Agrobacterium rhizogenes rolB gene affects photosynthesis and chlorophyll content in transgenic tomato (*Solanum lycopersicum* L.) plants

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Abstract

Insertion of *Agrobacterium rhizogenes rolB* gene into plant genome affects plant development, hormone balance and defence. However, beside the current research, the overall transcriptional response and gene expression of *rolB* as a modulator in plant is unknown. Transformed *rolB* tomato plant (*Solanum lycopersicum* L.) cultivar Tondino has been used to investigate the differential expression profile. Tomato is a well-known model organism both at the genetic and molecular level, and one of the most important commercial food crops in the world.

Through the construction and characterization of a cDNA subtracted library, we have investigated the differential gene expression between transgenic clones of *rolB* and control tomato and have evaluated genes specifically transcribed in transgenic *rolB* plants. Among the selected genes, five genes encoding for chlorophyll *a/b* binding protein, carbonic anhydrase, cytochrome b_6/f complex Fe-S subunit, potassium efflux antiporter 3, and chloroplast small heat-shock protein, all involved in chloroplast function, were identified. Measurement of photosynthesis efficiency by the level of three different photosynthetic parameters (F_v/F_m, rETR, NPQ) showed *rolB* significant increase in non-photochemical quenching and *a*, *b* chlorophyll content. Our results point to highlight the role of *rolB* on plant fitness by improving photosynthesis.

Abbreviations: CA, carbonic anhydrase; CAB, chlorophyll *a/b* binding protein; Chl, chlorophyll; Cyt b_6/f, cytochrome b_6/f; DIG, digoxigenin; F_m, maximum fluorescence yield obtained with a light-

saturating pulse after dark adaptation; F_v, variable fluorescence; F_v/F_m, maximum photochemical efficiency of PSII; KEA, potassium efflux antiporter; μE, microeinstein; NPQ, non-photochemical quenching; PAR, photosynthetically active radiation; PEc, photosynthesis-irradiance curve; rETR, relative electron transfer rate; RLC, rapid light curve; sHSP, small heat shock protein; SSH, suppression subtractive hybridization.

Introduction

The soil bacterium *Agrobacterium rhizogenes* is the causal agent of the hairy root disease of
Dicotyledonous plants. Virulence is associated with the transfer to the plant cell of the T-DNA
region of the Ri plasmid, containing several loci crucial for disease development such as the
r̲ooting-l̲ocus or *rol* genes, *rolA, B, C* and *D* (Costantino et al., 1994; Nilsson and Olsson, 1997). In
particular, the *rolB* gene was found to be essential for the induction of hairy roots on different plant
species (Spena et al., 1987).

rolB-transgenic plants show increased rooting, alterations in leaf and flower morphology,
adventitious root formation, reduction in internode length and apical dominance (Casanova et al.,
2005). This observation, together with the reported increase in auxin sensitivity of *rolB*-transformed
protoplasts (Maurel et al., 1991), suggested an altered response to auxins in transgenic plants and
tissues. This increase in auxin sensitivity was suggested to determine the induction of *de novo*
meristem formation by *rolB* both in tissue culture and *in planta* (Koltunow et al., 2001; Altamura
2004). It has been proposed that *rolB* could function in the signal transduction and/or perception
pathways of auxins, an hypothesis supported by the finding that it encodes a protein with tyrosine
phosphatase activity localized in the plasma membrane of transformed plant cells (Filippini et al.,
1996).

As for other *rol* genes, *rolB* has an effect on plant secondary metabolism. Shkryl et al. (2008)
observed an increased anthraquinone content in transgenic *Rubia cordifolia* callus, dependent on the
expression level of the key anthraquinone biosynthetic enzyme isochorismate synthase. In *Vitis
amurensis* an increased production of stilbene resveratrol was also positively correlated with *rolB*
expression (Kiselev et al., 2007). ~~In *rolB*-transformed ginseng tissues the production of
ginsenosides was decreased with respect to the untransformed controls (Bulgakov et al., 1998).~~
rolB has also been proposed to control tolerance to both abiotic and biotic stress. The expression of
rolB could promote scavenging of reactive oxygen species via enhanced expression of genes

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encoding antioxidant enzymes (Bulgakov et al., 2012), increased production of defence-related secondary metabolites such as phenolics (Arshad et al., 2014), and augmented activity of pathogenesis-related proteins (Veremeichik et al., 2012). Recently, *rolB* has been found to induce the expression of genes encoding components of the miRNA processing machinery, indicating a possible interaction in RNA-silencing network (Bulgakov et al., 2015).

