transferase genes function in both anthocyanins and proanthocyanidins. Some UDP glycosyl-transferase genes were up-regulated in a transcriptional study conducted on Huanglongbing infected citrus [13, 15]. Moreover, some regulation factor such as TT8 regulates the UDP glycosyl-transferase genes to accumulate the proanthocyanidins and anthocyanins in Arabidopsis [31].

Accumulation of proanthocyanidins is governed by TT2, TT8, and TTG1 genes, and some downstream PAs biosynthetic genes and these genes are also involved in the flavonoid biosynthetic pathway [31]. Generally, PAs were accumulated in the inner layer of seed testa and gives dark brown color to Arabidopsis seeds [31]. The terminal subunits of PAs react with vanillin and produces red color [29, 30]. PAs are colorless flavonoid polymers and PAs oxidation gives brown color to mature seeds [28]. In addition, PAs protect seeds from unfavorable environmental condition and enhance seed longevity [32]. Our vanillin assay showed that the PAs were accumulated in the transgenic seed coats (Figure 1D) and the seed showed dark brown color (Figure 1B) than wild type seed color (pale yellow) (Figure 1C) in addition, the proanthocyanidins contents were also high in seeds all transgenic lines (Figure 3A).

Our results showed that the TT8 and TTG1 both genes were up regulated in the transgenic lines as compared with wild type. TT8 is a regulation factor which is involved in regulating the biosynthesis of

flavonoids, proanthocyanidins and anthocyanins [31]. The brown color of Arabidopsis seeds are mainly due to PAs and their biosynthesis is governed by TT8 and TTG1 genes and these factors are involved in regulating the various biosynthetic gene of PAs pathway [31]. In addition TT8 also regulates the JAZ proteins to triggers the anthocyanin production. The TTG1 and TT8 expression are essential for BAN correct expression to accumulate PAs in seeds [31].

The Poinsettia (*Euphorbia pulcherrima)* leaves accumulates high level of water soluble anthocyanin pigments and withstand under high light stress, signifying the photo-defensive role of anthocyanin compounds [34]. Our results also showed high accumulation of TAC (Figure 4E) and showed dark purple color in leaves of all transgenic lines (Figure 5B) after HLS than wild type; which demonstrate that overexpression of *Cs-UGT78D3* gene is involved in anthocyanin accumulation and protects plants from HLS damage. In addition, the real time PCR also showed many folds increment of TT8, TT9, BAN genes in all transgenic lines (Figure 6) and these genes are involved in the biosynthesis of anthocyanins.

The high light stress led to rapid degradation of chlorophyll a and b contents in the leaves of soybean (*Glycine max)* plants with decreased efficiency of photosynthetic apparatus [39]. In a recent study, similar results (lower contents chlorophyll a and b) have been observed in Poinsettia (*Euphorbia pulcherrima)* plants when they were exposed to high light stress [34]. The plants who possess high chlorophyll contents and maintained high level of antioxidant activity and capacity can tolerate prolong abiotic stresses [40]. Our results also showed that high light stressed transgenic lines possesses significantly higher level of antioxidant activity, chlorophyll a and b contents than HLS wild type plants (Figure 4). Abiotic stress decreases the antioxidant potential, chlorophyll contents and also lowers the high harvesting ability of plants which ultimately triggers the production reactive oxygen species (ROS) and cause serve damage to cells (due to free radicals) [40].

Excessive light stress accumulates large amount of phenolic and flavonoid compounds such as flavonols in leaves, which has significant antioxidant capability to scavenging the ROS generated during abiotic [18, 23] and biotic stress [19]. Flavonols behaves as photo-protectants and possesses strong antioxidant activity that's helps to counters the ROS species to fight against the oxidative stress caused by excessive light stress [17]. Additionally, the Poinsettia accumulates anthocyanins in leaves after HLS, that indicates strong photoprotective role of anthocyanins [34]. Flavonoids and anthocyanin holds resilient antioxidant properties and contributes to photoprotection [41]. Our metabolic results showed high level of TFC and TAC in all transgenic lines compared to wild type (Figure 3C, B). These outcomes showed that the overexpression of citrus *Cs-UGT78D3* gene is strongly linked with the flavonoids and anthocyanin biosynthesis; moreover, the qRT-PCR data showed high expression of some genes involved in flavonol and anthocyanin biosynthesis (Figure 6), that also supports the metabolic results. The transgenic plants become acclimatized to stress situation by triggering the flavonoids and anthocyanin biosynthesis and makes transgenic lines more tolerant against the HLS.

Recent researches have reported some positive and key roles of H_2O_2, by stimulating the complex signaling transduction pathways leading to systemic acquired resistance and stress acclimation under

abiotic and biotic stresses [36, 37]. Moreover increment in H_2O_2 level will also induce some defense related genes [37]. Our results have showed high H_2O_2 contents in high light stress transgenic lines as compared with wild type (Figure 4H). In addition, the significantly high antioxidant activity and capacity (Figure 4F, G), after 14 days of HLS, in all transgenic lines also showed that the overexpressed lines possesses better and strong free radical scavenging properties than wild type. This clearly demonstrates that under high light stress the transgenic lines showed less oxidative stress with better free radical scavenging capability than wild type Arabidopsis plants. So, biochemical, metabolic, phenotypic and gene expression analysis supports that all transgenic lines were tolerant to high light stress due to high total anthocyanin and flavonoids contents with better antioxidant potential than wild type.

