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Arabidopsis thaliana MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants

Diana Lucia Zuluaga[A], *Silvia Gonzali*[A], *Elena Loreti*[B], *Chiara Pucciariello*[A], *Elena Degl'Innocenti*[C], *Lucia Guidi*[C], *Amedeo Alpi*[D] *and Pierdomenico Perata*[A,E]

[A]Plant and Crop Physiology Lab, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, 56127 Pisa, Italy.
[B]Institute of Biology and Agricultural Biotechnology, CNR, Via del Borghetto 80, 56124 Pisa, Italy.
[C]Department of Agricultural Chemistry and Biotechnology, University of Pisa, Via S. Michele degli Scalzi 2, 56124 Pisa, Italy.
[D]Department of Crop Plant Biology, University of Pisa, Via Mariscoglio 34, 56124 Pisa, Italy.
[E]Corresponding author. Email: p.perata@sssup.it

Abstract. Tomato (*Solanum lycopersicum* L.) cv. Micro-Tom plants were transformed with the *Arabidopsis thaliana* (L.) Heyhn. *MYB75*/*PAP1* (*PRODUCTION OF ANTHOCYANIN PIGMENT 1*) gene. This gene encodes for a well known transcription factor, which is involved in anthocyanin production and is modulated by light and sucrose. Transgenic tomato plants expressing *AtMYB75* were characterised by a significantly higher anthocyanin production in leaves, stems, roots and flowers under normal growth conditions. Further, they also exhibited anthocyanins in fruits. Anthocyanin accumulation was not widespread but took place in specific groups of cells located in epidermal or cortical regions or in proximity of vascular bundles. In all the organs of the transgenic plants, where *AtMYB75* overexpression was determined, a clear increase in the accumulation of *DFR* (*DIHYDROFLAVONOL 4-REDUCTASE*) transcript was also detected. The expression of the tomato *MYB*-gene *ANT1* (*ANTHOCYANIN1*), which had previously been identified as a transcriptional endogenous regulator of anthocyanin biosynthesis, was not altered. The higher basal content of anthocyanins in the leaves of the transgenic plants could be further increased in the presence of high light conditions and contributed to mitigate photobleaching damages under high irradiance.

Additional keywords: antioxidant, flavonoids, Micro-Tom, photoprotection, *Solanum lycopersicum*, transformation.

Introduction

Anthocyanins are an important class of water-soluble flavonoids that confer red, purple and blue pigmentations to flowers, seeds and fruits to attract pollinators or other dispersal agents. Owing to their chemical structure and antioxidant properties, anthocyanins can also act as protective agents in plants. More specifically, high irradiance, high or low temperatures, drought or other environmental stresses can induce the synthesis of anthocyanins, particularly in leaves and fruits (Merzlyak and Chivkunova 2000; Harvaux and Kloppstech 2001; Winkel-Shirley 2002; Gould 2004; Merzlyak *et al*. 2008). They also possess antiherbivory and antimicrobial effects, and are, thus, involved in the defence against pathogens (Karageorgou and Manetas 2006).

Enhancing the content of flavonoids and anthocyanins in food plants represents an important objective in crop genetic improvement owing to the well known antioxidant and health-promoting effects of these compounds (Duthie and Crozier 2000; Pietta 2000; Lee and Lee 2006; Boudet 2007). As a major crop, tomato (*Solanum lycopersicum* L.) is one of the species on which this kind of research has been conducted in recent years, especially to improve the flavonoid content of the fruit. The major flavonoids which accumulate during ripening are naringenin chalcone and the flavonols quercetin and kaempferol rutinosides (Verhoeyen *et al*. 2002; Torres *et al*. 2005). These flavonoids are synthesised in the peel, and negligible levels accumulate in flesh tissues (Verhoeyen *et al*. 2002). Anthocyanins are not normally produced in tomato fruits, and their red colour at ripening stage is due to the carotenoid lycopene. Some *Lycopersicon* species closely related to *S. lycopersicum* produce anthocyanins also in fruits and crosses with cultivated tomato introduced dominant genes, such as the *Anthocyanin fruit* (*Aft*), resulting in anthocyanin-pigmented tomato fruits (Jones *et al*. 2003).

The activity of the genes that are involved in anthocyanin biosynthesis (Holton and Cornish 1995; Winkel-Shirley 2001) is largely regulated at the transcriptional level. Multiple transcription factors and other regulatory proteins containing R2R3-MYB domains, basic helix-loop-helix (bHLH) motifs or WD40 repeat domains have been identified in plants, including *Arabidopsis* (Borevitz *et al*. 2000; Nesi *et al*. 2001; Davies and Schwinn 2003; Baudry *et al*. 2004, 2006; Matsui *et al*. 2004;

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Cominelli *et al*. 2008), maize (Goff *et al*. 1992; Procissi *et al*. 1997; Davies and Schwinn 2003) and *Petunia hybrida* Hort. (Quattrocchio *et al*. 1993, 1998, 1999, 2006; de Vetten *et al*. 1997; Mol *et al*. 1998; Spelt *et al*. 2000). Despite the existence of some specificities in different plant species, a common regulatory mechanism involving MYB proteins interacting with bHLH and WD40 proteins in a combinatorial way seems to be highly conserved, and able to regulate not only the flavonoid biosynthetic pathway but also some important developmental processes (Baudry *et al*. 2004; Zimmermann *et al*. 2004; Broun 2005; Lepiniec *et al*. 2006). This regulatory mechanism works in a hierarchical way, with a WD40 protein on the top, physically interacting with one from multiple bHLH proteins, each with particular but also partially overlapping functions, and finally with one from diverse MYB transcription factors which confer the specificity for the final effect (Zimmermann *et al*. 2004; Lepiniec *et al*. 2006). The physical interaction between MYB and bHLH proteins is mediated by the R3 domain of the MYB transcription factor and the N-terminal region of the bHLH partner, as was first demonstrated in maize (Goff *et al*. 1992). Recently, a conserved amino acid motif in the R3 repeat of a subset of MYB transcription factors, including those involved in anthocyanin biosynthesis, has been shown to represent the structural basis for the interaction with bHLH proteins (Zimmermann *et al*. 2004).

In tomato, the major genes involved in the anthocyanin biosynthetic pathway have recently been identified (De Jong *et al*. 2004); nevertheless, their genetic regulation is still poorly described. Therefore, the engineering strategies conducted to increase the flavonoid content of tomato mainly consisted in the heterologous expression of genes isolated in other species encoding biosynthetic enzymes or transcription factors (Bovy *et al*. 2007). For instance, anthocyanin pigmentation could be strongly increased in vegetative tissues by the ectopic expression of *DELILA* (*DEL*), a regulatory gene of *Antirrhinum majus* L. (Mooney *et al*. 1995), which encodes a bHLH factor. However, *DEL* expression mostly affected tissues that normally produce anthocyanins, such as seedlings, leaves, stems and also roots after exposure to light, whereas fruits did not show any detectable increase in normal pigmentation. Moreover, the ectopic expression of a selected number of biosynthetic genes isolated from *P. hybrida* resulted in tomato fruits with a higher content of flavonols (Verhoeyen *et al*. 2002). In the peel tissue, the overexpression of the single gene coding for chalcone isomerase (CHI) was sufficient to induce a dramatic increase in flavonol levels, suggesting that *CHI* expression might be a rate-limiting step in this tissue (Verhoeyen *et al*. 2002). In the fruit flesh, however, many genes involved in flavonoid synthesis show very low expression levels (Bovy *et al*. 2002). Therefore, a significant increase in flavonol production could only be achieved by the simultaneous expression of at least three different *Petunia* genes encoding the key biosynthetic enzymes CHI, chalcone synthase and flavonol synthase (Verhoeyen *et al*. 2002). A significantly higher content of flavonols in fruit flesh has also been obtained by the heterologous and simultaneous expression of the two maize transcription factor genes *LEAF COLOUR* (*LC*) and *COLORLESS1* (*C1*) (Bovy *et al*. 2002). In these transgenic tomato fruits, however, anthocyanins were not synthesised and their absence was primarily attributable to an insufficient expression of the gene encoding the flavanone 3′,5′-hydroxylase enzyme, whose product dihydromyricetin should preferentially be used as a substrate by the enzyme dihydroflavonol 4-reductase (DFR) in tomato fruit (Bovy *et al*. 2002).