To our knowledge no data are currently available on the overall transcriptional response of the plant to *rolB*, despite current research efforts in clarifying *rolB* gene function by morpho-physiological parameters, metabolism and stress response in different plant species.

The aim of the present study was therefore to isolate genes differentially expressed in tomato plants upon transformation with *rolB*, using the Suppression Subtractive Hybridization method (SSH) to generate a cDNA library enriched in sequences expressed in the transgenic plants versus the untransformed, *in vitro* regenerated controls.

Among the selected genes, we found the up-regulation of genes involved in photosynthesis. The indication that genes directly or indirectly induced by *rolB* participate in protection from light and oxidative stress, in capture and transfer of light energy, CO_2 diffusion, and cytochrome involvement in chloroplast electron transport chain suggested putative modification of photosynthesis in transgenic plants, that were further explored through measurement of chlorophyll fluorescence.

The measure of chlorophyll variable fluorescence allows a non-destructive plant determination of photosynthetic efficiency and heat dissipation (Baker, 2008; Murchie and Lawson, 2013). Chlorophyll fluorescence emission changes depending on developmental (Cordon et al., 2016) and environmental (Baba et al., 2016) plant status and upon stress (Hazrati et al., 2016; Bi et al., 2016). In normal physiological conditions photosynthetic pigments absorb more light energy than needed for driving photosynthesis. Excess light is either dissipated as heat or re-emitted as Chl fluorescence (Baker, 2008; Ruban, 2016). As a quantitative correlation exists between fluorescence changes observed upon exposure to light in dark-adapted leaves and changes in CO_2 assimilation, measurements of Chl fluorescence can be used to estimate photosynthetic efficiency and, in some

conditions, also photosynthetic rate (Baker, 2008; Enriquez and Borowitzka, 2010).

Relative electron transfer rate (rETR) is used as a descriptor of relative changes in the photosynthetic rates (Enriquez and Borowitzka, 2010). A tight correlation, even if variable according to the conditions of photoacclimation, has been in fact observed between rETR and photosynthetic rate in terms of O_2 evolution rate.

Non-photochemical quenching of Chl *a* fluorescence (NPQ) is a short-term acclimation process that defends the photosynthetic apparatus against oxidative damage and is fundamental in preserving the integrity of the photosynthetic reaction centers and the antenna pigments (Ruban, 2016). This parameter describes the plant photoprotective capacity to dissipate as heat and/or to balance between photosystems the excess of energy absorbed (Enriquez and Borowitzka, 2010). Evaluation of NPQ has shown modifications upon stress in a bryophyte (Azzabi et al. 2012) and in tomato plants (Gerganova et al., 2016).

Light absorption is the first stage in photosynthesis, which is carried out by pigments such as Chl and accessory pigments (Liu et al., 2004). The efficiency of this process depends on pigments concentration and structure (Horton and Ruban, 2005), and changes in the content of Chl *a* and *b* are related to adaptation processes to maximize light-harvesting by chloroplasts (Björkman, 1981). Taken together, these parameters obtained from the measure of Chl variable fluorescence allow assessing the photosynthetic performance of plants, gathering information on the efficiency of both the conversion of light into chemical energy by the photosynthetic apparatus and the mechanisms protecting it against excess light.

Materials and methods

Bacterial strains and media

rolB gene and its own promoter from *A. rhizogenes* pRi1855 was cloned into pBIN19 vector and transformed into *Agrobacterium tumefaciens* strain GV3101 via chemical transformation. Bacteria were grown at 28 °C in YEB medium supplemented with 50 mg L^{-1} rifampicin and 100 mg L^{-1} kanamycin.

Plant material

Agrobacterium-mediated transformation of *Solanum lycopersicum* cv. Tondino, kindly provided by Petoseed Italia (Parma, Italy), was performed as described (Bettini et al., 2003). Transgenic plants and the corresponding untransformed, *in vitro* regenerated controls were multiplied by micropropagation in Murashige and Skoog medium supplemented with Gamborg's B5 vitamins (Duchefa Biochemie B.V.) and 3% sucrose, and grown *in vitro* at 25 ± 1 °C with a 16 h light-8 h dark photoperiod. Plants were transferred to a containment greenhouse for morphological analysis and self-fertilized to obtain first generation progeny.