Conclusions

Overexpression of *Cs-UGT78D3* showed dark brown color in seeds of all transgenic Arabidopsis lines due to proanthocyanidins accumulation. Moreover, all transgenic lines showed significantly high level of total anthocyanin and total flavonoid contents in leaves. Additionally, after 14 days of high light treatment, the transgenic Arabidopsis lines showed significant anthocyanins accumulation and maintained high antioxidant activity and capacity as compared with wild type. The gene expression data also supported the up-regulation of many flavonoids (flavonol) and anthocyanin biosynthesis genes in transgenic Arabidopsis lines than wild type. We demonstrate that overexpression of *Cs-UGT78D3* gene accumulates proanthocyanidins in transgenic seed coats and confers high light stress tolerance by accumulating total anthocyanin and flavonoid contents with improved antioxidant potential than wild type. This study also provided an ideal candidate gene for future metabolic engineering to breed a cultivar with increased protective metabolites (phytochemicals).

2. Methods

2.1 Plant materials and growth conditions

In this study, wild type *Arabidopsis thaliana* (Arabidopsis) ecotype Columbia–0 (Col–0) (Arabidopsis seeds purchased online www.arashare.cn) was used to overexpress the citrus *UDP-GLUCOSYL TRANSFERASE* (Cs5g24820) gene. The wild type (WT) Arabidopsis seeds were sterilized with 70% (v/v) ethanol for 10 min followed by ethanol 100% (v) for 8 min, and then the seed were washed 4 times with double deionized water. Then the seeds were pour out on the Murashige and Skoog (MS) medium petri dishes plate containing 4.43g MS (dried basal medium with vitamins supplement phyto-technology laboratories); 25g sucrose; 10g agar for one liter 1% (w/v) and petri plates were left in the growth chamber having 20–22 °C and then after ten days the plants were transferred into the small pots (soil). Then the plants were grown in growth chamber for 3 weeks having an irradiance of 120 micromoles quanta m^{-2} per sec, with 70% relative humidity and temperature about 22 ± 3 °C, under 16/8 hours light and dark period.

2.2 Vector construction and Agrobacterium transformation

The T-DNA Gateway technology (Invitrogen) pK7WG2D vector, was constructed (to overexpress the flavonol transferase gene of Citrus in Arabidopsis), which contains green fluorescent protein (GFP) and also confers kanamycin resistance (that helps for plant visual or manual selection) due to neomycin phosphotransferase II (nptII) gene [42]. By using cDNA, coding region of *Cs-UGT78D3* was amplified by means of PCR using gene specific primers, then the plasmid was extracted and first cloned into pDONR221 and then intervened by L.R clonase enzymatic reactions (invitrogen) (as prescribed by manufactures instructions) and cloned into binary Gateway Vector pK7WG2D. After that the pK7WG2D was transferred into GV3101 (Agrobacterium strain) and then transformed into the Arabidopsis, by dipping the wild type Arabidopsis flowers in Agrobacterium solution via floral dip method [43].

2.3 Transgenic lines and light stress conditions

The transgenic Arabidopsis lines were developed via Agrobacterium-mediated transformation. The wild type (WT), transgenic Arabidopsis line 35S:PK7WG2D (empty vector), and three independent overexpressed 35S:PK7WG2D-UGT78D3 lines (OX–1, OX–5, OX–7) have been prepared by cross checking and selfing to get T_4 stage transgenic plants for 14 days of light stress experiment. In each stage, the Arabidopsis seeds were first sown on MS medium having Kanamycin (50 mg/L) to get positive plants and later it was confirmed by qRT-PCR expression analysis. So, for light stress experiment, the 25 days old wild type and transgenic plants were used and subjected to high light stress for 14 days in a growth camber with following conditions (Figure 5A); 50,000 Lux light stress having 16 hours light and 8 hours dark, with 70% relative humidity and temperature about 24 ± 2 °C. On day 1 and after 14 days of HLS, the leaves samples were collected and immediately frozen by means of liquid nitrogen and preserved at –80°C for biochemical, metabolic and gene expression analysis.

2.4 DNA extraction and PCR analysis

DNA was extracted by 2% CTAB method [44, 45]. 50–100 milligram (mg) fresh Arabidopsis leaves were crushed into fine powder and followed by addition of 700 microliter of DNA buffer than 90 min incubation at 65 °C. After that 800 microliter of chloroform-isoamyl alcohol (24:1) was added and then after 10 min of gentle inversions, centrifuged at 10,000 rpm for 10 min and DNA pellet was taken [45]. PCR was performed by using PCR-Master mix (dream tag green-Thermo Scientific) according to manufactures instructions.

2.5 RNA extraction and quantitative real time PCR analysis

Fresh leaves were immediately frozen into liquid nitrogen after harvesting and then used for RNA extraction by using TRIzol RNA (extraction kit) reagent (Takara). RNA was extracted as described by manufacture's instruction on TRIzol kit. After total RNA extraction, the complementary DNA (cDNA) was

synthesized by using 1 µg of total RNA by means of (Vazyme, R223–01) HiScript II QRT (reverse transcriptase) SuperMix for qPCR (+gDNA wiper) methodology. After cDNA synthesis all the cDNA samples were stored at –80 °C for further expression analysis (qPCR). For qRT-PCR was conducted by using SYBR Green (YEASEN Biotec Co.Ltd.) PCR Master mix and all standard procedure were adopted as described by producer's instructions. The qRT-PCR was done with three technical replicates (light cycler 480 multi-well plate 384-white) and performed by means of light cycler 480 II instrument (Roche). Relative expressions of pathway genes were calculated by means of 2−ΔΔCt methodology [46]. Arabidopsis β-actin gene was used as an internal reference gene. The qRT-PCR primers details are documented in additional file 1.