More recently, using an activation-tagging strategy, the MYB-protein ANT1 has been identified in tomato as a transcriptional regulator of anthocyanin biosynthesis (Mathews *et al*. 2003). This protein shows a high similarity to already known transcription factors controlling the same pathway in *Petunia*, *Arabidopsis* and maize and its overexpression both in tomato and tobacco was responsible for a purple phenotype of the transgenic plants. In tomato, in particular, the overexpression of *ANT1* caused an upregulation of genes involved in anthocyanin biosynthesis, glycosylation and transport into the vacuole, thereby resulting in plants displaying a purple coloration in their vegetative tissues and purple spotting on the epidermis and pericarp of fruits (Mathews *et al*. 2003).

In this paper, we show the ectopic production of anthocyanins in tomato plants, obtained by the heterologous expression of the *Arabidopsis thaliana* (L.) Heyhn. *MYB75*/*PAP1* gene. *AtMYB75* is a well known regulatory gene of *Arabidopsis* regulating in anthocyanin biosynthesis (Borevitz *et al*. 2000), which, as well as *MYB90*/*PAP2*, can work in a combinatorial way with TRANSPARENT TESTA GLABRA1 (TTG1), a WD40 protein, and different bHLH partners, such as TRANSPARENT TESTA8 (TT8), GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3) (Zhang *et al*. 2003; Lepiniec *et al*. 2006; Gonzalez *et al*. 2008). The expression of *AtMYB75* is particularly stimulated by light (Vanderauwera *et al*. 2005; Cominelli *et al*. 2008) or high sucrose concentrations (Teng *et al*. 2005; Solfanelli *et al*. 2006). The transgenic plants of tomato presented an anthocyanin-rich pigmentation. *AtMYB75* drove anthocyanin production not only in vegetative tissues, but also in organs that normally do not accumulate anthocyanins, as roots, flowers and fruits.

Materials and methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L.) cv. Micro-Tom was used in this work. Seeds were sowed in pots containing a peat-based substrate (Hawita Flor, Vechta, Germany). Plants were cultivated in the growth chamber for ~3 months with 80 μmol m^{-2} s^{-1} PAR, 16 h light photoperiod (GroLux, Sylvania, OH, USA), 24°C temperature. In these conditions, plants began to flower after ~40 days.

Cloning procedure and plasmid construction

For plant transformation, the 13.27 kb pBIN-*AtMYB75* binary vector was used. pBIN-*AtMYB75* is a pBIN19-derived plasmid carrying the *Cauliflower mosaic virus 35S promoter*::*AtMYB75*::*Nopaline synthase terminator* genetic cassette inserted between the *Eco*RI and *Cla* I restriction sites. *AtMYB75* was cloned by one-step RT–PCR, using the 'Titan One Tube RT–PCR Kit' (Roche Diagnostics, Mannheim, Germany), and starting from the total RNA extracted from *Arabidopsis thaliana* (L.) Heyhn. Col-0 seedlings germinated on a 90 mM sucrose-containing medium, as previously described (Solfanelli *et al*. 2006). Additionally,

pBIN-*AtMYB75* carries on its T-DNA the *NptII* gene coding for neomycin phosphotransferase II under the control of the 35S promoter conferring kanamycin resistance to plants. The plasmid was introduced into the *Agrobacterium tumefaciens* GV3101 strain, using the freeze–thaw method.

Tomato transformation

For plant transformation, tomato seeds were surface-sterilised by immersion into a 4% (v/v) solution of sodium hypochlorite for 15 min and rinsed four times in sterile distilled water. Afterwards, they were placed on 25 mL of half-strength MS medium (Murashige and Skoog 1962) (pH 5.7) containing 15 g L^{-1} sucrose and 7 g L^{-1} agar and incubated for 7 days at 24°C for germination. Single colonies of *Agrobacterium* harbouring the pBIN-*AtMYB75* vector were grown in 5 mL of Luria-Bertani (LB) liquid medium with 200 μM acetosyringone overnight. Cotyledon pieces (~0.25 cm^2) were cut from tomato seedlings and soaked for 10 min in *Agrobacterium* liquid culture ($OD_{600\,nm}$ = 0.5), and then placed on the co-cultivation medium consisting of MS containing 20 g L^{-1} glucose, 1 mM MES, 0.75 mg L^{-1} trans-zeatin, 1.0 mg L^{-1} IAA, and 200 μM acetosyringone, pH 5.7, and incubated at 24°C in the dark. After 2 days, the cotyledon pieces were transferred to the induction medium (same composition of the co-cultivation medium plus 300 mg L^{-1} cefotaxime and 100 mg L^{-1} kanamycin sulfate, but without acetosyringone). Tissues were sub-cultured every 21 days until shoot formation, incubated at 24°C under a 16 h light photoperiod with a light intensity of 80 μmol m^{-2} s^{-1}. Once shoots began to form, they were transferred to the elongation medium (same composition of the induction medium with 0.1 mg L^{-1} trans-zeatin and 0.05 mg L^{-1} IAA) and incubated as above. Once shoots reached 2–4 cm in height, they were harvested and transferred to the rooting medium consisting of MS containing 20 g L^{-1} glucose, 1 mM MES, 0.2 mg L^{-1} IBA and 400 mg L^{-1} carbenicillin. Regenerated plantlets were finally transferred in soil and cultivated in pots in the growth chamber with light and temperature conditions as described above. Single T0 transgenic plants were kept in the growth chamber until seed production. T1-generation plants from T0 individuals were grown to confirm the transgenic phenotypes. All the chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

RNA isolation and real time reverse transcription PCR

Total RNA from different tomato organs was extracted using the previously described method (Perata *et al*. 1997), which was modified (aurintricarboxylic acid omitted) to make the procedure compatible with the subsequent PCR procedures. To ensure a good quality, RNA was electrophoresed on a 1% agarose gel and quantity was spectrophotometrically measured. Contaminating DNA was eliminated using the TURBO DNA-free kit (Ambion, Austin, TX, USA). RNA (2 μg) from each sample were reverse-transcribed into cDNA using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The expression analysis of the genes *AtMYB75* (*At1g56650*) (forward primer: 5′-CCGCAAATGACGTCAAGAATTA CTG-3′; reverse primer: 5′-GGAATGGGCGTAATGTCTCTC TTTT-3′; TaqMan probe: 5′-ACACGGTTCATGTTTCT TAC-3′), *LeDFR* (*Lycopersicon esculentum DFR*; GenBank accession number Z18277) (forward primer: 5′-CAAGG CAGAGGGAAGATTCATTTG-3′; reverse primer: 5′-GCA CCATCTTAGCCACATCGTA-3′; TaqMan probe: 5′-ATC CCATCATGCTATCATC-3′) and *LeANT1* (*Lycopersicon esculentum ANT1*; GenBank accession number DD030645) (forward primer: 5′-AAGTGGATCTCATTTTGAGGCT TCA-3′; reverse primer: 5′-TCCTTCCGGGAAGTCTA CCA-3′; TaqMan probe: 5′-CAACAGATGGTCACTTA TTG-3′) was performed by real time PCR, using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Quantitative PCR was conducted using 100 ng of cDNA and TaqMan Universal PCR Master Mix (Applied Biosystems), and following the manufacturer's protocol. The expression of the gene *LeEF1A* was used as an endogenous control (*Lycopersicon esculentum ELONGATION FACTOR 1-ALPHA*; GenBank accession number X14449) (forward primer: 5′-TGCTTGCTTTCACCCTTGGT-3′; reverse primer: 5′-CGATTTCATCATACCTAGCCTTGGA-3′; TaqMan probe: 5′-CTGCTGTAACAAGATGGATGC-3′). Relative quantitation of *AtMYB75*, *LeDFR* and *LeANT1* expressions was performed with the comparative threshold cycle method, as described in the ABI PRISM 7700 Sequence Detection System User Bulletin # 2 (Applied Biosystems).

High-intensity light treatment

Transgenic and wild-type tomato plants were placed for 9 days under a high-intensity sodium lamp (LU400; General Electric, Fairfield, CT, USA), suspended at a 50-cm distance from the plants. Under the lamp, the light intensity was ~1000 μmol m^{-2} s^{-1}, and the ambient temperature was maintained at 24°C. Control plants for each genotype were kept under normal growth chamber conditions for the same period (80 μmol m^{-2} s^{-1} PAR, 16 h light photoperiod, 24°C temperature). For molecular and biochemical analyses, leaves from control and treated plants were collected at the beginning of the experiment and after 2, 4 and 7 days; plant tissues were immediately frozen in liquid nitrogen and stored at −80°C for use in either RNA preparation or anthocyanin quantification. For chlorophyll fluorescence imaging plants kept under low light or high light were analysed after 6 days of light treatment.