Molecular analysis

DNA and RNA extractions were carried out by using the NucleoSpin Plant II and NucleoSpin RNA Plant kits (Macherey-Nagel GmbH & Co. KG), respectively. The presence of *rolB* in the transformed clones was assessed by PCR with gene-specific primers *rolB*-fw and *rolB*-rev (Table 1). Amplificability of the samples was evaluated by performing control reactions with primers for the β-1,3-glucanase gene as in Bettini et al. (2015). One-step RT-PCR for transgene expression was carried out with the Titan One tube RT-PCR System (Roche Life Science) with primers *rolB*-fw and *rolB*-rev2 (Table 1). Control amplifications lacking the reverse transcriptase enzyme were included to confirm the absence of contaminating DNA. For *rolB* copy number determination, 5 μg of total DNA were digested with the restriction endonucleases *Nsi*I/*Sal*I or *Kpn*I/*Alw*44I, not cutting inside the transgene sequence, separated by agarose gel electrophoresis and transferred to positively

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charged nylon membranes (Roche Life Science). DNA hybridization with digoxigenin (DIG)-labelled *rolB* probe (Roche Life Science) was performed as described in Bettini et al. (2010).

Suppression subtractive hybridization and cDNA library construction

Total RNA was extracted from leaves of greenhouse-grown plants with the RNeasy Maxi kit (Qiagen), and polyA$^+$ RNA was isolated with the NucleoTrap mRNA kit (Macherey-Nagel GmbH & Co. KG). Suppression subtractive hybridization was performed according to the PCR-Select cDNA subtraction kit (Clontech Laboratories Inc.) user's manual. The tester sample contained mRNA isolated from first generation *rolB*-transgenic tomato plants and the driver sample contained mRNA extracted from the corresponding control plants. The cDNAs obtained were cloned in the pCR 2.1-TOPO vector (Invitrogen by Thermo Fisher Scientific) and plasmid DNA was purified with the NucleoSpin Plasmid kit (Macherey-Nagel GmbH & Co. KG). Screening of positive clones was performed by differential hybridization against two complex DIG-labelled probes made from driver (control plants) and tester (transgenic plants) cDNAs to identical dot blot membranes as described in Salvianti et al. (2007). Only the clones showing no or weak hybridization with the cDNAs from the control plants and a strong signal with the cDNAs from the transgenic plants were retained for subsequent analysis.

cDNA sequencing and sequence analysis

DNA sequencing was performed at the Centro Interdipartimentale di Servizi per le Biotecnologie di Interesse Agrario, Chimico e Industriale (C.I.B.I.A.C.I.) of the University of Florence using an automated sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems by Thermo Fisher Scientific). M13 primers were used for sequencing. Each sequence was manually edited to correct ambiguities and remove vector and primer sequences. Sequence similarities were determined by BLASTx searches versus the GenBank non-redundant protein sequence database. Sequences were

deposited in the GenBank dbEST database under accession numbers JZ916953 to JZ916974 (Table 2).

Validation of differential gene expression by real-time PCR

For the determination of the level of differentially expressed transcripts, 1 µg of total RNA was reverse transcribed (QuantiTect Reverse Transcription kit, Qiagen) in a final volume of 20 µL. PCR reactions, containing 5 µL of a 1:5 dilution of the cDNA template, were carried out with QuantiNova Sybr Green PCR master mix (Qiagen) as per user manual recommendations. Primers were designed with the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). *Solanum lycopersicum* tubulin gene (GenBank acc. no. XM_004237998.2) was used as endogenous reference for gene expression normalization. Amplification was performed in an Applied Biosystems StepOne Plus Real-Time PCR System (ThermoFisher Scientific Inc.) with the following programme: 1 cycle of 2 min at 95 °C, 40 cycles of 5 s at 95 °C and 10 s at 60 °C. Melting curve analysis was performed at the end of each run to confirm the absence of primer-dimers and non-specific amplification products. PCR efficiency was assessed by the standard curve method and was 90-110% for all primer combinations, with a correlation coefficient (R^2) between 0.98 and 0.99. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical significance of the observed differences was assessed by the Student's *t* test within the Past 3.x software package (Hammer et al., 2001). Two biological and three technical replicas were performed.