2.6 Vanillin Assay for proanthocyanidins determination

For vanillin assay, wild type and over-expressed seeds were taken in 1.5 milliliter of centrifuge tube followed by addition of I milliliter dye solution 1% vanillin (w/v) in 6 M of hydrochloric acid (HCl) [28]. About one layer of seeds has been taken to cover the bottom of 1.5 milliliter tube. Incubate the mature seeds for one hour at room temperature. After incubation, the seeds coats were gently separated with dissecting needle and tweezers on glass-slide by using stereomicroscope (OLYMPUS, SZ61 model). Then the light microscope (OLYMPUS, BX61 model) was used to photograph the stained seeds coats [47].

2.7 Determination of proanthocyanidins contents (PAC)

Proanthocyanidins concentration was estimated by grinding 20 milligram of Arabidopsis seeds with liquid nitrogen followed by adding 1 milliliter of extraction buffer (acetone 70%: water 29.5%: acetic acid 0.5%) with slightly modifications [48]. Then the samples were centrifuges at 4000 rpm and supernatant was taken for proanthocyanidins quantification. The reaction mixture contained 200 microliter of above prepared solution followed by addition of 3 milliliter of 0.5% (w/v) vanillin dissolved in methanol and 1.5 milliliter of 4% (v/v) HCl. Then after 15 min the absorbance was taken at 550 nm on spectrophotometer (model UV–1800, Shimadzu corporation, Japan) whereas pure methanol was used as blank. The standard curve was generated by using catechin and the PAC value was expressed in milligrams of catechin equivalents (mg CE/gram of sample).

2.8 Determination of chlorophyll content

For chlorophyll 'a' and 'b' estimation, the Arabidopsis leaves tissues (500mg) were grounded into fine powder by using 10 milliliter (mL) of 80% (v/v) acetone [49], followed by 4 hours of incubation at room temperature (RT) in dark. After that the sample tubes were centrifuged for 5 min at 12,000 rpm, and the supernatant was collected into a new tube then its absorbance was measured at 645 nm and 663 nm on spectrophotometer (80% (v/v) acetone was used as blank). Chlorophyll content was expressed in milligram per liter and calculated by using following formula:

Chlorophyll a = OD663 × 12.7 − OD645 × 2.69 (mg/L),

Chlorophyll b = OD645 × 22.9 − OD663 × 4.68 (mg/L).

2.9 Determination of total contents of phenolics and flavonoids

2.9.1 Extraction

100mg of leaves tissues were crushed into powdered by using pestle and mortar followed by addition of 5 mL of 80% methanol and samples were left for 2 hours at RT on an orbital shaker at 200 rpm followed by centrifugation [50]. The supernatant mixture was collected into a new tube while the remaining pellet was again extracted with the same procedure (with similar conditions) as described earlier and both supernatants were combined in 15mL tube, and then used for estimation of total phenolics and total flavonoid contents.

2.9.2 Total phenolics contents (TPC)

Folin–Ciocalteu reagent (FCR) based methodology was used for determination of total phenolics content [50]. 300 microliters of above prepared extract was taken in a fresh 10 mL tube and mixed with (10-fold diluted FCR with distilled water) 2.25 mL of FCR followed by 5–10 gentle inversions and 5 min incubation at RT. Then I added 2.25 mL of sodium carbonate (Na_2CO_3) (60 g/L) solution into the reaction mixture. Then after 2 hours of incubation at RT the absorbance was taken at 725 nm by means of spectrophotometer (model UV–1800, Shimadzu corporation, Japan). Standard curve was generated by using gallic acid (GA) and results were defined as milligram of GA-equivalents (GAE) per one gram of dried weight of plant leaves (mg GAE/g).

2.9.3 Determination of total flavonoid content (TFC)

Total flavonoid content was measured by means of colorimetric method with minor modification [51]. About 500 microliter of the prepared methanolic extract was taken into 10 mL new tube followed by addition of 2.25 mL of distilled water and mix well then added 150 microliter of 5% sodium nitrite ($NaNO_2$) solution followed by 6 min incubation at RT. After that 300 microliter solution of 10% aluminum chloride hexahydrate ($AlCl_3{\cdot}6H_20$) was added into the reaction mixture with 5 min of incubation at RT and then 1000 microliter of one Molar (M) sodium hydroxide (NaOH) was added followed by vertex for 30 seconds. The reaction mixture absorbance was taken instantly at 510 nm by means of spectrophotometer (model UV–1800, Shimadzu corporation, Japan). Standard curve was generated by using rutin compound and results were defined as milligram rutin equivalents (RE) per one gram of dried plant leaves sample (mg RE/g).

2.10. Total Anthocyanin contents (TAC)

For determination of total anthocyanin content 100 mg of leaves tissues were grounded (using liquid nitrogen) by means of mortar and pestle [52, 53]. After that the samples were re-suspended in five volumes (based on fresh weight) of extraction solution (having 45% methanol (v/v) and 5% acetic acid v/v) followed by gentle inversion and then centrifuged at (10,000 rpm) for 10 min at RT. The supernatant solution was collected into a new tube to check the absorbance at 530 nm and 657 nm by anthocyanin measurement and chlorophylls respectively thru spectrophotometer (model UV–1800, Shimadzu corporation, Japan). Then by using the following formula the anthocyanin contents were measured by correction in the 530 nm absorbance by chlorophylls:

TAC *(mg/ 100g of dried weight)* = (absorbance at 530 nm - (0.25 × absorbance at 657 nm) × extraction volume (mL) × 1 /weight of leave tissue sample (g).

*For anthocyanin we used 5 times extraction volume and 0.1 gram leave tissue sample.