Anthocyanin quantification

Approximately 100 mg of frozen tomato tissues was used to perform the extraction of anthocyanins, following the protocol described by Ronchi *et al*. (1997) with the modifications brought by Solfanelli *et al*. (2006).

Microscopy and photography

Photos of freehand cross-sections from the different tomato organs were taken using a DS-U2 Nikon digital sight camera (Nikon, Tokyo, Japan) together with a Nikon TMS-F microscope or a Nikon SMZ-2T stereomicroscope. For some pictures (e.g. Fig. 6*D*, *E*) the image contrast was adjusted, using the logarithmic image processing model of the NIS-elements F2.20 imaging software (Laboratory Imaging, Nikon, Tokyo, Japan).

Chlorophyll fluorescence imaging

The imaging technique was performed by using an IMAGING-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany). Details of the capture of chlorophyll fluorescence imaging are reported by Guidi *et al*. (2007). The current fluorescence yield (F_t) was continuously measured and the F_o images were recorded in a quasi-dark state. The maximum fluorescence yield F_m was determined with a saturating pulse of 8000 µmol m^{-2} s^{-1} PPFD for 1−2 s. The images of F_o and F_m were subtracted and divided $[(F_m - F_o)/F_m]$ to generate the image of the maximum quantum efficiency of PSII photochemistry F_v/F_m. The current fluorescence yield (F_t) and the maximum light-adapted fluorescence (F_m') were determined in the presence of an actinic illumination of 400 µmol m^{-2} s^{-1}, then Φ_{PSII} was computed as the quotient $(F_m' - F_t)/F_m'$ (Genty *et al*. 1989). The coefficient of non-photochemical quenching $[q_{NP} = (F_m - F_m')/(F_m - F_o')]$ was calculated according to Schreiber *et al*. (1994). Correct F_o' determinations require the application of a far-red light, which would disturb the fluorescence imaging. Therefore, instead of measuring F_o', we estimated it using the approximation of Oxborough and Baker (1997) $[F_o' = F_o/(F_v/F_m + F_o/F_m)]$. Images of the fluorescence parameters were displayed by means of a false colour code ranging from 0.00 (black) to 1.00 (purple). Single leaflets in triplicate for each light condition and genotype and in corresponding positions were used for the analyses. In every leaflet analysed, an internal square portion of the lamina was chosen and, inside it, the value of each single parameter was measured in different points and an average value was calculated. The area, the position and the number of points were the same in all the leaflets analysed. Finally, an average value of each parameter for each genotype and light condition was calculated.

Results

Transformation of tomato plants with the A. thaliana *MYB75 gene*

The *A. thaliana MYB75* gene was introduced in tomato Micro-Tom plants via *Agrobacterium*-mediated transformation. Ninety-eight different regenerated plantlets were obtained from the explants grown on the kanamycin selective medium. PCR analysis was performed to verify the presence of the transgene and all the T0 lines resulted positive (data not shown). Furthermore, the expression of *AtMYB75* was confirmed by real time RT−PCR in more than 90% of the lines (data not shown). Most of the transgenic lines displayed an anthocyanin-rich phenotype, characterised by visible accumulation of these pigments in vegetative organs (leaves, stems, roots) and at different levels. This was also observed in the flowers. We noted that some of the lines presented anthocyanin-spotted fruits. A single transgenic line (line n. 23) showing a significantly higher amount of anthocyanins in all the organs, including flowers and fruits, and a homogeneous darker coloration of the foliage (Fig. 1*A*) was selected by visual screening and chosen for subsequent characterisation and analyses. Morphology, size, life-cycle duration and fertility were monitored in plants from the selected line and compared with wild-type Micro-Tom plants, without observing significant differences.

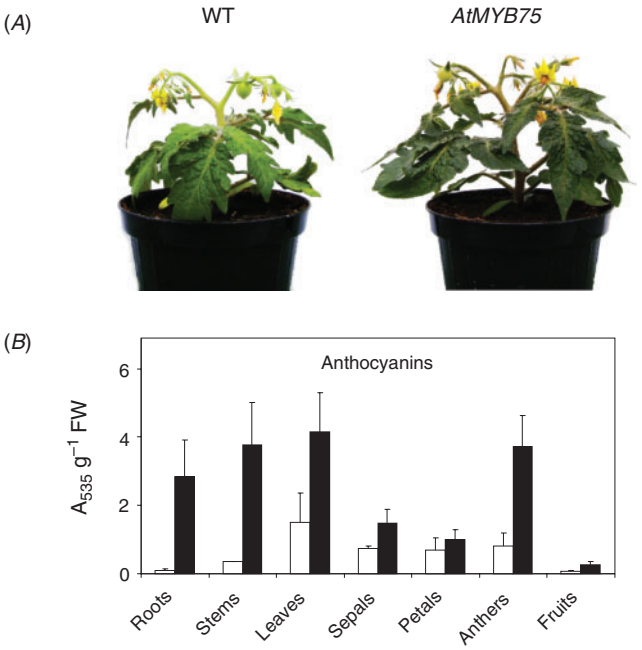

Fig. 1. High-anthocyanin phenotype of *AtMYB75* transgenic tomato plants. (*A*) Phenotypes under normal growth conditions of two representative Micro-Tom wild-type (WT) and *AtMYB75* transgenic plants from the selected line. (*B*) Anthocyanin levels measured in the organs of WT (white bars) and transgenic plants (black bars). Each value is mean ± s.d. ($n = 3$).

As shown in Fig. 1*B*, biochemical analysis confirmed that higher anthocyanin levels were present in the organs of the transgenic plants than in the wild type. The highest level of pigments was observed in transgenic leaves, but transgenic roots, stems and anthers also displayed an anthocyanin content remarkably higher than the wild type (Fig. 1*B*). The difference was less evident for sepals and petals, and in fruits anthocyanin levels were low in both genotypes, although slightly higher in the transgenic (Fig. 1*B*). The presence of anthocyanins in wild-type flowers (Fig. 1*B*) is not visually evident since it is likely masked by the high concentration of carotenoids.

Morphological analysis and anatomical localisation of anthocyanins

The presence of a higher amount of anthocyanins in the transgenic plants represented a clearly observable phenotypic trait since the seedling stage (data not shown) and was even more evident later on when anthocyanin-rich leaves and stems conferred to these plants a dark green coloration (Fig. 1*A*). In transgenic leaves, anthocyanins were synthesised both in the adaxial (Fig. 2*A*) and the abaxial sides (Fig. 2*B*), and were particularly concentrated in leaflet veins, rachis, and petioles (Fig. 2*B*). In the leaflet blades, anthocyanins were localised at the epidermal level, but their highest accumulation was observed in the midrib, whose cross-section showed purple spots located in the collenchyma surrounding the vascular bundles (Fig. 2*C*). Also, in petioles, anthocyanins were accumulated in parenchymatic cells around the vascular tissues (Fig. 2*D*).

