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Hormonal modulation of photomorphogenesis-controlled anthocyanin accumulation in tomato (*Solanum lycopersicum* L. cv Micro-Tom) hypocotyls: Physiological and genetic studies

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ABSTRACT

Hormones are likely to be important factors modulating the light-dependent anthocyanin accumulation. Here we analyzed anthocyanin contents in hypocotyls of near isogenic Micro-Tom (MT) tomato lines carrying hormone and phytochrome mutations, as single and double-mutant combinations. In order to recapitulate mutant phenotype, exogenous hormone applications were also performed. Anthocyanin accumulation was promoted by exogenous abscisic acid (ABA) and inhibited by gibberellin (GA), in accordance to the reduced anthocyanin contents measured in ABA-deficient (*notabilis*) and GA-constitutive response (*procera*) mutants. Exogenous cytokinin also enhanced anthocyanin levels in MT hypocotyls. Although auxin-insensitive *diageotropica* mutant exhibited higher anthocyanin contents, pharmacological approaches employing exogenous auxin and a transport inhibitor did not support a direct role of the hormone in anthocyanin accumulation. Analysis of mutants exhibiting increased ethylene production (*epinastic*) or reduced sensitivity (*Never ripe*), together with pharmacological data obtained from plants treated with the hormone, indicated a limited role for ethylene in anthocyanin contents. Phytochrome-deficiency (*aurea*) and hormone double-mutant combinations exhibited phenotypes suggesting additive or synergistic interactions, but not fully espistatic ones, in the control of anthocyanin levels in tomato hypocotyls. Our results indicate that phytochrome-mediated anthocyanin accumulation in tomato hypocotyls is modulated by distinct hormone classes via both shared and independent pathways.

1. Introduction

An important strategy allowing seedling survival during early development is the photoprotection promoted by anthocyanin accumulation in hypocotyls [1]. Light is the major factor controlling anthocyanin accumulation, and, therefore, the role of photoreceptors in the biosynthesis of the flavonoid pigment has been closely investigated [2–5]. Similarly, it has also been reported the effect of certain hormone classes on anthocyanin accumulation [5–7]. Current evidence suggests that hormones participate in light transduction pathways controlling anthocyanin accumulation; or, alternatively, that hormones and photoreceptors share common molecular targets regulating the response. Although the presence of several crosstalk points between hormones and light signaling is well characterized for some developmental responses, such as seed germination, hypocotyls elongation, de-etiolation and flowering [8–14], little is known about the role of the main hormone classes in anthocyanin accumulation and its interplay with light.

Tomato is an attractive model system to study light and hormone interactions controlling anthocyanin accumulation, due to the promptly observed anthocyanin-pigmented hypocotyls and the availability of several phytochrome [15] and hormone mutants [16–22]. To allow in-depth studies in a uniform genetic background, we recently introgressed several mutations associated to light- and hormone-responses into the tomato Micro-Tom (MT) cultivar [23]. Initially developed as an ornamental cultivar [24], MT exhibits many common features to the current model-plant *Arabidopsis thaliana*, such as reduced size and short life-cycle [25,26]. The available MT mutant collection represents a community genetic resource, proved to be useful in the study of the role of hormones in plant responses to environmental factors such as heavy metals [27], herbivory [28] and mycorrhizal association [29]. In the present work, we used the mentioned mutant collection in MT genetic background to perform single and double-mutant analyses, as well as exogenous hormone application, in the investigation of the role of the major hormone classes in

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Table 1
Photomorphogenic and hormonal mutants introgressed in the Micro-Tom cultivar used in this work.

Mutant[a]	Class[b]	Genetic function	Main phenotypical features	Reference
diageotropica (*dgt*)	AUX	Reduced sensitivity. Defective for a cyclophilin	Agravitropic roots lacking lateral initiation. Hyponastic leaves	[16]
notabilis (*not*)	ABA	Reduced levels of ABA. Defective in the carotenoid cleavage enzyme (NCED)	Severe loss of water under high temperatures. Dark leaves	[18]
sitiens (*sit*)	ABA	Reduced levels of ABA. Defective in ABA-aldehyde oxydase	Phenotype similar to *not* mutant, but more severe	[17]
epinastic (*epi*)	ET	Ethylene high-producer. Unknown gene function	Severely epinastic leaves	[20]
Never ripe (*Nr*)	ET	Reduced sensitivity. Defective for an ethylene receptor	Impaired fruit ripening. Delayed petal abscission	[21]
procera (*pro*)	GA	GA-constitutive response. Loss-of-function in the DELLA repressor domain	Increased plant height. Reduced lobe formation in the main leaflets	[22]
aurea (*au*)	–	Phytochrome-deficient. Defective for the *PHYTOCHROMOBILIN SYNTHASE* gene	Chlorotic leaves and elongated stem	[62]
high pigment1 (*hp1*)	–	Increased response to light. Defective for the *DDB1A* protein, a repressor of photomorphogenesis	Dark green leaves and reduced height of light-grown plants	[74]

[a] Name of genotype initialized in capital letters denote dominant alleles.
[b] AUX: auxin; ET: ethylene; ABA: abscisic acid; GA: gibberellin.

phytochrome-driven anthocyanin accumulation in tomato hypocotyls.

2. Materials and methods

2.1. Plant material and growth conditions

Tomato (*Solanum lycopersicum* L.) lines of single mutants exhibiting hormonal and photomorphogenic alterations (Table 1) were kindly provided by R. Chetelat (The C.M. Rick Tomato Genetics Resource Center, Davis, USA). These genotypes were introgressed into the cultivar Micro-Tom (kindly provide by A. Levy from Weizmann Institute of Science, Israel) through successive backcrosses (BCs), which results in near-isogenic lines after the BC6F2 generation [30]. The crosses, backcrosses and phenotypical screening procedures used in the introgression of mutations and double mutant production were as described previously [29,31,32].

General-purpose growth of mutant plants for seed production was carried out in a greenhouse under automatic irrigation (four times a day), average mean temperature of 28 °C, 11.5 h/13 h (winter/summer) photoperiod, and 250–350 $\mu mol\ m^{-2}\ s^{-1}$ PAR irradiance (natural radiation reduced with a reflecting mesh (Aluminet–Polysack Industrias Ltda, Itápolis, Brazil). Seeds were sown in trays containing a 1:1 mixture of commercial substrate (Plantmax HT, Eucatex, Brazil) and expanded vermiculite, supplemented with $1\ g\ L^{-1}$ 10:10:10 NPK and $4\ g\ L^{-1}$ lime ($MgCO_3$ + - $CaCO_3$). Ten days after germination, plants were transferred to 150-mL pots containing the described soil mix and fertilizer.

