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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.

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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

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