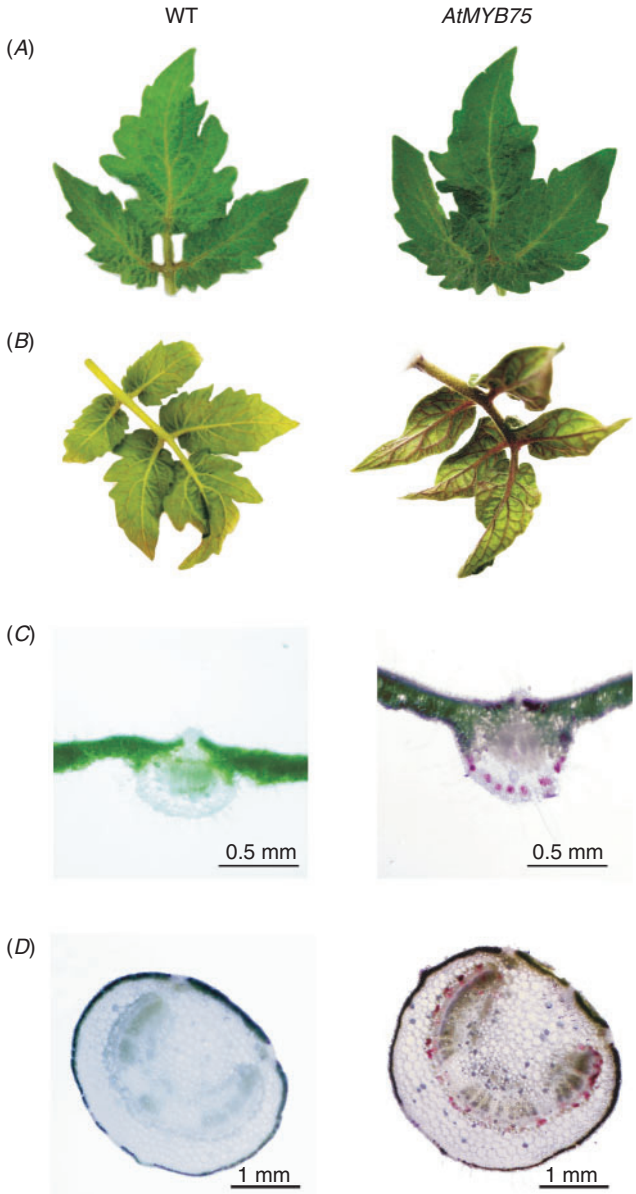

Fig. 2. Wild-type (WT) and *AtMYB75* transgenic leaves from tomato. (*A*) Upper side showing a darker coloration in the transgenic plant. (*B*) Abaxial side showing a red coloration in the veins, rachis, and petiole of the transgenic. (*C*) Cross-section of the leaflet showing purple spots in the epidermis and in the collenchyma surrounding the vascular bundles of the transgenic. (*D*) Cross-section of the petiole showing an accumulation of anthocyanins in the parenchymatic cells around the vascular tissues of the transgenic.

The stems of the transgenic plants showed two main differences compared with control wild-type plants: a clear reddish-purple pigmentation (Fig. 3*A*) and a slight increase in the number of trichomes (data not shown). The cross-section of transgenic tomato stems showed a high accumulation of pigments not only in epidermal or subepidermal cells, but also in the tissues inside the cortex (Fig. 3*B*), and particularly in intervascular parenchymatic cells (Fig. 3*C*).

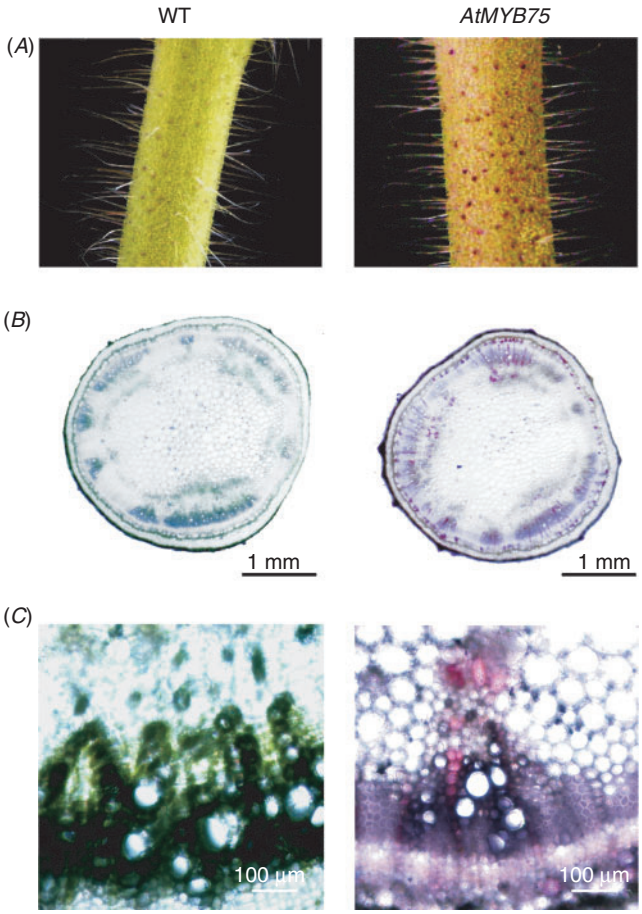

Fig. 3. Wild-type (WT) and *AtMYB75* transgenic stems from tomato. (*A*) Reddish-purple anthocyanin pigmentation in the transgenic stem compared with WT. (*B*) Cross-section showing an accumulation of anthocyanins in the parenchymatic cells close to vascular bundles of the transgenic. (*C*) Close-up of the parenchymatic cells associated with the xylematic elements, with evident purple areas in the transgenic.

The roots of the transgenic plants were purple under normal growth conditions in soil (Fig. 4*A*). Pigmentation was located in groups of parenchymatic cells in the cortex region, but it was apparently absent within the vascular cylinder (Fig. 4*B*).

The anthocyanin pigmentation of flowers was the most peculiar phenotype observed in *AtMYB75* transgenic tomato plants. The presence of anthocyanins was particularly evident in the anther tube, which displayed a purple coloration (Fig. 5*A*, *B*, *D*). Enhanced pigmentation was also observed in sepals (Fig. 5*C*), where an anthocyanins distribution similar to that of leaves was observed, as well as in petals, especially associated with the main veins and tips (Fig. 5*B*), and in pistils (data not shown). Pigments were produced both in the outer and in the inner walls of the anthers (Fig. 5*E*).

Most of the transgenic plants showed uniform anther tube pigmentation (Fig. 5*B*, *D*). However, although plants obtained from independent transformation events shared a common high anthocyanin phenotype in the vegetative tissues, some individual plants displayed anthocyanin pigmentation restricted to specific

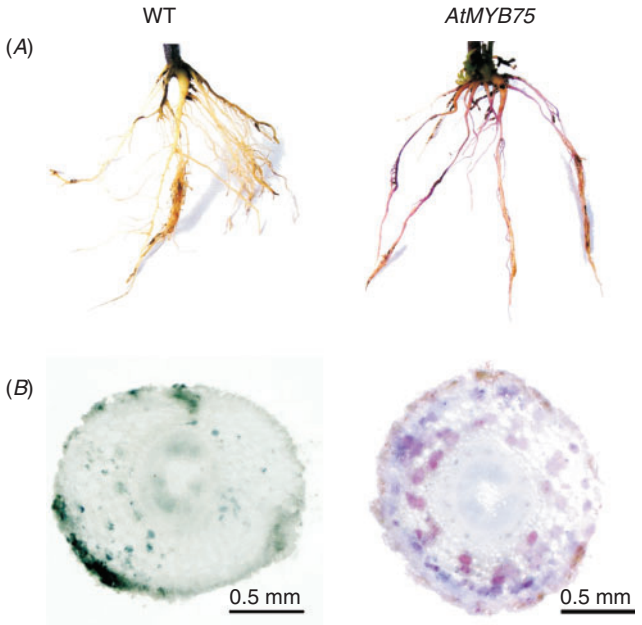

Fig. 4. Wild-type (WT) and *AtMYB75* transgenic roots of tomato. (*A*) Root system showing an anthocyanin pigmentation in the transgenic under normal growth conditions in soil. (*B*) Cross-section of roots showing the presence of anthocyanins in the cortex region of the transgenic.

areas, such as the stamen tip (line n. 41; Fig. 5*F*), the bottom of the anther sutures (line n. 57; Fig. 5*G*), along the junction between each single stamen locule (line n. 47; Fig. 5*H*) or the anther sutures (line n. 39; Fig. 5*I*).

The presence of anthocyanins in fruits was observed only in some independent transgenic lines. When present, as in the line selected for the characterisation, pigmentation was observed both in fruit peel and flesh (Fig. 6*A*–*C*). In the exocarp, anthocyanins accumulated as small purple spots (Fig. 6*A*). In the flesh, anthocyanins were synthesised in cells located in the inner wall of seed locular cavities (Fig. 6*B*), in small groups of pericarp parenchymatic cells (Fig. 6*C*) and also in the columella tissue (Fig. 6*C*). In many cases, these purple spots were close to vascular bundles (Fig. 6*D*, *E*). Transgenic fruits also displayed a clear trichome overproduction in their epidermal tissues compared to control wild type ones (Fig. 6*A*).