2.2. Hormone treatment

Seeds were germinated on filter paper moistened with distillated water. After radicle protrusion, the seeds were transferred to light (16 h photoperiod, $55\ \mu mol\ m^{-2}\ s^{-1}$ PAR, 25 °C) and incubated for 10 days on filter paper soaked with hormone solutions: 10^{-7} to 10^{-4} M of auxin (naphthalene acetic acid – NAA, SIGMA), auxin transport inhibitor (2,3,5-triiodobenzoic acid - TIBA, SIGMA), cytokinin (Thidiazuron, TDZ, Bayer CropScience) and abscisic acid (ABA, SIGMA); 10^{-6} to 10^{-4} M of gibberellin (GA_3, ProGibb, SUMITOMO) and 10^{-4} to $10^{-1}\ g\ L^{-1}$ of CEPA (chloroethylphosphonic acid, Ethrel, Bayer CropScience).

2.3. Anthocyanin contents determination

Anthocyanin determination was performed according to Peters et al. [2]. Briefly, five hypocotyls from 10-day-old seedlings were excised and extracted with 0.48 mL acidified methanol (1% HCl, w/v) for 48 h in the dark with shaking. Liquid-liquid partitioning was performed by adding 0.36 mL H_2O and 0.96 mL chloroform to the extracts and centrifuging the mixture for 30 min at 2000 × *g*. The absorbance of the top phase was determined by spectrophotometry at 535 nm (A_{535}). Anthocyanin measurements were performed using whole hypocotyls and the results were expressed as means of at least three replicates ($n = 3$), consisting of five hypocotyls. Data were compared using the Student's *t* test.

3. Results

3.1. Anthocyanin accumulation in hypocotyls of tomato mutants

Hypocotyls are advantageous and intensively used in anthocyanin analysis [2,3], partially due to the rapid and easy growth of seedlings under controlled conditions in comparison to adult plants. Moreover, hypocotyls facilitate sampling of the entire organ, thus, avoiding indirect pigment dilution or concentration due to variations in cell size and number. We have analyzed the anthocyanin content in hypocotyls of mutants exhibiting alterations in four hormone classes; namely, ABA (*not* and *sit*), auxin (*dgt*), GA (*pro*) and ethylene (*Nr* and *epi*). We also used the photomorphogenic mutants *au* (defective in phytochrome-mediated light perception) and *hp1* (exaggerated photoresponse), shown to exhibit, respectively, reduced and increased anthocyanin accumulation [15]. The mutations investigated in this study were introgressed in MT genetic background and their main phenotypical features and affected gene functions are summarized in Table 1.

The mutations *hp1* and *au* significantly ($p < 0.001$) increased and reduced anthocyanin accumulation in comparison to MT, respectively (Fig. 1), as observed in previous works [2,15,33]. The auxin-insensitive *dgt* mutation caused a significant ($p < 0.05$) increase in anthocyanin contents in comparison to MT (Fig. 1). In the original description of the *dgt* mutant by Zobel [34], increased anthocyanin contents in hypocotyls were observed, although in-depth investigation was still lacking. Three hormonal mutations significantly reduced anthocyanin contents in comparison to MT: *not* (ABA-deficient), *Nr* (ethylene-insensitive) and *pro* (GA-constitutive). Anthocyanin contents in the hypocotyls of ethylene over-producer mutant *epi* were not significantly distinct from those of MT (Fig. 1).

3.2. Anthocyanin accumulation upon exogenous hormone application

In order to further dissect the hormonal modulation of light-controlled anthocyanin accumulation in tomato hypocotyls, we

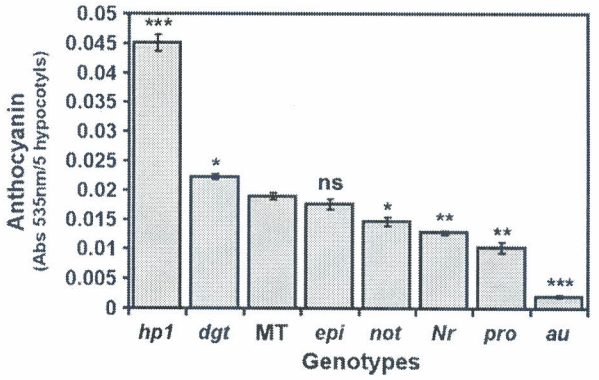


Fig. 1. Anthocyanin accumulation in hypocotyls of hormone and photomorphogenic mutants. Seedlings were grown for 10 days in the light. Data shown represent means ± SE ($n = 3$ dosages using five hypocotyls per treatment). Significant differences, according to Student's t-test comparing each mutant to the Micro-Tom (MT) control are indicated as follows: $^{*}p < 5\%$; $^{**}p < 1\%$; $^{***}p < 0.1\%$ and ns = non-significant. A description of hormone alterations exhibited by the genotypes is displayed in Table 1.

have applied exogenous hormones to light-grown (55 μmol m^{-2} s^{-1} white light, 16 h photoperiod) seedlings of the photomorphogenic mutants *hp1* and *au*. We used the high-anthocyanin producing mutant *hp1* to test exogenous NAA and GA_3 (Fig. 2), which are predicted to reduce the pigment contents,

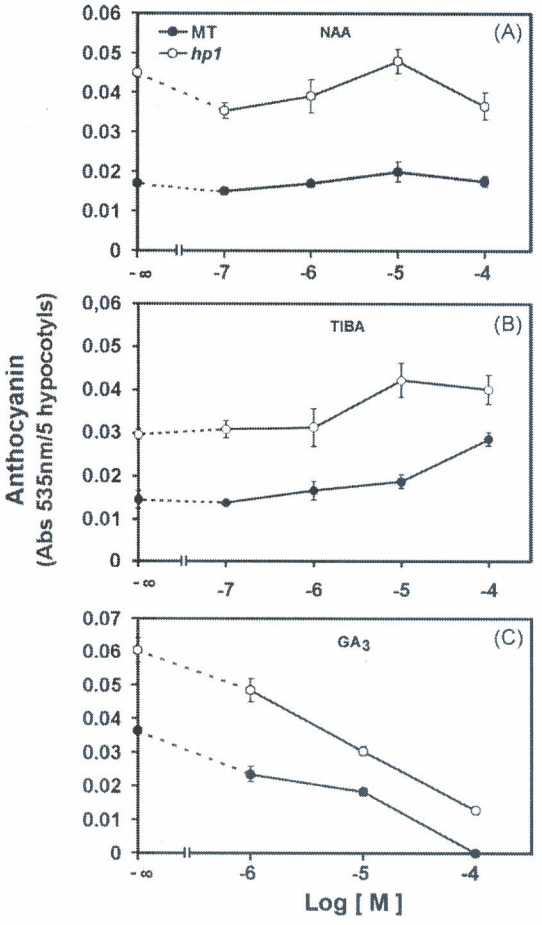


Fig. 2. Anthocyanin accumulation in hypocotyls of Micro-Tom (MT) and the photomorphogenic mutant *high pigment1* (*hp1*) upon exogenous application of auxin (A), auxin transport inhibitor (B) and gibberellin (C). After radicle protusion, seedlings were incubated during 10 days in filter papers soaked with naphthalene acetic acid (NAA), 2,3,5-triiodobenzoic acid (TIBA) and gibberellic acid (GA_3). Data are means ± SE ($n = 3$ replicates consisting of five hypocotyls).