Determination of photosynthetic parameters

Photosynthetic parameters i.e. maximum photochemical efficiency of photosystem II, relative electron transfer rate (rETR), and non-photochemical fluorescence quenching (NPQ), were determined through variable fluorescence kinetics (Schreiber at al., 1986; Enriquez and Borowitzka,

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2010). Maximum photochemical efficiency of PSII, related to the number of moles of O_2 produced, or CO_2 assimilated, per mole of photons absorbed by the photosynthetic apparatus, was estimated as F_v/F_m, where F_v represents variable fluorescence and F_m maximum fluorescence yield obtained with a light-saturating pulse after dark adaptation. Relative electron transfer rate was calculated as $(\Delta F/F_m')$ multiplied by PAR, where PAR is the photosynthetically active radiation, ΔF the increase in fluorescence yield induced by a light-saturating pulse during the exposure to actinic light, and F_m' the maximum fluorescence in light-adapted state (Kromkamp and Forster, 2003). NPQ was calculated as $(F_m - F_m')/F_m'$.

Leaf discs were obtained by cutting with a cork borer (17 mm diameter) from three transgenic first generation plants of the clone *rolB*-22 and three control plants (at least two discs per plant). Measures were taken with a PAM 101 (Heinz Walz GmbH, Germany) equipped with the Optical Unit ED101-US as a detector, the L655 red led to supply the pulsed measuring light and two white KL-1500 lamps to supply both the actinic and the saturating (saturation pulse width 0.8 s, saturation intensity ≥ 2000 μE m^{-2} s^{-1}) lights. Measurements of photsynthetic parameters were taken with an actinic light gradient of six steps ranging from 0 to 2200 μmol photons m^{-2} s^{-1}. The actinic light duration was 10 s (Rapid Light Curve, RLC) (White and Critchley, 1999; Ralph and Gademann, 2005) and 1 min (Photosynthesis-Irradiance curve, PEc) (Lazzara et al., 2010), with the purpose to distinguish between the actual and the potential/optimal ability of both the control and the *rolB* samples to photosynthesize.

Chlorophyll extraction and quantification

The concentrations of chlorophyll *a* and *b* were determined spectrophotometrically according to the equation of Porra et al. (1989). The same leaf discs used for the measurement of photosynthetic parameters were ground with a glass pestle in 5 mL 100% acetone, the extracts were incubated for 24 h at 4 °C and centrifuged for 15 min at 2500 x *g*. The extracts were then diluted with distilled water to an acetone concentration of 80% (v/v) and absorbance spectra were recorded at 664 and

647 nm for Chl *a* and *b*, respectively. Statistical analysis was performed with the Past 3.x software package (Hammer et al., 2001) on three replicates.

3. Results

Molecular analysis and phenotype of the rolB-transgenic plants

Eight-hundred seventy-five cotyledons from 11 day-old *Solanum lycopersicum* cv. Tondino plantlets were used for transformation with the *A. rhizogenes rolB* gene. Regeneration frequencies of 2.06% and 43.9% were observed, respectively, for *rolB* and the untransformed, *in vitro* regenerated controls. The regenerated plantlets obtained were micropropagated on culture medium supplemented with 100 μg mL^{-1} kanamycin prior to PCR and RT-PCR analysis for the presence and the expression of the inserted gene. The transgene was found to be present and expressed in the clones analyzed (Fig. 1a, b). Transgene copy number determination on clones *rolB*-10 and *rolB*-22 showed the presence of one and two copies of the transgene, respectively (Fig. 1c).

The two transgenic clones (ten plants each) and five untransformed regenerated controls (three plants each) were used for morphological analysis and self-fertilization. The phenotype of the transformed plants was consistent with previous data on the effect of *rolB* in tomato (van Altvorst et al., 1992), having a significantly reduced height, fruit number and fruit weight ($P \leq 0.01$) (Supplementary Table 1).

The same molecular controls were performed on the first generation progeny of transgenic plants in order to identify the individuals harbouring and expressing the transgene (Fig. 2a, b).