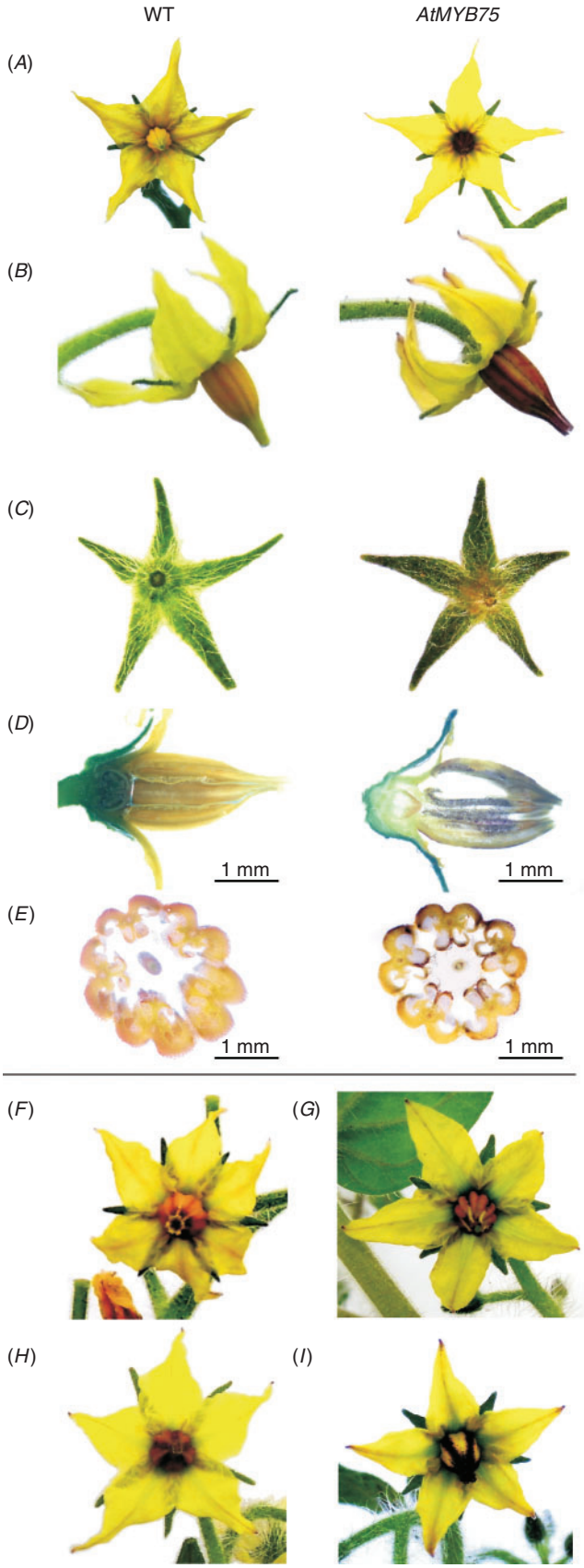

Fig. 5. (*A*–*E*) Wild-type (WT) and *AtMYB75* transgenic flowers of tomato from the line chosen for the characterization (line n. 23). (*A*) Front view showing an evenly distributed reddish-purple pigmentation in transgenic anthers and the normal yellow pigmentation in WT stamens. (*B*) Side view showing a darker green coloration in the flower stalk, and a reddish-purple pigmentation in the main veins and tips of petals and in the anther tube of the transgenic. (*C*) Light green WT and green-reddish transgenic sepals. (*D*) Longitudinal section of the flower, showing a clear accumulation of purple anthocyanins in the stamen compartments of the transgenic. (*E*) Transversal section of the anther tube showing a dark purple coloration in both the outer and inner walls of the transgenic. (*F*–*I*) Flowers from individual plants showing different anthocyanin distribution patterns in the anther tube: (*F*) stamen tip (line n. 41), (*G*) bottom of the anther sutures (line n. 57), (*H*) along the junction between each single stamen locule (line n. 47), (*I*) the anther sutures (line n. 39).

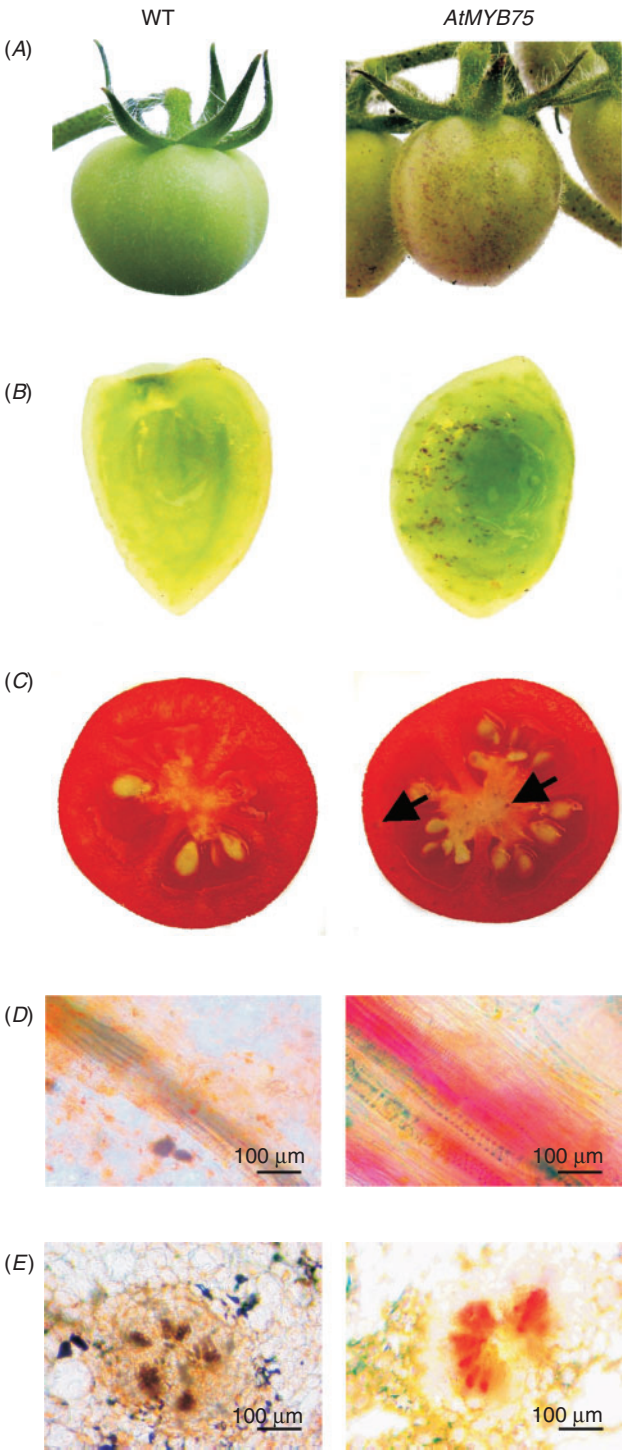

Fig. 6. Wild-type (WT) and *AtMYB75* transgenic fruits of tomato. (*A*) Small purple spots in the exocarp of the green transgenic tomato and absence of them in WT. (*B*) Mesocarp of the green tomato showing red spots in the inner wall of a seed locular cavity in the transgenic. (*C*) Cross-section of a ripening tomato fruit showing some scattered dark pigmentation spots (indicated by the arrows) in the pericarp and in the columella regions, in both cases close to vascular bundles, in the transgenic. (*D*) Microscopic close-up of a longitudinal section of a central vascular bundle. (*E*) Microscopic close-up of a transverse view of a central vascular bundle.

Effects of AtMYB75 *on the expression of other genes*

To confirm the existence of a link between transgenic phenotypes and the expression of *AtMYB75* and to dissect the possible effects of the transgene, we analysed the expression of *AtMYB75*, *LeDFR* (one of the possible targets of *AtMYB75* in the later steps of the anthocyanin biosynthetic pathway) and *LeANT1* (an endogenous transcriptional regulator of anthocyanin biosynthesis in tomato). Since *AtMYB75* was absent in wild-type plants, its expression could only be detected in the organs of the transgenic plants (Fig. 7*A*). *LeDFR* showed very different expression levels in the organs of both control and transgenic plants, with the highest relative expression values detected in leaves and sepals and the lowest in fruits (Fig. 7*B*). We noted that the transcription of this gene was always up-regulated in the organs of the transgenic plants compared with the wild-type ones, and very high relative differences between transgenic and control plants were observed in anthers (149-fold), stems (112-fold), roots (47-fold) and fruits (21-fold), and lower differences were recorded in leaves (10-fold), petals (10-fold) and sepals (2-fold) where a certain transcript level of the gene was detected also in wild-type plants (Fig. 7*B*). *LeANT1* expression levels largely varied in the different organs of the plants with the highest values detected in leaves and sepals and the lowest in fruits, but without significant differences between wild-type and transgenic plants (data not shown).