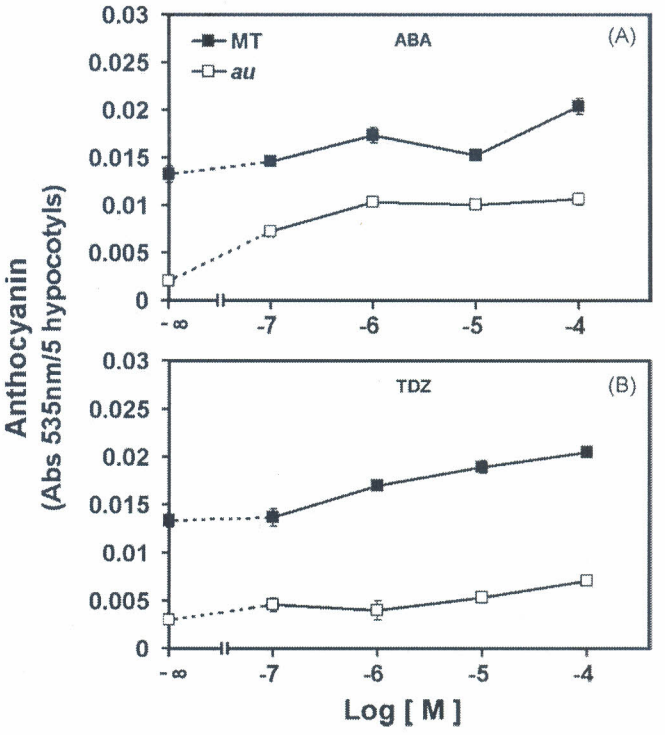


Fig. 3. Anthocyanin accumulation in hypocotyls of Micro-Tom (MT) and the photomorphogenic mutant *aurea* (*au*) upon exogenous application of abscisic acid (ABA) (A) and cytokinin (B). After radicle protusion, seedlings were incubated during 10 days in filter papers soaked with ABA or Thidiazuron (TDZ). Data are means ± SE ($n = 3$ replicates consisting of five hypocotyls).

according to the results for *dgt* and *pro* (Fig. 1), respectively. Similarly, the mutant *au*, which displays reduced anthocyanin contents, received exogenous ABA (Fig. 3A), as the reduced anthocyanin levels observed in *not* hypocotyls (Fig. 1) indicates that ABA application may enhance anthocyanin accumulation. Since exogenous auxins, such as NAA, usually do not recapitulate the effect of the endogenous forms of the hormone [35], we also used the specific auxin-transport inhibitor TIBA (Fig. 2B). In TIBA-treated *hp1* seedlings, the hypocotyls would be devoid of endogenous auxin, due to the impairment of the transport from the cotyledons. Although, fully-characterized cytokinin mutants in tomato are still lacking, previous evidences indicate that cytokinin induces anthocyanin in *Arabidopsis* hypocotyls [7]. Thus, we treated *au* and MT seedlings with exogenous TDZ (Fig. 3B). Ethrel was used in both photomorphogenic mutants *hp1* and *au*, as well as in *Nr* mutant and MT controls (Fig. 4), to further investigate the results observed for ethylene-mutants *epi* and *Nr* (Fig. 1).

The exogenous hormones tested were unable to recapitulate the wild-type phenotype of *au* and *hp1* mutants, as evidenced by the parallel dose-response curves in comparison to MT (Figs. 2 and 3). These observations indicate that light is the major regulator of anthocyanin accumulation in tomato hypocotyls and suggest that the hormones function as modulators of the response, probably acting on parallel pathways or sharing molecular signaling partners. Exogenous NAA was unable to reduce anthocyanin contents in MT and *hp1* (Fig. 2A); thus, failing to corroborate the increased anthocyanin levels observed in auxin-insensitive *dgt* mutant (Fig. 1). However, TIBA treatments increased anthocyanin contents in both *hp1* and MT (Fig. 2B). The application of 10^{-4} M TIBA phenocopied the anthocyanin accumulation of *dgt* mutant in wild-type MT seedlings (Fig. 1). GA application caused a prominent reduction in hypocotyl anthocyanin in MT and *hp1* (Fig. 2C). These observations are in accordance with the results observed for the GA-constitutive response *pro* mutant (Fig. 1), suggesting that this

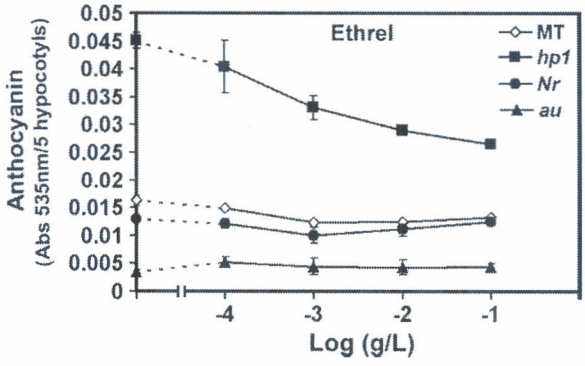


Fig. 4. Anthocyanin accumulation in tomato hypocotyls upon exogenous application of an ethylene releaser (Ethrel). The effect of ethylene was tested in Micro-Tom (MT), the photomorphogenic mutants *hp1* and *au*, as well as in the ethylene insensitive mutant *Never ripe* (*Nr*). Data are shown as means ± SE ($n = 3$ replicates consisting of five hypocotyls).

hormone mediates a repression pathway for anthocyanin accumulation in tomato hypocotyls. Applications of ABA at 10^{-6} M increased the levels of anthocyanin in MT plants, and even more substantially at 10^{-4} M (Fig. 3A), which is consistent with the low contents of the pigment observed in hypocotyls of the ABA-deficient mutant, *not* (Fig. 1). In phytochrome mutant *au*, anthocyanin levels were induced by the application of 10^{-6} M ABA, until stabilization at 10^{-4} M (Fig. 3A), providing evidence of an ABA-mediated induction pathway that modulates anthocyanin accumulation in tomato hypocotyls. The application of exogenous cytokinins increased anthocyanin accumulation in MT (Fig. 3B), reaching levels similar to those observed in *dgt* (Fig. 1). Anthocyanin accumulation was unaffected by ethylene application in MT and *au* (Fig. 4). A similar response was observed in the ethylene-insensitive mutant *Nr* (Fig. 4). However, in *hp1* hypocotyls the decrease in anthocyanin contents was proportional to the increase in Ethrel concentration (Fig. 4).