2.11. Antioxidant capacity and activity (DPPH free radical scavenging assay)

For antioxidant capacity and activity fresh Arabidopsis leaves were ground (100 mg) and homogenized in 1 mL of extraction solution (ethanol, water, and acetic acid, 70%, 29%, and 1% respectively) and then centrifuged [54] with slight modifications. The supernatant was used to calculate antioxidant capacity by using the 30 microliter of above prepared solution followed by addition of 2.97 mL of 0.1-mM 2,2-diphenyl–1-picrylhydrazyl (DPPH) followed by 30 min incubation in dark (in RT). Then the sample absorbance was taken at 517 nm by means of spectrophotometer (model UV–1800, Shimadzu corporation, Japan). 30 microliter of extraction solution (without plant sample) in 2.97 mL of DPPH is used as control. The antioxidant capacity was calculated by generating standard curve of trolox and samples were expressed in (mM Trolox/ 100 mg). While the antioxidant (free radical scavenging) activity is described by using the following formula:

Antioxidant activity (%) = [1–{ sample OD/control OD}] × 100.

2.12 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide was measured by using trichloro-acetic acid (TCA) method [55]. Ground leaves samples (0.1 g) were re-suspended in 1000 microliter of TCA (0.1%) solution in an ice bath and then centrifuged for 10 min at 10,000 rpm. the 500 microliter of supernatant was taken into a new tube and added 500 microliter of 10-mM potassium phosphate buffer followed by addition of 1000 microliter of 1 M potassium iodide (KI) then mix well and checked the absorbance reading at 390 nm by means of spectrophotometer (model UV–1800, Shimadzu corporation, Japan). The sample absorbance was

calculated by comparing the standard curve absorbance of commercial H_2O_2. The H_2O_2 contents were expressed in micromoles/g of dried samples.

2.13 Statistical analysis

The Statistix 8.1 (Tallahassee Florida, USA) statistical package was used to analyze the data. The standard error and graphs were made by using Microsoft Excel 2010 program (Microsoft Corp., Redmond, WA, USA). Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

List Of Abbreviations

ROS: Reactive oxygen species

PAs: Proanthocyanidins

qRT-PCR: Quantitative real time PCR

HLS: High light stress

OX: Overexpression

DPPH: 2,2-diphenyl–1-picrylhydrazyl

H_2O_2: Hydrogen peroxide

TAC: Total Anthocyanin contents

TFC: Total flavonoid content

TPC: Total phenolics contents

FCR: Folin–Ciocalteu reagent

PAC: Proanthocyanidins contents

GA: Gallic acid

CE: Catechin equivalents

µg: microgram

RT: Room temperature

rpm: Rounds per minute

G: gram

Mg: milligram

mL: Milliliter

M: Molar

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

MJR planned and designed the research; YX produced plant material; MJR performed the experiments, MJR and TX, YHcollected and analyzed the data; MJR, XD, QX interpreted the results, MJR wrote the manuscript, QX revised the manuscript. All authors have read and approved the final manuscript.

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Authors' information (optional)