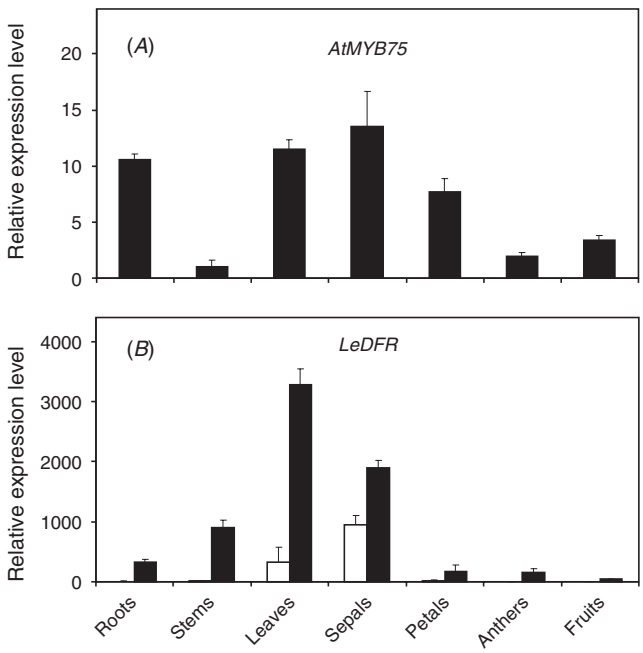

Fig. 7. Real time RT–PCR analysis performed in different organs from wild-type and transgenic tomato plants. Transcript levels of (*A*) *AtMYB75* in the organs of the transgenic plants and (*B*) *LeDFR* in the organs of wild-type (white bars) and transgenic plants (black bars). Transcript levels are indicated in relative units, assuming as unitary the organ with the lowest value (transgenic stems for *AtMYB75*, wild-type fruits for *LeDFR*). Each value is mean ± s.d. ($n = 3$). The expression of *LeEF1A* gene was used as endogenous control to normalise the data.

Effects of higher anthocyanins levels on the photosynthetic efficiency in transgenic tomato leaves

Anthocyanins in leaves are often produced as photoprotective agents in response to high irradiances. To test whether a higher amount of these pigments in the leaves of transgenic plants might confer them some kind of defence against high light stress, wild-type and transgenic mature plants were exposed to a 1000 µmol photon m^{-2} s^{-1} continuous light for 9 days. As a control, wild-type and transgenic plants were kept for the same period under 80 µmol photons m^{-2} s^{-1}. As shown in Fig. 8, high light induced an evident photobleaching in wild-type leaves, as well as a marked production of anthocyanins that was more concentrated in the leaflet abaxial sides (Fig. 8*A*–*F*, WT). In transgenic plants high light further increased the anthocyanin content in the vegetative organs exposed (leaves, petioles, stems), and a significantly lower photobleaching was observed (Fig. 8*A*–*F*, *AtMYB75*).

The induction of anthocyanin synthesis by high light either in wild-type or transgenic leaves and the higher levels of anthocyanins in *AtMYB75*-overexpressing plants were confirmed by measuring the concentration of pigments in extracts from leaves analysed at different times of the treatment (Fig. 9*A*). Real rime RT–PCR analysis confirmed the increase of *LeDFR* transcript both in wild-type and transgenic plants until the fourth day of light treatment, with expression levels in *AtMYB75* plants always higher than in the wild type (Fig. 9*B*). *LeANT1* gene appeared to be transcriptionally induced by high light during the first four days of treatment but, in this case, with comparable expression levels in wild-type and transgenic leaves (Fig. 9*C*).

We verified whether plants exposed to high irradiance displayed differences in photosynthetic efficiency at a time-point earlier than the appearance of evident photobleaching symptoms (Fig. 8). Chlorophyll fluorescence was monitored to determine the maximum efficiency of PSII (F_v/F_m), the proportion of absorbed light that is utilised for photosynthetic electron transport (Φ_{PSII}), and the q_{NP} coefficient, which estimates the amount of energy non-photochemically dissipated as heat (Maxwell and Johnson 2000). In low light pre-treated wild-type and transgenic plants we detected closely similar values for the three parameters analysed (Fig. 10, low light). In high light pre-treated wild-type leaves, F_v/F_m values were heterogeneous in the leaf lamina and lower than those recorded in plants grown in low light (Fig. 10), indicating photoinhibition attributable to high light exposure. Conversely, in transgenic leaves the average F_v/F_m ratio showed only a slight decrease and maintained an overall homogeneity in the response of the leaf lamina (Fig. 10). Similarly, the high-light dependent decline in the steady-state values of Φ_{PSII} was stronger in wild-type than in transgenic leaves (Fig. 10). Finally, in high light pre-treated plants, the q_{NP} coefficient showed a slight decrease in the wild type, and in transgenic leaves an increase of this coefficient

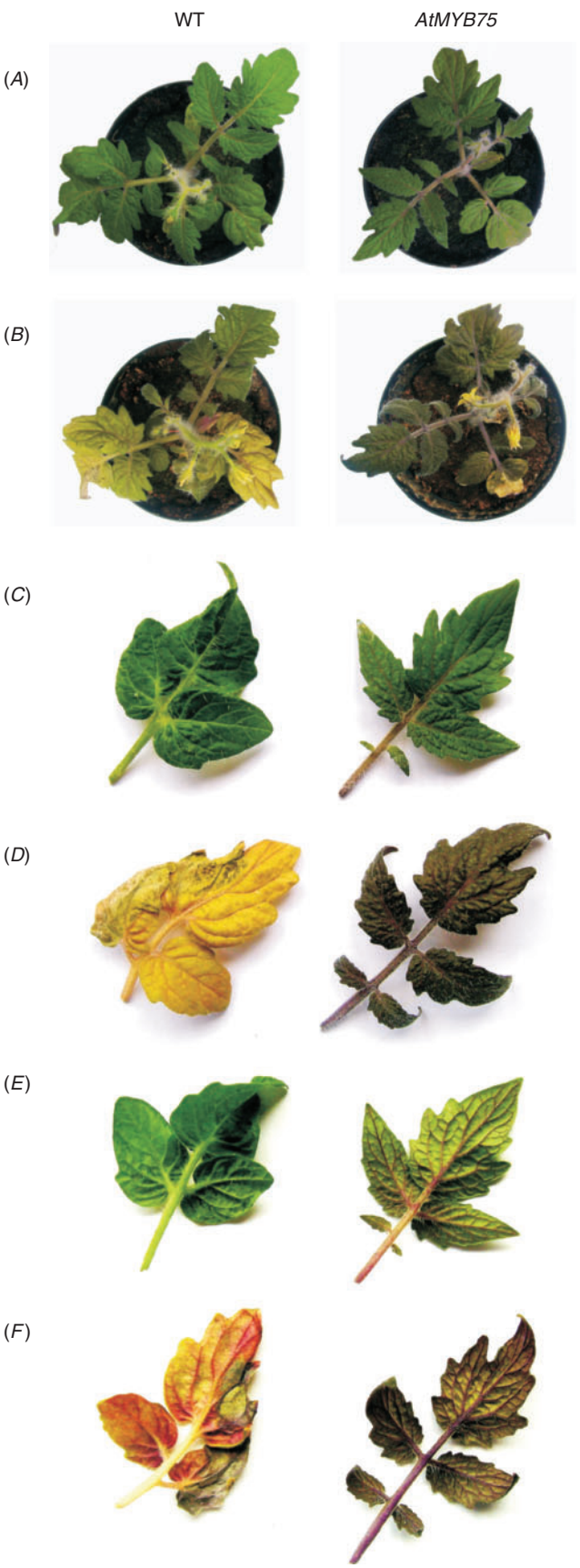

Fig. 8. Effects of high light treatment on wild-type (WT) and *AtMYB75* transgenic tomato plants. WT and *AtMYB75* plants (*A*) at the beginning of the treatment, and (*B*) after 9 days of continuous high light. Single representative leaves detached from WT and transgenic plants at the beginning of the treatment [(*C*) adaxial sides, (*E*) abaxial sides] and after 9 days of continuous high light [(*D*) adaxial sides, (*F*) abaxial sides].