3.3. Anthocyanin accumulation in double mutants

Mutant analysis and exogenous hormone application indicate that GA and ABA are important modulators of anthocyanin accumulation in tomato hypocotyls. Moreover, we have confirmed that light functions as the main factor controlling anthocyanin contents in tomato hypocotyls. Therefore, to further dissect the hormonal modulation of light-controlled anthocyanin accumulation, double-mutant combinations of phytochrome-defective *au* and hormone mutants were employed (Fig. 5).

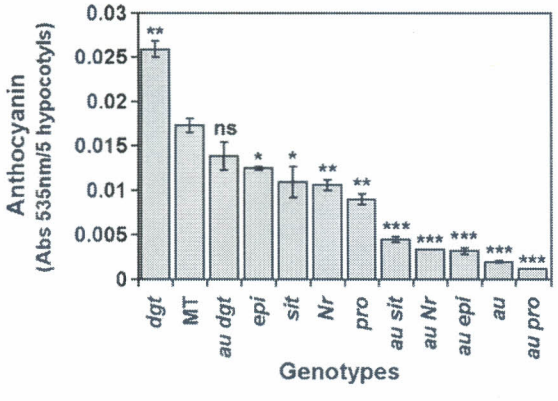
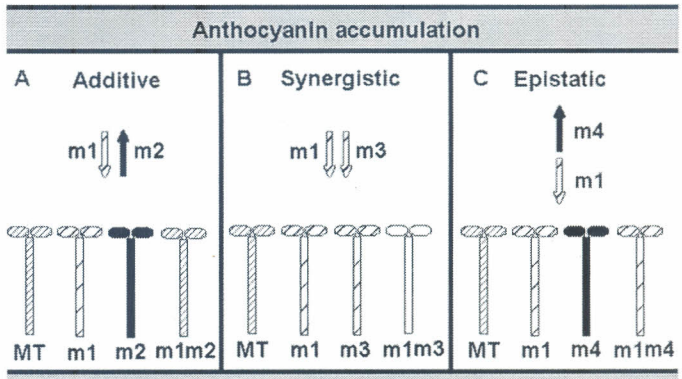


Fig. 5. Anthocyanin accumulation in hypocotyls of tomato single- and double-mutants, the later combining phytochrome-deficiency (*au*) and hormonal alterations. Data are shown as means ± SE ($n = 3$ dosages using five hypocotyls per treatment). Significant differences, according to Student's *t*-test, comparing each mutant with the Micro-Tom (MT) control were indicated as follows: $^{*}p < 5\%$; $^{**}p < 1\%$; $^{***}p < 0.1\%$ and ns = non-significant. For comparisons between single- and double-mutants, please refer to Table 2.



Genotype	Type of mutation	Type of interaction
au epi	phy⁻ and ET⁺	Additive
au pro	phy⁻ and GA⁺	Synergistic
au Nr	phy⁻ and ET⁻	Additive
au dgt	phy⁻ and AUX⁻	Additive
au sit	phy⁻ and ABA⁻	Additive

Fig. 6. Summary of the interactions between phytochrome and hormones as observed in double-mutants combining phytochrome-deficiency (*au*) and hormonal alterations. Additive (A) and synergistic (B) interactions indicate independent (parallel) pathways. In both cases, the double mutant combines the effects of each single mutant. In epistatic interactions (C), the double mutant will have the phenotype of one of the single mutants, meaning that both mutations control the response via a shared pathway. In the present work, only additive and synergistic interactions were observed. phy = phytochrome; AUX = auxin; ET = ethylene; ABA = abscisic acid; GA = gibberellin.

In the physiological interpretation of double-mutant phenotypes, we have used the terms additive or synergistic interactions to denote phenotypes distinct from the parents. Additive phenotypes are well observed when parents have opposite effects (Fig. 6A) and synergistic ones when parents have similar effects (Fig. 6B). Both interactions are best interpreted as the effect of different perturbations caused by each mutant in parallels (independent) pathways (Fig. 6A and B). On the other hand, when the double mutant assumes the phenotype of one of the single mutant parent, the interaction is termed epistatic, which may indicate perturbations in the same or in interdependent pathways (Fig. 6C).

As shown in Fig. 5, the presence of *au* mutation markedly decreases anthocyanin levels in all double-mutant combinations

Table 2
Summary of Student's *t*-test for the anthocyanin accumulation in the hypocotyls of tomato double mutants in comparison to single mutants. *p*-values lower than 0.05 denote significant differences.

Comparisons between genotypes	*p*-value
au pro × *au*	0.005
au pro × *pro*	<0.005
au sit × *au*	<0.05
au sit × *sit*	>0.05
au Nr × *au*	<0.001
au Nr × *Nr*	<0.005
au epi × *au*	<0.001
au epi × *epi*	<0.001
au dgt × *au*	<0.05
au dgt × *dgt*	0.005

tested. Interestingly, a significant decrease in anthocyanin content, in comparison to the *au* single mutant, was only observed in hypocotyls of the double mutant *au pro* (Fig. 5, Table 2); thus, indicating a synergistic interaction (Fig. 6B) between the mutations *pro* and *au*. Anthocyanin contents in double mutants *au dgt*, *au Nr* and *au epi* (Fig. 5) were significantly different from both parents (Table 2), suggesting additive interactions (Fig. 6A). The accumulation of the investigated pigment in *au sit* double mutant, although indistinct from the observed in the *sit* parent (Table 2), was slightly different from that found in the *au* parent, also suggesting an additive interaction.

4. Discussion

4.1. ABA positively and GA negatively modulate anthocyanin accumulation in tomato hypocotyls

Genetic and physiological approaches, such as hormone mutants and exogenous applications, indicated that ABA and GA function as positive and negative modulators, respectively, of anthocyanin accumulation in tomato hypocotyls. In contrast, our results provided no evidence of a direct role for auxin and ethylene in modulating the process.