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Figures

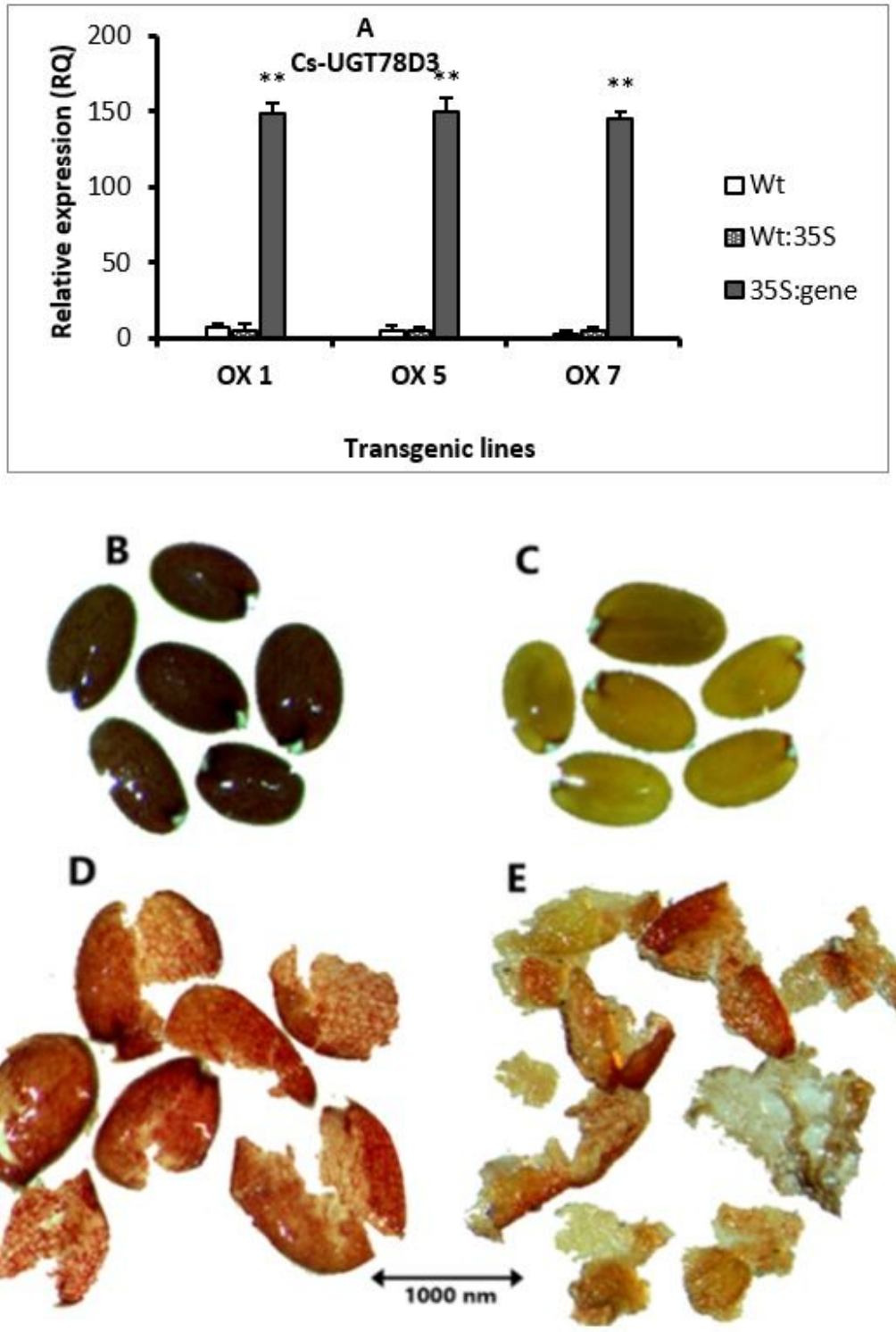

Figure 1

(A) Showing the overexpression of Cs-UGT78D3 genes in three individual lines (B) Group of OX T4 generation seeds showing dark brown color (C) Group of wild type pale yellow color of seed (D) Vanillin assay of OX seed coats showing red color on each segment of seed coat (E) Wild type seed coats showing little red color on margins while the rest of seed coat color unchanged/pale yellow.

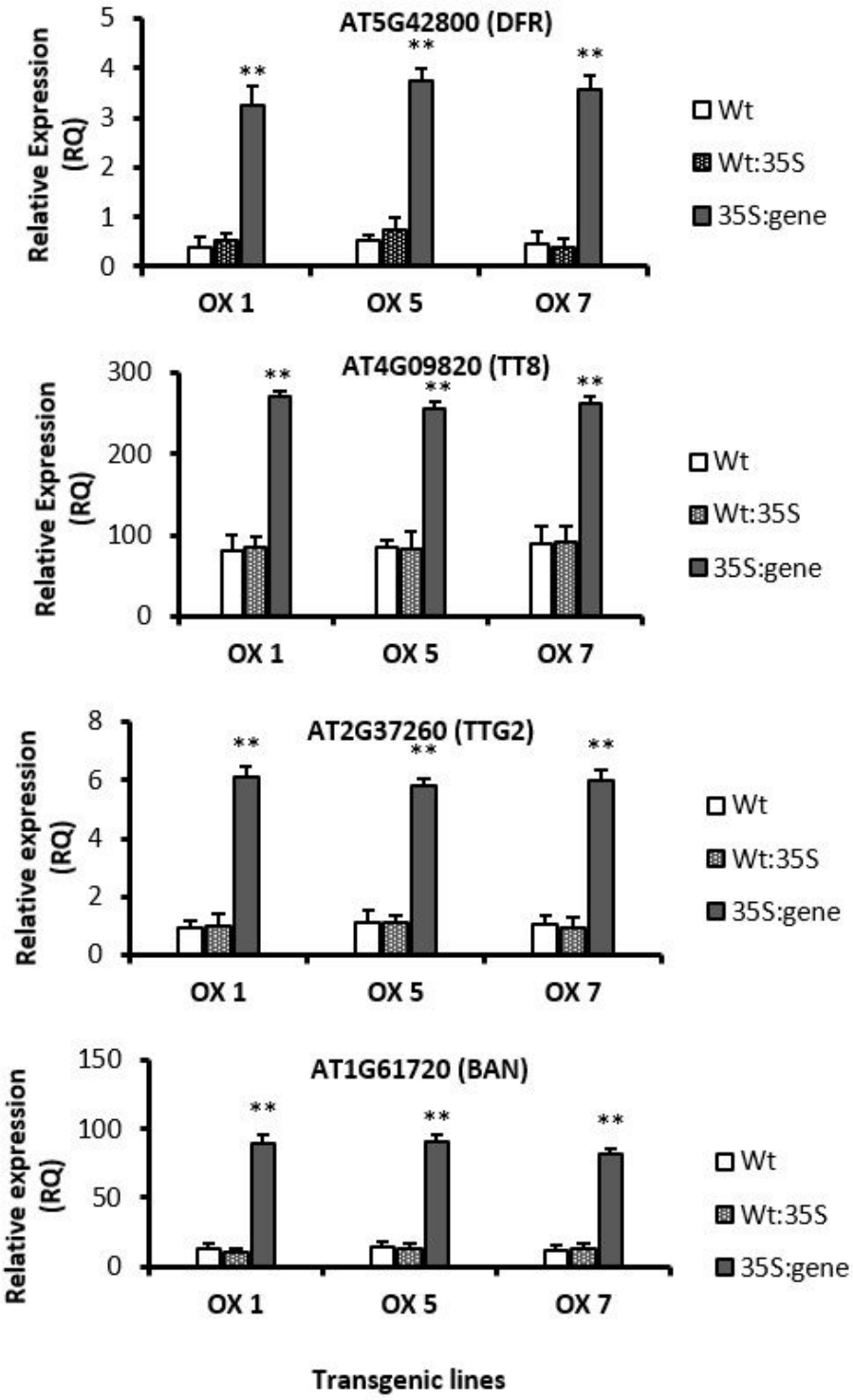

Figure 2

Genes involved in the regulation of Anthocyanins and proanthocyanidins biosynthesis upregulated in the transgenic Arabidopsis. Values are mean of three replicates ± SE at $p < 0.05$. Gene IDs were taken from Arabidopsis genome website TAIR (https://www.arabidopsis.org/). TT3 or DFR: Dihydroflavonol 4-reductase; TT8: Transparent testa 8; TTG2: Transparent testa glabra 2; BAN: BANYULS; OX: Overexperessed lines.