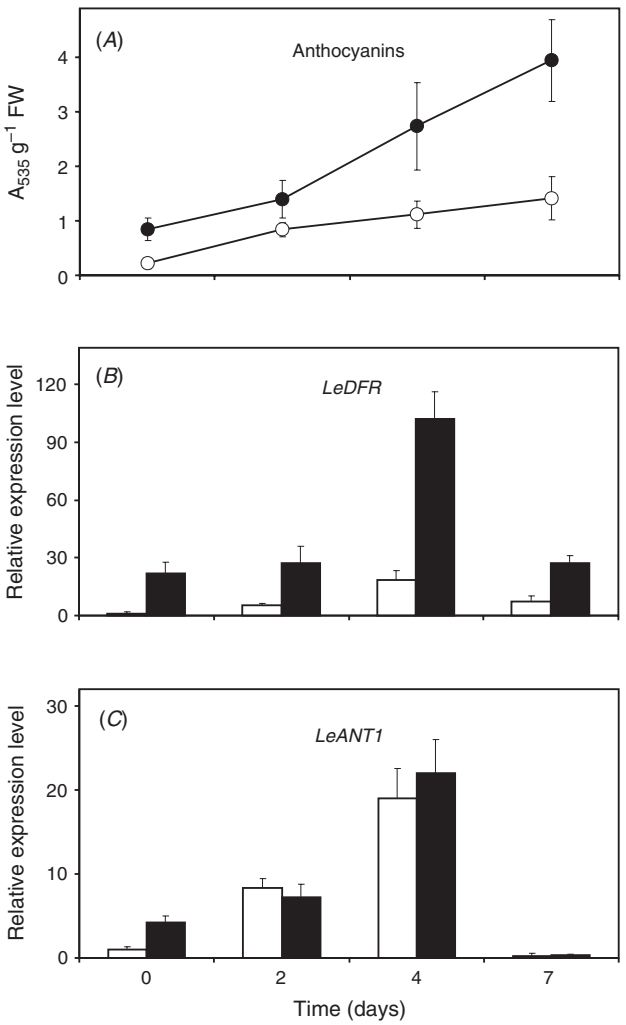

Fig. 9. Anthocyanin content and gene expression analyses in wild-type and transgenic tomato leaves during the high light treatment. (*A*) Anthocyanin levels measured in extracts from leaves analysed after 0, 2, 4 and 7 days of high light treatment. Wild-type, open circles; transgenic, filled circles. Each value is mean ± s.d. ($n = 3$). Real time RT–PCR analyses of (*B*) *LeDFR* and (*C*) *LeANT1* transcript levels in wild-type (white bars) and transgenic (black bars) leaves after 0, 2, 4 and 7 days of high light treatment. Transcript levels are indicated in relative units, assuming as unitary the lowest value (wild type $t = 0$ for *LeDFR* and wild type $t = 7$d for *LeANT1*). Each value is mean ± s.d. ($n = 3$). The expression of *LeEF1A* gene was used as endogenous control to normalise the data.

was measured when compared with the values recorded in low light leaves (Fig. 10).

Discussion

The heterologous expression of *AtMYB75* induced a significant basal production of anthocyanins in transgenic tomato plants. Not only vegetative tissues responded to *AtMYB75*, but also flowers and even fruits, which usually do not synthesise anthocyanins at significant levels in tomato. Previous works showed that ectopic flavonoid production could be induced in tomato plants by expressing genes encoding specific transcription factors isolated from other species. However, their effects appeared limited to some specific organs or restricted parts of the plant (Mooney *et al*. 1995; Bovy *et al*. 2002). *AtMYB75* expression in tomato results in reddish-purple anthocyanin pigmentations in vegetative and reproductive tissues as well as at different developmental stages. We noted that the *AtMYB75* plants resemble, both in their vegetative and reproductive organs, plants overexpressing the endogenous ANT1 transcription factor (Mathews *et al*. 2003). Since ANT1 represents the only MYB transcription factor clearly involved in anthocyanin biosynthesis currently characterised in tomato, it is still unknown whether it can be the AtMYB75 orthologous protein. Certainly, structural characteristics, such as the common presence in the R3 repeat of the amino acid signature ([DE] Lx_2[RK]$x_{3\ L}x_{6\ L}x_3$R) shown to be responsible for the interaction with bHLH proteins (Zimmermann *et al*. 2004), as well as the similarities in the pigmented phenotype of their respective overexpressing lines (Mathews *et al*. 2003; see earlier) strongly suggest this convergence. Furthermore, the anthocyanin-spotted fruits displayed by *AtMYB75* transgenic lines were quite similar to those produced by tomato accessions with the *Aft* gene, which has been recently shown to be associated with a DNA polymorphism on chromosome 10 in a MYB transcription factor corresponding to *AN2* of *Petunia* (Boches and Myers 2007; Mes *et al*. 2008). AN2 can be considered the protein of *Petunia* orthologous to AtMYB75 (Quattrocchio *et al*. 1999) and its counterpart in tomato is likely involved in similar functions. However, the correspondence of *Aft* with a putative *AN2* gene of tomato is still under debate, since another group has recently identified *Aft* trait with a single locus on chromosome 10 fully associated with *ANT1* gene (Sapir *et al*. 2008). The tomato genes *ANT1* and *AN2* apparently correspond to separate genetic loci on chromosome 10, being their nucleotide sequences rather different (data not shown). Nevertheless, both of them are strongly similar to *Petunia AN2* (data not shown). Therefore, whatever the real nature of *Aft* mutation, the presence of common phenotypic characteristics in *ANT1* and *AN2* overexpressing or mutant lines as well as in our *AtMYB75* transgenic lines would be not surprising and could indicate a common set of target genes and the likely involvement in the same MYB-bHLH-WD40 complex.

LeDFR is one of the possible targets of *AtMYB75*, since its transcript levels were significantly increased compared with wild-type plants in all the organs of the transgenic plants (Fig. 7*B*). DFR plays a crucial role in the later steps of the anthocyanin biosynthetic pathway and the induction of its transcription by *AtMYB75* in *A. thaliana* is well known (Borevitz *et al*. 2000; Matsui *et al*. 2004; Zimmermann *et al*. 2004). On the contrary, *LeANT1* expression appeared to be very similar in wild-type and transgenic plants (data not shown), as it could be expected since this gene codifies for a MYB transcription factor likely involved in functions similar to those of AtMYB75 and unlikely regulated by this latter one (see above).

Both *LeDFR* and *LeANT1* were induced by high light treatment in wild-type plants (Fig. 9*B*, *C*) and these inductions correlate well with the increase in anthocyanin production (Fig. 9*A*). In transgenic plants high light inducibility of these two genes was retained (Fig. 9*B*, *C*), suggesting that the ectopic expression of *AtMYB75* did not fully saturate the ability of the