Several lines of evidence support the induction of anthocyanin biosynthesis by ABA. Our results are in accordance with observations that ABA application enhances anthocyanin contents in grapevine leaves [36] and torenia shoots [37]. Moreover, in beans [38] and strawberry [39], ABA induces phenylalanine ammonia-lyase (PAL), a key enzyme for the biosynthesis of anthocyanin and other phenolic compounds. However, Guo and Wang [40] demonstrated that the expression of a tomato *PAL* gene (*SlPAL5*) was not increased by ABA. This indicates that some PAL genes might be regulated by ABA and others might be ABA-independent. In Arabidopsis, it has been demonstrated that *fus3* and *abi3-4* mutants, which present reduced ABA levels and sensitivity [41], shown enhanced levels of anthocyanins [42]. Although this may indicate that ABA is as negative regulator of anthocyanin accumulation in Arabidopsis, the ABA-hypersensitive *elongator* mutant, which downregulates a *MYB* transcription factor that is a negative regulator or anthocyanin biosynthesis, presents a high anthocyanin accumulation [43]. Thus, it is likely that other developmental or environmental responses present in *abi3-4* and *fus3* mutants may be influencing anthocyanin accumulation. In tomato, an enhanced anthocyanin accumulation was also observed in the hypocotyls of a mutant thought to be ABA-hypersensitive and/or overproducer [44,45], corroborating our hypothesis that ABA is a positive regulator of anthocyanin biosynthesis.

The role of GA in hypocotyl anthocyanin accumulation has been the object of a smaller number of studies. In tomato, the effect of GA in the reduction of the anthocyanin in hypocotyls was initially shown by Perez et al. [6]. Later, van Tuinen et al. [46] have also provided evidences that GA is involved in anthocyanin production in tomato, since reduced levels of the flavonoid were observed in young leaves of the mutant *pro*. In other species, the application of GA has been shown to reduce [47,48] or to increase [49] anthocyanin contents, indicating that the response may be species-specific.

Interestingly, the antagonism between ABA and GA in anthocyanin accumulation resembles that often described for seed germination [50]. One possible explanation for the antagonism may involve the function of DELLA proteins, whose degradation is induced by GA and whose stabilization is possibly mediated by ABA [51]. Accordingly, a positive role for DELLA proteins in anthocyanin accumulation was demonstrated during phosphorus starvation in Arabidopsis [52].

4.2. Hormonal modulation of phytochrome-controlled anthocyanin contents in tomato hypocotyls

Light, as a main environmental regulator, plays a central role in controlling plant development [53]. Many light responses are controlled by manipulating the plant hormones, which function as master controllers of physiology, growth and developmental responses [54]. A large number of studies have identified links between light and several hormone classes, including auxin, gibberellins (GA), ethylene, cytokinin and brassinosteroids [55]. In this context, the coinciding phenotypes caused by light regimes and hormone treatments (e.g. the well-known similar etiolated aspect of GA-treated and dark-grown seedlings) are indicative of crosstalk between these signaling pathways [56]. Hypocotyls are excellent organs to study this question, considering their quick and obvious response to light/dark and hormones. Although distinct hormones can influence hypocotyl growth, GA has attracted the attention of scientists due to recent works showing that hypocotyl growth is positively regulated by phytochrome-interacting transcription factors (PIFs) and negatively regulated by DELLA proteins, which are GA-response repressors [12,13,57]. Thus, the increased hypocotyl growth in GA-treated and dark-grown seedlings can be interpreted, respectively, by the occurrence of GA-mediated DELLA degradation [58] and the absence of active phytochrome B (phyB)-mediated PIF inactivation [59]. Since PIFs are also repressed by DELLAs [12,13] and the repressor accumulates in light-grown hypocotyls due to the reduction of GA levels [58,60], it has now became clear that light and GA use shared pathways in the control of hypocotyl growth [12,13]. This crosstalk may also function for other GA and light responses, such as seed germination and anthocyanin accumulation. However, in *Arabidopsis* seeds, light induces GA accumulation [61], which is the opposite of the observed reduction of this hormone in light-growing hypocotyls [58]. For anthocyanin accumulation, our results showing that the GA-constitutive-response *pro* mutant and the phytochrome-deficient *au* mutant exhibited the coinciding phenotype of low anthocyanin, suggest the existence of shared signaling partners between light and GA in this response. This light-mediated anthocyanin accumulation may involve PIF proteins, as observed for Arabidopsis hypocotyl growth [12,13]. Thus, we can speculate that the reduced anthocyanin contents in *au*, which is deficient in all photoactive phytochromes due to the lack of the chromophore [62], may involve tomato PIF orthologs. Conversely, the low anthocyanin accumulation in *pro*, which is devoid of active DELLA proteins [22], indicates that the repressor is also important for anthocyanin accumulation in tomato. Consistently, Jiang et al. [52] demonstrate that active DELLAs are positive regulators of anthocyanin accumulation during phosphorous starvation in Arabidopsis. Our results showing a synergistic interaction between *au* and *pro* mutations in double mutants suggest that the perturbations leading to low anthocyanin accumulation in these mutants may be also acting on distinct signaling partners (Fig. 6). However, since some of the genotypes used in the present work may represent not complete loss-of-function alleles, the possibility that the observed synergistic phenotype is due to the sum of the effects of weak alleles in a shared pathway cannot be rule out.

Despite the evidences that light, via phytochrome, can negatively regulate the ABA activity [63,64] and that this hormone and light display interaction points in the control of gene expression [65], a few works have been dedicated to study the interaction for responses such as: de-etiolation [11] and anthocyanin accumulation [49]. Our results using *au* and ABA-deficient mutants, as well as double-mutants, indicate that anthocyanin accumulation in tomato hypocotyls is controlled by both ABA and phytochrome via, at least, partially independent pathways. The intermediary levels of anthocyanin in the double-mutant *au sit* indicate that, despite the fact that the final result in *sit* mutant is

the anthocyanin reduction in comparison to MT, ABA deficiency may also lead to anthocyanin accumulation, a response that is not reverted by phytochrome absence in the double *au sit*. Thus, it is possible to observe significantly higher anthocyanin contents in the double-mutant *au sit* in comparison to the levels found in the *au* single mutant (Table 2). Since ABA deficiency is known to induce stress responses [66], which also often lead to anthocyanin biosynthesis [1], it is likely that anthocyanin accumulation in *sit* single mutant is partially due to stress-related effects, not directly mediated by phytochrome.

In the present work, we postulated that the differences in anthocyanin accumulation exhibited by ethylene and auxin tomato mutants are indirect and likely attributed to other hormones or to developmental effects. For ethylene, the reduced levels of anthocyanin upon Ethrel application in *hp1* and the absence of observable effect in MT, *Nr* and *au* suggest the occurrence of unaccounted and ethylene-related developmental effects specific to *hp1*. Regarding auxin, since the increased anthocyanin levels in *dgt* hypocotyls was only corroborated by exogenous TIBA, but not by NAA, it is likely to involve other factor, such as an altered auxin-to-cytokinin balance. This is consistent with previous observations that constant cytokinin application in wild-type tomato plants replicates the phenotype of *dgt* mutant [67] and the observations that both auxin reduction (via TIBA application) and cytokinin application caused anthocyanin accumulation in tomato hypocotyls (Figs. 2B and 3B). In the case of NAA application, a favorable auxin-to-cytokinin balance might not be reached, due to other effects of the exogenous hormone [35].