Characterization of the subtracted cDNA library

Suppression subtractive hybridization was performed on cDNA populations from transgenic tomato plants of the *rolB*-22 clone first generation progeny (tester) and the corresponding untransformed,

in vitro regenerated controls (driver). Three hundred thirty independent positive clones were randomly selected and PCR amplification with M13 universal primers was carried out to verify the presence and the size of inserts. Average insert size was 460 bp, ranging from 196 bp to 1583 bp. Further screening to remove false positives was performed by dot blotting plasmids DNAs onto a nylon membrane and probing with DIG-labeled driver cDNA. This procedure would allow excluding the clones corresponding to mRNAs present in the control plants. Identical dot blot membranes were also hybridized against DIG-labeled tester cDNAs, in order to identify the clones highly expressed only in the *rolB*-22 transgenic plants. After the first screening, 81 clones were retained that showed no hybridization with the driver probe, and among these 20 clones showed the strongest signal with the probe corresponding to the tester. These 20 clones were sequenced and their putative functions identified by homology searches against the GenBank database. All cDNAs matched with high significance to sequences involved in stress response, basal metabolism, signal transduction and gene expression (Table 2). Genes associated with the response to biotic and abiotic stress were: SRE1A (A-92), cystathionine-γ-synthase (C-11), catalase (C-17), Avr9/Cf-9 rapidly elicited protein 194 (C-66) and vacuolar pyrophosphatase (D-37), while ribosomal proteins (A-76, C-55, D-50), transcription factor 18 (D-6), elongation factor 1α (E-23) and High Mobility Group 1/2 protein (A-33) were mainly engaged in gene expression.

NADH dehydrogenase (ubiquinone) (D-70) is a respiratory chain enzyme localized in the inner mitochondrial membrane that catalyzes electron transfer from NADH to coenzyme Q10, while DDB is a transmembrane protein (E-15) and nucleoside diphosphate kinase III (E-34) a signal transduction factor.

In particular, we have identified five sequences encoding proteins engaged in chloroplast function: chlorophyll *a/b* binding protein, carbonic anhydrase, cytochrome *b₆/f* complex Fe-S subunit, potassium efflux antiporter 3, and chloroplast small heat-shock protein.

The chlorophyll *a/b*-binding proteins (CAB-4, A-6) are part of the light harvesting complexes I and II, whose function is to capture and transfer light energy to the photosynthetic reaction centers in

photosystems I (PSI) and II (PSII), respectively (Green and Durnford, 1996). In tomato the *Cab* gene family comprises at least 19 members (Green et al., 1991). The *Cab-4* (*Lhb2a*) gene product is localized in PSII and, as for the other *Cab* genes, is regulated by environmental and developmental stimuli, such as light, oxidative stress, and abscisic acid (Bartholomew et al., 1991; Kellmann et al., 1993).

Carbonic anhydrases (CA, EC 4.2.1.1) (C-43 and C-57) are zinc-metalloenzymes present in all living organisms, where they catalyze the reversible hydration of CO_2 to $HCO_3^- + H^+$ (Moroney et al., 2001). In plants CAs have been studied mainly for their roles in photosynthetic process. In C3 plants, such as tomato, they facilitate CO_2 diffusion into the chloroplast as a supply for RuBisCO activity (Tiwari et al., 2005). Moreover, CAs could play indirect roles in photosynthesis by regulating chloroplast pH during rapid changes in light intensity and photosynthetic electron transport (Graham et al., 1984; Stemler, 1997). In clones C-43 and C-57 a putative conserved domain was found corresponding to β-CA superfamily, that takes part in stomatal response to CO_2 in *Arabidopsis thaliana* (Hu et al., 2015).

Homology searches for clone C-63 detected significant similarity to the nuclear-encoded Rieske Fe-S subunit, a protein belonging to the cytochrome b_6/f complex where it has a key role in the chloroplast electron transport chain (Molik et al., 2001). The Cyt b_6/f complex, involved in transmembrane signalling, participates in photosynthetic electron transport leading to ATP and NADPH generation. A positive correlation has been found between chloroplast electron transfer rate and Cyt b_6/f content (Yamori et al., 2011), thus an increased Cyt b_6/f content could improve photosynthetic efficiency.

Potassium efflux antiporter 3 (KEA, E-6) belongs to the cation proton antiporter family (Véry and Sentenac, 2003). Presence of a putative chloroplast transit peptide and subcellular localization experiments predicted chloroplast targeting for KEA both in rice and *A. thaliana* (Aranda-Sicilia et al., 2012; Sheng et al., 2014). Loss-of-function of KEA was implicated in rice chlorophyll-deficient

phenotype and in altered chloroplast ultrastructure providing evidence for KEA proteins taking part in chloroplast development (Sheng et al., 2014).