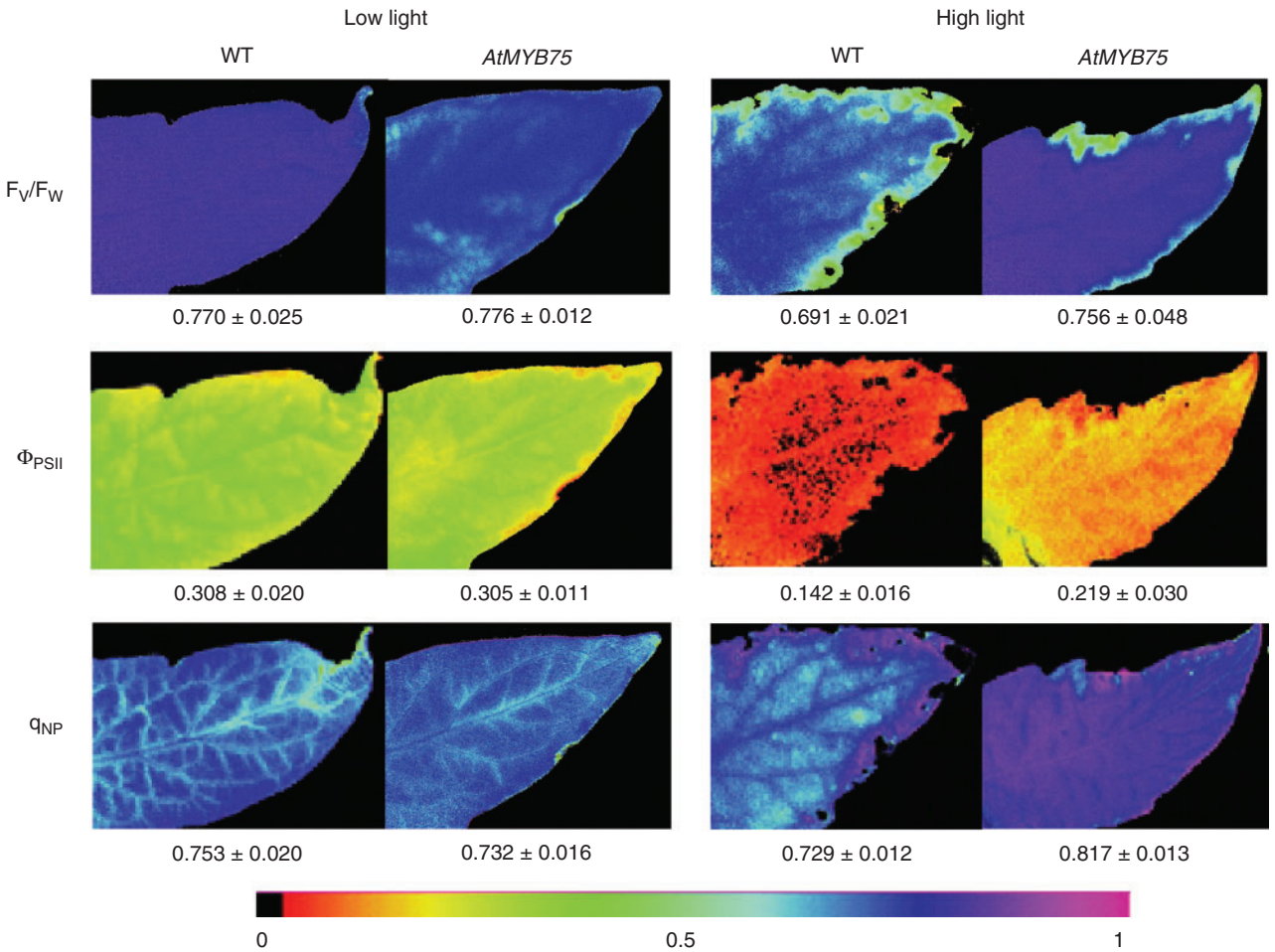

Fig. 10. Analysis of chlorophyll fluorescence parameters in wild-type (WT) and transgenic leaves (*AtMYB75*) from tomato plants grown under low light (80 µmol m^{-2} s^{-1}) or high light (1000 µmol m^{-2} s^{-1}) for 6 days. Fluorescence images of the maximum efficiency of PSII (F_v/F_m), the proportion of absorbed light, which is utilised for photosynthetic electron transport (Φ_{PSII}), and the non-photochemical quenching coefficient (q_{NP}), in representative leaves from both genotypes, are shown. Under each figure, the average value of the corresponding parameter ± s.d. ($n = 3$) is shown.

transgenic plants to enhance anthocyanin synthesis in response to environmental triggers.

Anthocyanins in *AtMYB75* plants were not evenly distributed, but restricted to specific cells or small groups of cells, suggesting that cell-autonomous mechanisms can operate and combine their action with the expression of the transgene. The specific sites of anthocyanin localisation changed within the different organs, but three main accumulation patterns were observed: (i) in epidermal or sub-epidermal cells (leaflet blades, stems, sepals, petals, stamens, fruits); (ii) in parenchymatic cells (stems, roots, fruits); and (iii) in close proximity to vascular tissues (midribs, petioles, fruits). We speculate that, in these particular areas, the presence of other factors made the action of *AtMYB75* possible, with subsequent anthocyanin biosynthesis. Such factors should be necessary, but not sufficient, to induce anthocyanin production *per se*, and could be related to the availability of biosynthetic precursors or the activation or the replacement of specific genes, which are involved at different roles in the biosynthetic pathway and which are normally repressed or not transcribed. Actually, *AtMYB75* could have a specific regulatory role stimulating the transcription of single biosynthetic genes, which are normally not expressed: this could be the case of fruits, where it had already been proposed that the negligible level of expression of some structural genes [*CHI* (Verhoeyen *et al.* 2002), *F3′5′H* (Bovy *et al*. 2002)] might represent a rate-limiting step in anthocyanin biosynthesis. The transcription factors involved in flavonoid biosynthesis, including AtMYB75, often work in a complex combinatorial way and can also modulate the expression of other regulatory factors, allowing a cell-specific accumulation of pigments (Baudry *et al*. 2006). It is, therefore, also possible that the ectopic expression of AtMYB75 in transgenic tomato plants could ultimately regulate the expression of specific *bHLH* genes in a manner similar to that recently described in *Arabidopsis* (Baudry *et al*. 2006), or replace endogenous transcription factors (AN2, ANT1?) in cells where they are absent but these bHLH proteins are synthesised. This could also explain the presence of anthocyanins in organs that normally do not produce these pigments, such as the roots, where anthocyanins are produced in transgenic plants even in the absence of light (Fig. 4*A*, *B*). It is

likely, therefore, that some other MYB-regulatory factors orthologous to *AtMYB75* are light-stimulated and, hence, affect the production of these pigments when tomato wild-type roots are exposed to light (Mooney *et al*. 1995). *ANT1*, whose expression is clearly induced by light (Fig. 9*C*), could be a good candidate gene. In transgenic roots, the constitutive expression of the transgene likely replaced these endogenous MYB factors in the activation of the anthocyanin biosynthetic pathway, allowing constitutive anthocyanin pigmentation.

The increased number of trichomes (Fig. 6*A*) was not unexpected, since an intimate link between flavonoid metabolism and specific cellular differentiation programs, including trichomes production, was demonstrated in *Arabidopsis* (Broun 2005). In this plant species, the specific molecular interaction between the WD40 containing protein TTG1 and different MYB (e.g. AtMYB75) or bHLH partners can regulate multiple processes, including flavonoid biosynthesis, as well as trichome or root hair initiation or seed coat mucilage production (Zhang *et al*. 2003; Broun 2005). It is possible that similar regulatory networks operate in tomato: in this context, the ectopic production of AtMYB75 could interfere with some of them, for example, replacing specific endogenous MYB proteins involved in trichome initiation.

The presence of a higher constitutive level of anthocyanin pigments in transgenic plants could give them some advantage, in terms of adaptation and defence against environmental stresses. To test this hypothesis, a high light experiment was carried out exposing wild type and transgenic tomato plants to a strong light irradiance for about ten days and monitoring the respective phenotypic and molecular changes. The light intensity used was very high and likely not similar to normal environmental conditions (at least for such a prolonged period). Nevertheless, it was chosen to strengthen and amplify the physiological differences that the two genotypes present in terms of photosynthetic efficiency. Actually, wild-type plants were severely injured by high light stress, showing discolored leaves with anthocyanin production, but also marked necrotic areas, thus indicating strong photoinhibition and consequent photobleaching damages (Fig. 8*A*–*F*, WT). Transgenic plants appeared less stressed than wild type at the end of the high light treatment, with anthocyanin-rich and consequently dark leaves and a lesser degree of photobleaching (Fig. 8*A*–*F*, *35S-AtMYB75*). Chlorophyll fluorescence imaging on control and stressed leaves from both genotypes suggest that, in transgenic leaves, the apparent tolerance to photoinhibition was probably not due to an increased capacity for PSII to repair, but reflected instead the ability of these leaves to protect their photosynthetic apparatus. Further research is required to clarify the exact role of anthocyanins in this context. In particular, the accumulation of anthocyanins in the epidermal tissues could act as a light screen attenuating visible radiation by absorbing UV wavelengths and consequently shading the photosynthetic apparatus from excessive light (Steyn *et al*. 2002; Gould 2004; Liakopoulos *et al*. 2006). Moreover, anthocyanins could act as scavengers for free radicals and reactive oxygen species (ROS) produced under photoinhibitory conditions (Yamasaki *et al*. 1997; Neill and Gould 2003; Tattini *et al*. 2005), especially if they were located in close vicinity to the oxy-radical sources, as in mesophyll cells (Gould 2004; Kytridis and Manetas 2006; Agati *et al*. 2007). Further characterisations of transgenic leaves in terms of anthocyanin subcellular localisation and ROS production will help to elucidate these aspects.

In conclusion, our results show how the heterologous expression of the *AtMYB75* regulatory gene from *A. thaliana* can significantly affect anthocyanin production in tomato plants, by remodulating the anthocyanin biosynthetic pathway also in terms of pigment localisation. The expression of the transgene led to a high basal anthocyanin level in the vegetative organs of the plant, but also conferred an ectopic pigmentation to flowers and fruits by the local activation of the biosynthetic pathway. Even though the transgene is constitutively expressed, anthocyanin production could be induced only in distinct groups of cells, suggesting the existence of specific and complex cell-autonomous mechanisms likely involving other endogenous regulatory factors. Whatever the mechanism of action, in *AtMYB75* transgenic plants, the higher constitutive levels of anthocyanins conferred an advantage in terms of photoprotection during high irradiance stress, reducing photobleaching damages.

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