Indirect effects of ethylene and auxin may interfere with pathways independent of those induced by light to promote anthocyanin accumulation; thus, explaining the additive phenotype observed between mutations affecting these hormones and phytochrome, in the *au* mutant background (Fig. 6). However, ethylene and phytochrome interactions have been demonstrated in other species controlling responses distinct from anthocyanin accumulation. Light has been demonstrated to function as a negative regulator of ethylene in the development of the plumular portion of pea seedlings [68] and of stem elongation in tobacco [69] and sunflower [70].

5. Concluding remarks

Here we have used tomato hypocotyls to dissect the role of four hormone classes, and their interaction with phytochrome and light signaling in anthocyanin accumulation. Our results showed that ABA and GA are important modulators of anthocyanin accumulation in tomato hypocotyls and that the response is likely to use phytochrome-controlled and independent pathways. The occurrence of independent pathways was also manifested in other pleiotropic responses controlled by hormones and phytochrome, since all double mutants produced here combined, in a single plant, distinct characteristics described for each parent (Table 1). These findings may also provide tools to combine the advantages of different pathways to manipulate agronomically important traits. Considering that flavonoids are currently regarded as highly desirable nutraceutical compounds [71], and that the anthocyanin phenotypes of tomato hypocotyls reflect the pigment accumulation in other plant organs [72,73], the dissection of the role of different hormone classes and their interactions exploiting the easiness of working with hypocotyls may aid further fine tuning of flavonoids in tomato fruits [74,75].

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References

[1] L. Chalker-Scott, Environmental significance of anthocyanins in plant stress responses, Photochem. Photobiol. 70 (1999) 1–9.
[2] J.L. Peters, A. Van Tuinen, P. Adamse, R.E. Kendrick, M. Koornneef, High pigment mutants of tomato exhibit high sensitivity for phytochrome action, J. Plant Physiol. 134 (1989) 661–666.
[3] L.H.J. Kerckhoffs, M.E.L. Schreuder, A. van Tuinen, M. Koornneef, R.E. Kendrick, Phytochrome control of anthocyanin biosynthesis in tomato seedlings: analysis using photomorphogenic mutants, Photochem. Photobiol. 65 (1997) 374–381.
[4] S.S.H. Husaineid, R.A. Kok, M.E.L. Schreuder, M. Hanumappa, M. Cordonnier-Pratt, L.H. Pratt, L.H.W. Van der Plas, A.R. Van der Krol, Overexpression of homologous phytochrome genes in tomato: exploring the limits in photoperception, J. Exp. Bot. 58 (2007) 615–626.
[5] F. Vandenbussche, Y. Habricot, A.S. Condiff, R. Maldiney, D.V. Straeten, M. Ahmad, HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in *Arabidopsis thaliana*, Plant J. 49 (2007) 428–441.
[6] A.T. Perez, W.H. Lachman, H.V. Marsh Jr., Physiology of the *yellow-green 6* gene in tomato: a possible interrelationship between the phenotypic expressions of the *yellow-green 6* gene mutation and the gibberellins 12, Plant Physiol. 53 (1974) 192–197.
[7] J. Deikman, P.E. Hammer, Induction of anthocyanin accumulation by cytokinins in Arabidopsis thaliana, Plant. Physiol. 108 (1995) 47–57.
[8] J. Chory, D. Reinecke, S. Sim, T. Washburn, M. Brenner, A role of cytokinins in de-etiolation in *Arabidopsis*: *det* mutants have an altered response to cytokinins, Plant. Physiol. 104 (1994) 339–347.
[9] J. Chory, M. Chatterjee, R.K. Cook, T. Elich, C. Fankhauser, J. Li, P. Nagpal, M. Neff, A. Pepper, D. Poole, J. Reed, V. Vitart, From seed germination to flowering, light controls plant development via the pigment phytochrome, Proc, Natl. Acad. Sci. U.S.A. 93 (1996) 12066–12071.
[10] S. Yamaguchi, Y. Kamiya, Gibberellins and light-stimulated seed germination, J. Plant Growth Regul. 20 (2002) 369–376.
[11] G.M. Symons, J.B. Reid, Interactions between light and plant hormones during de-etiolation, J. Plant Growth Regul. 22 (2003) 3–14.
[12] S. Feng, C. Martinez, G. Gusmaroli, Y. Wang, J. Zhou, F. Wang, L. Chen, L. Yu, J.M. Iglesias-Pedraz, S. Kircher, E. Schäfer, X. Fu, L.M. Fan, X.W. Deng, Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins, Nature 451 (2008) 475–480.
[13] M. de Lucas, J.M. Davière, M. Rodríguez-Falcón, M. Pontin, J.M. Iglesias-Pedraz, S. Lorrain, C. Fankhauser, M.A. Blázquez, E. Titarenko, S. Prat, A molecular framework for light and gibberellin control of cell elongation, Nature 451 (2008) 480–486.
[14] E. Mutasa-Göttgens, P. Hedden, Gibberellin as a factor in floral regulatory networks, J. Exp. Bot. 60 (2009) 1979–1989.
[15] R.E. Kendrick, L.H. Kerckhoffs, A. van Tuinen, M. Koornneef, Photomorphogenic mutants of tomato, Plant Cell Environ. 20 (1997) 746–751.
[16] K. Oh, M.G. Ivanchenko, T.J. White, T.L. Lomax, The *diageotropica* gene of tomato encodes a cyclophilin: a novel player in auxin signaling, Planta 224 (2006) 133–144.
[17] I.B. Taylor, A. Burbidge, A.J. Thompson, Control of abscisic acid synthesis, J. Exp. Bot. 51 (2000) 1563–1574.
[18] A. Burbidge, T.M. Grieve, A. Jackson, A. Thompson, D.R. McCarty, I.B. Taylor, Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize *Vp14*, Plant J. 17 (1999) 427–431.
[19] M. Koornneef, T.D.G. Bosma, C.J. Hanhart, J.H. Van der Veen, J.A.D. Zeevaart, The isolation and characterization of gibberellin-deficient mutants in tomato, Theor. Appl. Genet. 80 (1990) 852–857.
[20] D.W. Fujino, D.W. Burger, S.F. Yang, K.J. Bradford, Characterization of an ethylene overproducing mutant of tomato (*Lycopersicon esculentum* Mill. cultivar VFN8), Plant Physiol. 88 (1988) 774–779.
[21] J.Q. Wilkinson, M.B. Lanahan, H.C. Yen, J.J. Giovannoni, H.J. Klee, An ethylene-inducible component of signal transduction encoded by *Never-ripe*, Science 270 (1995) 1807–1809.
[22] G.W. Bassel, R.T. Mullen, J.D. Bewley, procera is a putative DELLA mutant in tomato (*Solanum lycopersicum*): effects on the seed and vegetative plant, J. Exp. Bot. 59 (2008) 585–593.
[23] R.F. Carvalho, Analysis of the interactions between phytochrome and plant hormones in plant development. Ph.D. Thesis, Universidade de São Paulo, 2008 (in Portuguese – abstract in English).
[24] J.W. Scott, B.K. Harbaugh, Micro-Tom: a miniature dwarf tomato, Florida Agr. Expt. Sta. Circ. 370 (1989) 1–6.
[25] R. Meissner, Y. Jacobson, S. Melamed, S. Levyatuv, G. Shalev, A. Ashri, Y. Elkind, A. Levy, A new model system for tomato genetics, Plant J. 12 (1997) 1465–1472.
[26] E. Martí, C. Gisbert, G.J. Bishop, M.S. Dixon, J.L. García-Martínez, Genetic and physiological characterization of tomato cv. Micro-Tom, J. Exp. Bot. 57 (2006) 2037–2047.
[27] P.L. Gratão, C.C. Monteiro, M.L. Rossi, A.P. Martinelli, L.E.P. Peres, L.O. Medici, P.J. Lea, R.A. Azevedo, Differential ultrastructural changes in tomato hormonal mutants exposed to cadmium, Environ. Exp. Bot. 67 (2009) 387–394.
[28] M.L. Campos, M. de Almeida, M.L. Rossi, A.P. Martinelli, C.G.L. Junior, A. Figueira, F.T. Rampelotti-Ferreira, J.D. Vendramim, V.A. Benedito, L.E.P. Peres, Brassinos-