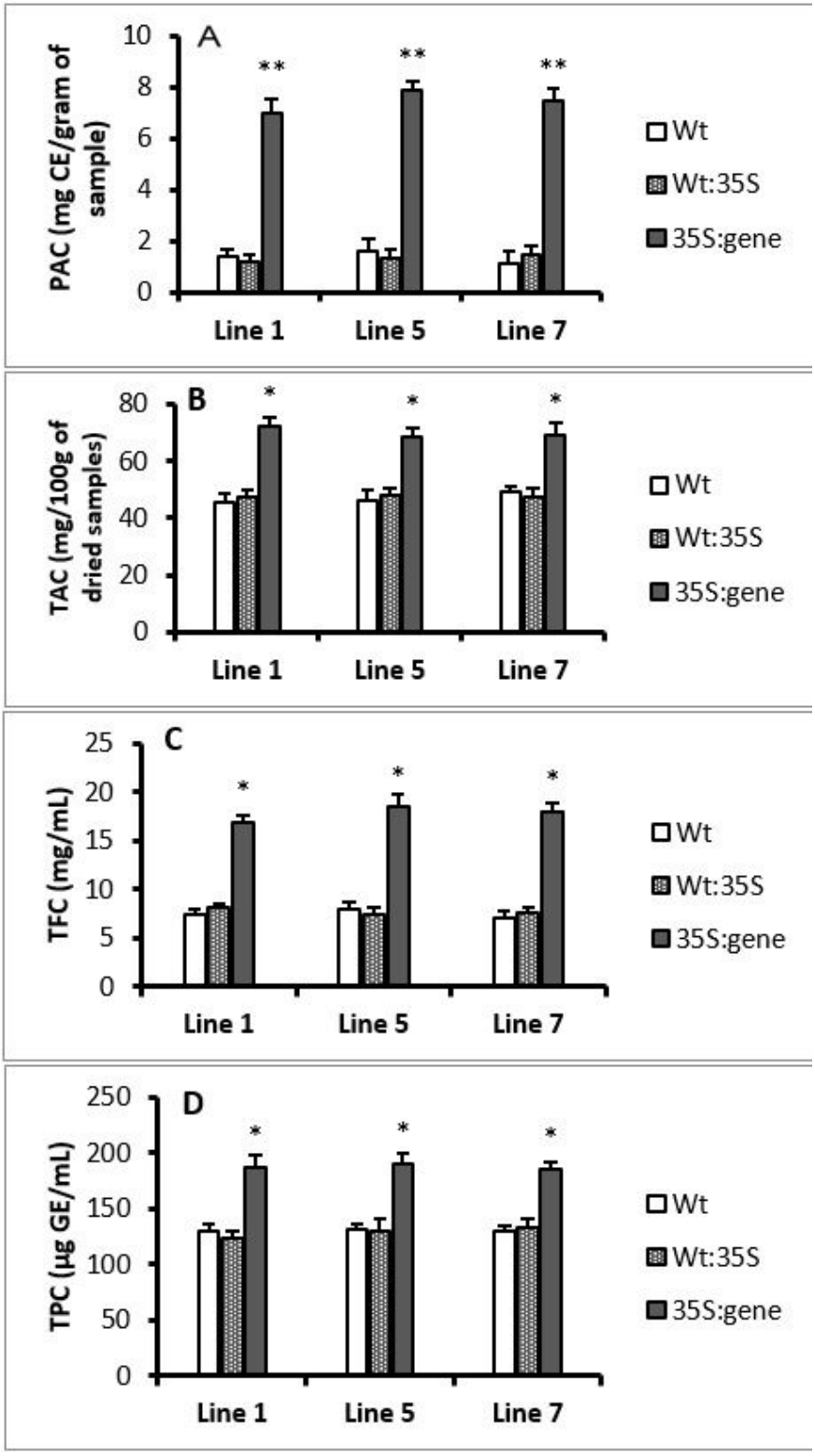

Figure 3

Proanthocyanins, anthocyanins, flavonoid and phenolic were significantly increased in transgenic lines. Values are mean of three replicates ± SE at $p < 0.05$. (*) Significant. A; Total anthocyanin contents (TAC) in leaves, B; Total flavonoid contents (TFC) in leaves, C; Total phenolic contents (TPC) in leaves, D; Proanthocyanidins contents (PAC) in seeds.