Finally, low molecular weight heat-shock proteins (sHSPs, A-5) are known to be involved in plant thermotolerance and are induced in additional abiotic stress conditions such as osmotic, oxidative and cold stress, heavy metals, ozone, UV (Sun et al., 2002). In particular, sHSPs protect thermolabile PSII and whole-chain electron transport during heat stress in both higher plants and cyanobacteria (Heckathorn et al., 1998; Nakamoto et al., 2000).

Validation of differential gene expression by real-time PCR

To confirm up-regulation of the photosynthetic genes isolated through SSH, the expression of the sHSP, *Cab*, CA, Cyt b_6/f, and KEA genes was analyzed in first generation *rolB*-22 and control plants by quantitative real-time PCR. The tubulin gene was used as endogenous reference to normalize gene expression. As expected on the basis of the SSH results, the expression of all the genes tested was significantly higher in the transgenic than in the control plants (Fig. 3).

Photosynthetic parameters and chlorophyll content

The presence in the *rolB* SSH library of five up-regulated genes encoding proteins that, as above described, are involved in chloroplast metabolism, encouraged us to measure the photosynthetic parameters F_v/F_m, rETR and NPQ, together with *a* and *b* chlorophyll content. Transgenic and control plants were compared.

No differences in F_v/F_m nor rETR were found between the *rolB*-transformed and the control individuals with either 10 s (RLC) or 1 min (PEc) actinic light duration (Fig. 4). F_v/F_m is an indicator of the status of the photosynthetic apparatus, in particular of PSII quantum efficiency, and in higher plants the rate of photosynthesis measured by fluorescence parameters has been demonstrated to be tightly linked to the rate of CO_2 fixation (Genty et al., 1989). Our results therefore indicate that the overall efficiency of the photosynthetic process was not modified in the

transgenic plants with respect to the controls. The different profiles obtained with 10 s or 1 min

pulses could be due because the system is rapidly saturated by shorter light pulses.

The measure of fluorescence quenching was also performed. Non-photochemical quenching allows

to analyze the efficiency of the mechanisms that protect the photosynthetic apparatus against

oxidative damage through the dissipation of excess absorbed light energy as heat within the light-

harvesting complexes. Interestingly, results show that the behavior of the transgenic plants is

significantly different from the corresponding controls both with 10 s (RLC) and 1 min (PEc)

actinic light duration (Fig. 5). The mechanisms acting in the dissipation of excess energy seemed

therefore to be more efficient in the *rolB* plants in comparison to controls.

When the chlorophyll content was measured, a significant increase in both Chl *a* and *b* in the

transgenic plants was observed, while the Chl *a/b* ratio was significantly lower in the *rolB* plants

when compared with the control (Table 3).

Discussion

Results obtained from the subtractive cDNA library showed that in *rolB* tomato plants chloroplast

functional genes were over-expressed when compared with the control (Table 2). In fact, 25% of

the screened cDNA clones had significant homology to proteins engaged, directly or indirectly, in

photosynthesis efficiency and protection.

Cytochrome b_6/f complex (clone C-63) is a major determinant of the rate of photosynthesis (Price et

al., 1995). In transgenic tobacco plants, reduced expression of the Rieske Fe-S subunit (a

component of Cyt b_6/f complexes) led to high levels of steady-state chlorophyll fluorescence and a

reduction in NPQ. Similarly, in *Arabidopsis pgr1* mutation of the Rieske subunit gene impaired

quenching of chlorophyll fluorescence by limiting electron transport through the Cyt b_6/f complex

and preventing the acidification of thylakoid lumen needed for thermal energy dissipation

(Munekage et al., 2001). Also the small heat shock proteins (clone A-5), generally induced upon abiotic stress conditions and with chloroplast localization, have a role in the protection of PSII from photoinhibition during thermal stress (Heckathorn et al., 1988; Neta-Sharir et al., 2005).

Not much data are at present available on tomato *Cab-4* gene (clone A-6). However, the chlorophyll binding proteins family, including Chl *a/b* binding proteins and fucoxanthin chlorophyll *a/c*-binding proteins, has been proposed to be involved in NPQ (Dittami et al., 2010).

These findings were consistent with the analysis of photosynthetic parameter NPQ, showing that the mechanisms protecting the photosynthetic apparatus from excess energy were more efficient in the *rolB*-transgenic plants than in the controls (Fig. 5).