teroids interact negatively with jasmonates in the formation of anti-herbivory traits in tomato, J. Exp. Bot. 60 (2009) 4347–4361.
[29] A. Zsögön, M.R. Lambais, V.A. Benedito, A.V.O. Figueira, L.E.P. Peres, Reduced arbuscular mycorrhizal colonization in tomato ethylene mutants, Sci. Agric. 65 (2008) 259–267.
[30] J.B. Reid, Plant hormone mutants, J. Plant Growth Regul. 12 (1993) 207–226.
[31] J.E. Lima, R.F. Carvalho, A.T. Neto, A. Figueira, L.E.P. Peres, Micro-MsK: a tomato genotype with miniature size, short life cycle, and improved *in vitro* shoot regeneration, Plant Sci. 167 (2004) 753–757.
[32] J.E. Lima, V.A. Benedito, A. Figueira, L.E.P. Peres, Callus, shoot and hairy root formation in vitro as affected by the sensitivity to auxin and ethylene in tomato mutants, Plant Cell Rep. 28 (2009) 1169–1177.
[33] M Koornneef, J.W. Cone, R.G. Dekens, E.G. O'Herne-Robers, C.J.P. Spruit, R.E. Kendrick, Photomorphogenic response of long-hypocotyl mutants of tomato, J. Plant Physiol. 120 (1985) 153–165.
[34] R.W. Zobel, Genetics of the *diageotropica* mutant in the tomato, J. Hered. 63 (1972) 91–97.
[35] L.E.P. Peres, S. Amar, G.B. Kerbauy, A. Salatino, G.R. Zaffari, H. Mercier, Effects of auxin, cytokinin and ethylene treatments on the endogenous ethylene and auxin-to-cytokinin ratio related to direct root tip conversion of *Catasetum fimbriatum* Lindl. (Orchidaceae) into buds, J. Plant Physiol. 155 (1999) 551–555.
[36] A. Pirie, M. Mullins, Changes in anthocyanin and phenolics content of grapevine leaf and fruit tissues treated with sucrose, nitrate, and abscisic acid, Plant Physiol. 58 (1976) 468–472.
[37] Y. Nagira, K. Ikegami, T. Koshiba, Y. Ozeki, Effect of ABA upon anthocyanin synthesis in regenerated torenia shoots, J. Plant Res. 119 (2006) 137–144.
[38] D.C. Walton, E. Sondheimer, Effects of abscisin II on phenylalanine ammonia-lyase activity in excised bean axes, Plant Physiol. 43 (1968) 467–469.
[39] Y.M. Jiang, D.C. Joyce, ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit, Plant Growth Regul. 39 (2003) 171–174.
[40] J Guo, M.-M. Wang, Characterization of the phenylalanine ammonia-lyase gene (SlPAL5) from tomato (*Solanum lycoperscicum* L.), Mol. Biol. Rep. 36 (2009) 1579–1585.
[41] S. Gazzarrini, Y. Tsuchiya, S. Lumba, M. Okamoto, P. McCourt, The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid, Dev. Cell 7 (2004) 373–385.
[42] F. Parcy, C. Valon, A. Kohara, S. Miséra, J. Giraudat, The *ABSCISIC ACID-INSENSITIVE3*, *FUSCA3*, and *LEAFY COTYLEDON1* loci act in concert to control multiple aspects of Arabidopsis seed development, Plant Cell 9 (1997) 1265–1277.
[43] X. Zhou, D. Hua, Z. Chen, Z. Zhou, Z. Gong, Elongator mediates ABA responses, oxidative stress resistance and anthocyanin biosynthesis in Arabidopsis, Plant J. 60 (2009) 79–90.
[44] M Fellner, R. Zhang, R.P. Pharis, V.K. Sawhney, Reduced de-etiolation of hypocotyl growth in a tomato mutant is associated with hypersensitivity to, and high endogenous levels of, abscisic acid, J. Exp. Bot. 52 (2001) 725–738.
[45] I.S. Sheoran, T. Dumonceaux, R. Datla, V.K. Sawhney, Anthocyanin accumulation in the hypocotyl of an ABA-over producing male-sterile tomato (*Lycopersicon esculentum*) mutant, Physiol. Plant. 127 (2006) 681–689.
[46] A. van Tuinen, A.H.L.J. Peters, R.E. Kendrick, J.A.D. Zeevaart, M. Koornneef, Characterisation of the *procera* mutant of tomato and the interaction of gibberellins with end-of-day far-red light treatments, Physiol. Plant. 106 (1999) 121–128.
[47] Y. Ozeki, A. Komamine, Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures, Plant Cell Physiol 27 (1986) 1361–1368.
[48] H. Mizukami, K. Tomita, H. Ohashi, N. Hiraoka, Anthocyanin production in callus cultures of roselle (*Hibiscus sabdariffa* L.), Plant Cell Rep. 7 (1988) 553–556.
[49] D. Weiss, A. Van Der Luit, E. Knegt, E. Vermeer, J.N.M. Mol, J.M. Kooter, Identification of endogenous gibberellins in petunia flowers. Induction of anthocyanin biosynthetic gene expression and the antagonistic effect of abscisic acid, Plant Physiol. 107 (1995) 695–702.
[50] T-H.D. Ho, A. Gomez-Cadenas, R. Zentella, J. Casaretto, Crosstalk between gibberellin and abscisic acid in cereal aleurone, J. Plant Growth Regul. 22 (2003) 185–194.
[51] P. Achard, H. Cheng, L. De Grauwe, J. Decat, H. Schoutteten, T. Moritz, D. Van Der Straeten, J. Peng, N.P. Harberd, Integration of plant responses to environmentally activated phytohormonal signals, Science 331 (2006) 91–94.