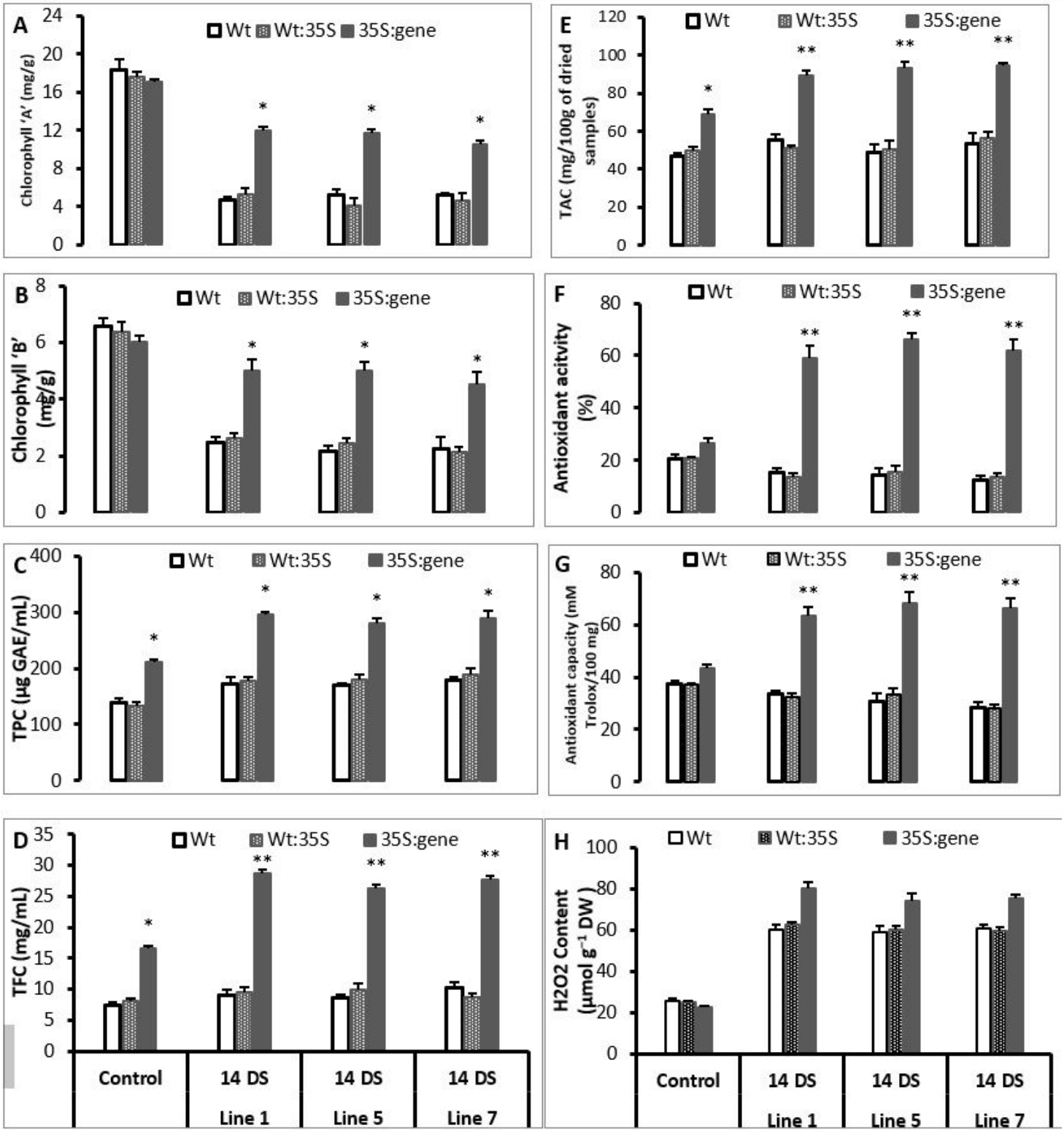

Figure 4

High light treatment differentially altered the biochemical and metabolic related parameters. Values are mean of three replicates ± SE at $p < 0.05$. (*) Significant: (**) Highly significant. A; Chlorophyll A, B; Chlorophyll B, C; Total phenolic contents (TPC), D; Total flavonoid contents (TFC), E; Total anthocyanin contents (TAC), F; Antioxidant activity, G; Antioxidant capacity, H; Hydrogen peroxide (H2O2 content); Control: Day 1; 14 DS: after 14 days light stress.