The alteration of photosynthesis metabolic cascade has been found in plant to cope with biotic and abiotic stress (Berger et al., 2007; Ashraf and Harris, 2013). In particular, a down regulation of photosynthetic genes and photosynthesis efficiency was found in plant-pathogen interaction as maize-*Stenocarpella macrospora* (Bermúdez-Cardona et al., 2015). In potato plants infected with Potato Virus Y a transient up-regulation of photosynthesis-related genes and photosynthetic activity was detected before viral multiplication, followed by down-regulation (Stare et al., 2015). The enhancement of photosynthetic protection and chlorophyll content in *rolB* plants could be a strategy to buffer the negative effect (reduced photosynthesis) exterted in response to adverse conditions. In this frame it is worth mentioning that *rolB*-tomato plants are more tolerant to fungal pathogens (Arshad et al., 2014; Bettini et al., unpublished results).

rolB plants contain significantly more Chl *b* when compared with the control (Table 3). As Chl *b* is preferentially involved in low light conditions, it could provide shade tolerance for *rolB* plants. The lower Chl *a*/Chl *b* ratio in *rolB* plants could mean these plants are more capable to acclimate to low light conditions, as the increased Chl *b* enhances the range of wavelengths absorbed by the shade-acclimated chloroplasts. Moreover, plants grown under canopy possess a lower PSI to PSII ratio compared to plants grown under high light (Björkman, 1981; Sato et al., 2015). The increased

expression of *Cab-4* gene (clone A-6), localized in PSII, and also of sHSP (clone A-5) having a role in the protection of PSII from photoinhibition, further supports this hypothesis.

Interestingly, in potato the insertion of *A. rhizogenes rolC* gene shows the opposite effect, by reducing photosynthetic rate and decreasing content of both Chl *a* and *b* (Fladung et al., 1993). Finally, previous research demonstrated that *rolB* confers an increased tolerance to stress and improves the antioxidant metabolism and the production of secondary metabolites, known to cope with various stresses in transgenic plants and/or cells (Bulgakov et al., 2012, 2013; Arshad et al., 2014). Consistently with these data, we have found over-expression of stress-responding genes, such as catalase, SRE1A, Avr9/Cf-9 rapidly elicited protein 194, vacuolar-type H+ pyrophosphatase and cystathionine-γ-synthase (Table 2).

Concluding, our results add a new competence for *rolB* when integrated into the tomato plant genome. This plant alters the ability to respond to the environment regulating a primary and central plant metabolism such as photosynthesis.

Conflict of interest statement

The authors declare no conflict of interests relevant to the subject of this study.

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Figure legends

Fig. 1. Molecular analysis of the *rolB*-transgenic plants. (a) PCR amplification with *rolB*-specific primers. MW marker, molecular weight marker VI (Roche Life Science). (b) RT-PCR for *rolB* expression. MW marker, molecular weight marker (GeneRuler 100 bp DNA ladder Plus, Thermo Scientific). (c) Copy number determination for the transgenic clones *rolB*-22 and *rolB*-10 by DNA hybridization with a DIG-labelled *rolB* probe. N-S, *Nsi*I-*Sal*I; K-A, *Kpn*I-*Alw*44I. Molecular weight marker, GeneRuler 1 kb DNA ladder (Thermo Scientific).

Fig. 2. Molecular analysis on 10 plants of the *rolB*-22 first generation progeny. (a) PCR amplification with *rolB*-specific primers. M, molecular weight marker (GeneRuler 1 kb DNA ladder, Thermo Scientific). (b) RT-PCR for *rolB* expression. M, molecular weight marker (GeneRuler 100 bp DNA ladder Plus, Thermo Scientific).

Fig. 3. Quantitative real-time PCR of five clones identified through SSH library. The fold-change of each transcript in the transgenic plants relative to the control plants was normalized with the tomato tubulin gene. Two biological and three technical replicas were performed. Significance of the differences between the *rolB*-22 and control plants, determined by the Student's *t* test, is indicated with one ($P \leq 0.005$) or two ($P \leq 0.001$) asterisks.

Fig. 4. Photosynthetic efficiency of *rolB*-22 and control first generation tomato plants measured with actinic light duration of 10 s (Rapid Light Curve) and 1 min (Photosynthesis-Irradiance curve). Relative electron transfer rate is reported as a function of incident PAR.

Fig. 5. Non photochemical quenching curves for *rolB*-22 and control first generation tomato plants with 10 s (Rapid Light Curve) or 1 min (Photosynthesis-Irradiance curve) actinic light duration. Non photochemical quenching values are reported as a function of incident PAR.