[52] C. Jiang, X. Gao, L. Liao, N.P. Harberd, X. Fu, Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in Arabidopsis, Plant Physiol. 145 (2007) 1460–1470.
[53] J.J. Casal, Phytochromes, cryptochromes, phototropin: Photoreceptor interactions in plants, Photochem. Photobiol. 71 (2000) 1–11.
[54] E.M. Josse, J. Foreman, K. Halliday, Paths through the phytochrome network, Plant Cell Environ. 31 (2008) 667–678.
[55] K.J. Halliday, C. Fankhauser, Phytochrome-hormonal signaling networks, New Phytol. 157 (2003) 449–463.
[56] D. Alabadí, J. Gil, M.A. Blázquez, J.L. García-Martínez, Gibberellins repress photomorphogenesis in darkness, Plant Physiol. 134 (2004) 1050–1057.
[57] J.L. Weller, V. Hecht, J.K. Vander Schoor, S.E. Davidson, J.J. Ross, Light regulation of gibberellin biosynthesis in pea is mediated through the COP1/HY5 pathway, Plant Cell 21 (2009) 800–813.
[58] P. Achard, L. Liao, C. Jiang, T. Desnos, J. Bartlett, X. Fu, N.P. Harberd, DELLAs contribute to plant photomorphogenesis, Plant Physiol. 143 (2007) 1163–1172.
[59] B. Al-Sady, W. Ni, S. Kircher, E. Schafer, P.H. Quail, Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteosome-mediated degradation, Mol. Cell 23 (2006) 439–446.
[60] J.L. García-Martínez, J. Gil, Light regulation of gibberellin biosynthesis and mode of action, J. Plant Growth Regul. 20 (2002) 354–368.
[61] E. Oh, S. Yamaguchi, Y. Kamiya, G. Bae, W.I. Chung, G. Choi, Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in *Arabidopsis*, Plant J. 47 (2006) 124–139.
[62] T. Muramoto, C. Kami, H. Kataoka, N. Iwata, P.J. Linley, K. Mukougawa, A. Yokota, T. Kohchi, The tomato photomorphogenetic mutant, aurea, is deficient in phytochromobilin synthase for phytochrome chromophore biosynthesis, Plant Cell Physiol. 46 (2005) 661–665.
[63] Y Kraepiel, P. Rousselin, B. Sotta, L. Kerhoas, J. Einhorn, M. Caboche, E. Miginiac, Analysis of phytochrome- and ABA-deficient mutants suggests that ABA degradation is controlled by light in *Nicotiana plumbaginifolia*, Plant J. 6 (1994) 665–672.
[64] M. Seo, A. Hanada, A. Kuwahara, A. Endo, M. Okamoto, Y. Yamauchi, H. North, A. Marion-Poll, T. Sun, T. Koshiba, Y. Kamiya, S. Yamaguchi, E. Nambara, Regulation of hormone metabolism in *Arabidopsis* seeds: phytochrome-regulation of abscisic acid metabolism and abscisic acid-regulation of gibberellin metabolism, Plant J. 48 (2006) 354–366.
[65] S.C. Weatherwax, M. Ong, J. Degenhardt, E. Bray, E.M. Tobin, The interaction of light and abscisic acid in the regulation of plant gene expression, Plant Physiol. 111 (1996) 363–370.
[66] W. Hartung, D. Schraut, F. Jiang, Physiology of abscisic acid (ABA) in roots under stress–a review of the relationship between root ABA and radial water and ABA flows, Aust. J. Agric. Res. 56 (2005) 1253–1259.
[67] C. Coenen, M. Christian, H. Lüthen, T.L. Lomax, Cytokinin inhibits a subset of diageotropica-dependent primary auxin responses in tomato, Plant Physiol. 131 (2003) 1692–1704.
[68] J.D. Goeschl, H.K. Pratt, B.A. Bonner, An effect of light on the production of ethylene and the growth of the plumular portion of etiolated pea seedlings, Plant Physiol. 42 (1967) 1077–1080.
[69] R. Pierik, E.J.W. Visser, H. de Kroon, L.A.C.J. Voesenek, Ethylene is required in tobacco to successfully compete with proximate neighbours, Plant Cell Environ. 26 (2003) 1229–1234.
[70] L.V. Kurepin, L.J. Walton, D.M. Reid, Interaction of red to far red light ratio and ethylene in regulating stem elongation of *Helianthus annuus*, J. Plant Growth Regul. 51 (2007) 53–61.
[71] E. Butelli, L. Titta, M. Giorgio, H.P. Mock, A. Matros, S. Peterek, E.G. Schijlen, R.D. Hall, A.G. Bovy, J. Luo, C. Martin, Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors, Nat. Biotechnol. 26 (2008) 1301–1308.
[72] C.M. Rick, P. Cisneros, R.T. Chetelat, J.W. Deverona, *Abg*, a gene on chromosome 10 for purple fruit derived from *S. lycopersiciodes*, Rep, Tomato Genet. Coop. 44 (1994) 29–30.
[73] C.M. Jones, P. Mes, J.R. Myers, Characterization and inheritance of the *Anthocyanin fruit* (*Aft*) tomato, J. Hered. 94 (2003) 449–456.
[74] Y. Liu, S. Roof, Z. Ye, C. Barry, A. van Tuinen, J. Vrebalov, C. Bowler, J. Giovannoni, Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9897–9902.
[75] R.J. Bino, C.H.R. de Vos, M. Lieberman, R. Hall, A. Bovy, H.H. Jonker, Y. Tikunov, A. Lommen, S. Moco, I. Levin, The light-hyperresponsive *high pigment-2^{dg}* mutation of tomato: alterations in the fruit metabolome, New Phytol. 166 (2005) 427–438.