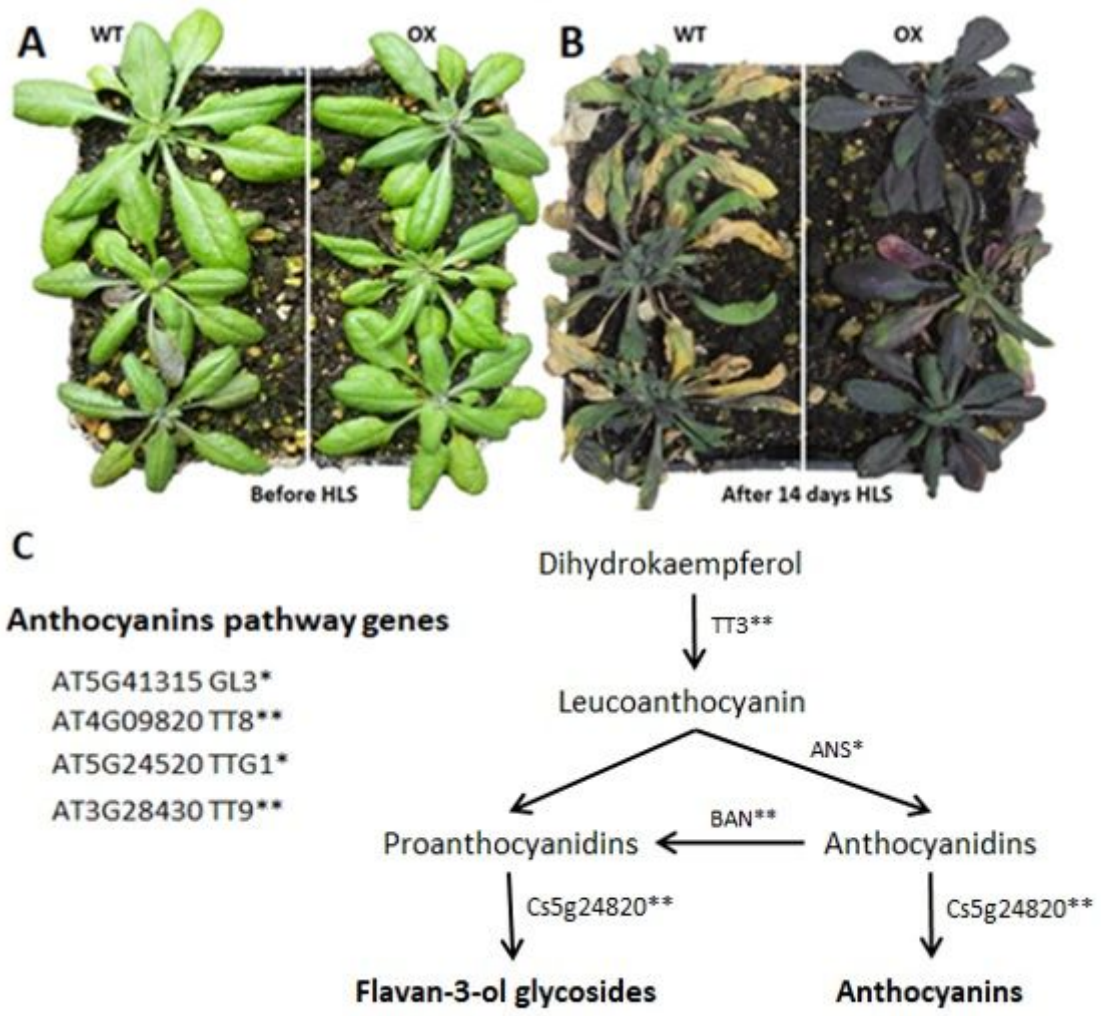

Figure 5

(A) 25 days old three wild type (WT) and three overexpressed (OX) Arabidopsis plants (B) After 14 days of high light stress WT and OX plants (C) Hypothesized functional step of citrus UDP-GLUCOSYL TRANSFERASE gene (Cs5g24820). (Metabolic pathway has been made from KEGG www.genome.jp/kegg/pathway; TAIR https://www.arabidopsis.org/ and [19]. (*) Significant: (**) Highly significant. TT3 or DFR: Dihydroflavonol 4-reductase; TT8, TT9: Transparent Testa 8, 9; ANS: anthocyanidin synthase; BAN: Banyuls; TT9 and TTG1: triggers the accumulation of brown colored proanthocyanidins; GL3: TT8 and GL3 are basic helix-loop-helix (bHLHs) transcription factors that influence anthocyanin accumulation.

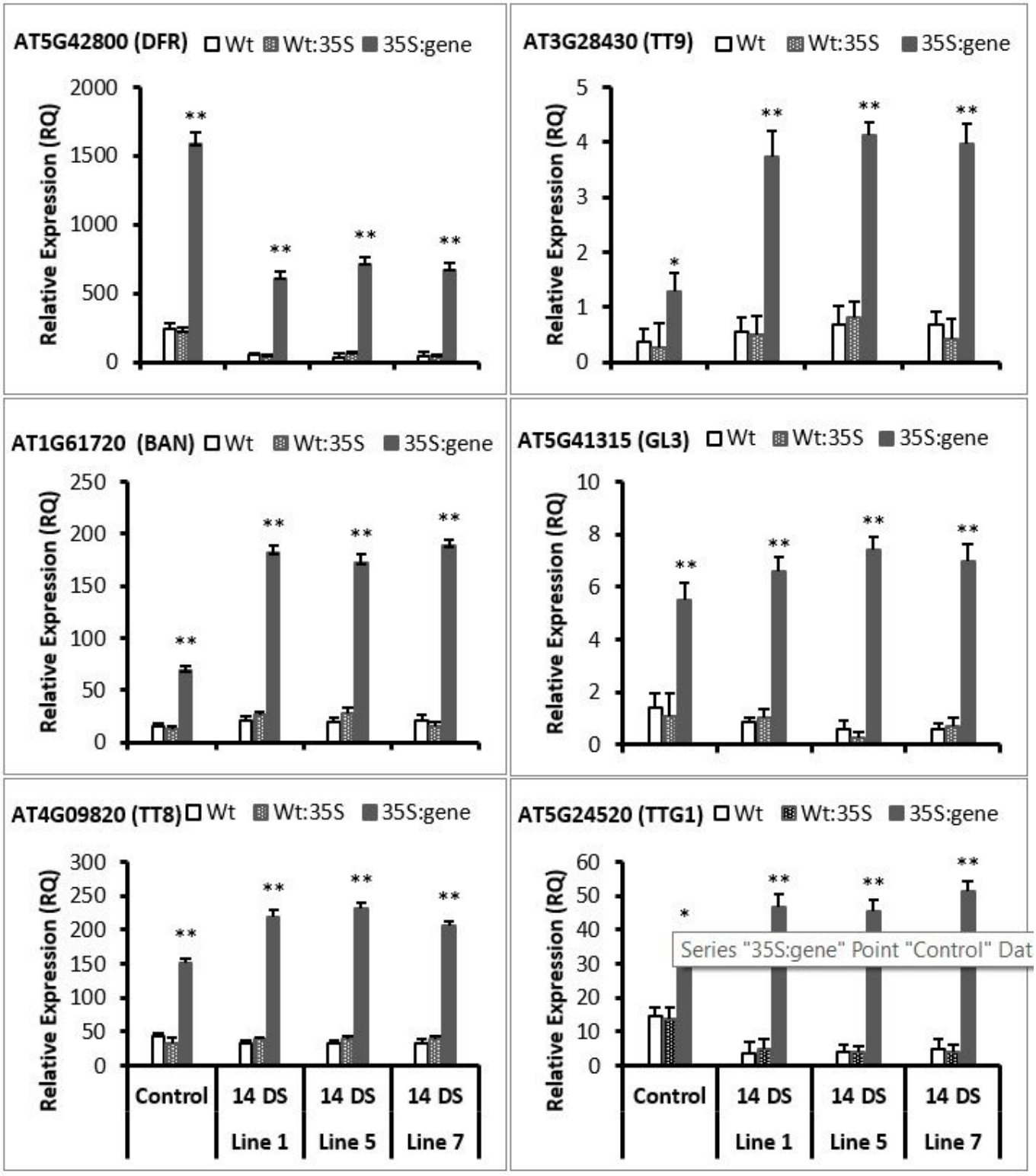

Figure 6

Genes involved in the regulation of Flavonoids and Anthocyanins biosynthesis were upregulated in the transgenic Arabidopsis after light stress treatment. Values are mean of three replicates ± SE at $p < 0.05$. (*) Significant: (**) Highly significant. Gene IDs were taken from Arabidopsis genome website TAIR (https://www.arabidopsis.org/).

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