Silencing of the Mitochondrial Ascorbate Synthesizing Enzyme \( \text{L-Galactono-1,4-Lactone Dehydrogenase} \) Affects Plant and Fruit Development in Tomato\(^{1,[W],[OA]} \)

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\( \text{L-Galactono-1,4-lactone dehydrogenase (EC 1.3.2.3)} \) catalyzes the last step in the main pathway of vitamin C (\( \text{L-ascorbic acid} \)) biosynthesis in higher plants. In this study, we first characterized the spatial and temporal expression of \( \text{SlGalLDH} \) in several organs of tomato (\( \text{Solanum lycopersicum} \)) plants in parallel with the ascorbate content. \( P_{\text{SS}} \text{Slgalldh}\text{RNAi} \) silenced transgenic tomato lines were then generated using an RNAi strategy to evaluate the effect of any resulting modification of the ascorbate pool on plant and fruit development. In all \( P_{\text{SS}} \text{Slgalldh}\text{RNAi} \) plants with reduced \( \text{SlGalLDH} \) transcript and activity, plant growth rate was decreased. Plants displaying the most severe effects (dwarf plants with no fruit) were excluded from further analysis. The most affected lines exhibited up to an 80% reduction in \( \text{SlGalLDH} \) activity and showed a strong reduction in leaf and fruit size, mainly as a consequence of reduced cell expansion. This was accompanied by significant changes in mitochondrial function and altered ascorbate redox state despite the fact that the total ascorbate content remained unchanged. By using a combination of transcriptomic and metabolomic approaches, we further demonstrated that several primary, like the tricarboxylic acid cycle, as well as secondary metabolic pathways related to stress response were modified in leaves and fruit of \( P_{\text{SS}} \text{Slgalldh}\text{RNAi} \) plants. When taken together, this work confirms the complexity of ascorbate regulation and its link with plant metabolism. Moreover, it strongly suggests that, in addition to ascorbate synthesis, \( \text{GalLDH} \) could play an important role in the regulation of cell growth-related processes in plants.

Vitamin C (\( \text{L-ascorbic acid} \)) has a huge importance for all living eukaryotic cells. In higher plants, ascorbate is the most abundant water-soluble antioxidant, acting to scavenge reactive oxygen species that are generated during photosynthesis, oxidative metabolism, and a wide range of stresses. In addition, ascorbate is involved in the regulation of several fundamental cellular processes such as photoprotection, the cell cycle, cell expansion, and in pathways of secondary metabolism such as the recycling of lipid-soluble \( \alpha \)-tocopherol and ethylene biosynthesis. Although generally accepted, the mechanism by which ascorbate participates in the cell developmental processes is not clearly established (for review, see Smirnoff, 2000). Much evidence supports the correlation between ascorbate content and its redox state and cell division and/or cell expansion (Horemans et al., 2003 and refs. therein).

Microorganisms, plants, and most animals produce ascorbate, however, a loss of the ability to synthesize ascorbate has occurred in primates and some other animals. Since the 1960s, the animal pathway has been completely characterized (Burns, 1960). It involves 2-\( \text{D-Glc} \) as the initial precursor and the last step is catalyzed by a microsomal \( \text{L-gulono-1,4-lactone oxidase} \), which oxidizes \( \text{L-gulono-1,4-lactone} \) to produce ascorbate. In plants, the ascorbate pathway is different from animals and has remained unsolved until recently, when convincing evidence in support of a novel pathway was established (Wheeler et al., 1998). To date, the pathway proposed by Wheeler et al. (1998) is the most commonly described in plants. Recently the last missing intermediate steps of the pathway have been elucidated (Laing et al., 2007; Linster et al., 2007). The last step in this pathway clearly involves the conversion of \( \text{L-galactono-1,4-lactone (L-GalL)} \) to ascorbic acid, a reaction catalyzed by the \( \text{L-galactono-1,4-lactone dehydrogenase} \) (here referred to as \( \text{L-GalLDH} \)),

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an enzyme located in the inner membrane of the mitochondria (Bartoli et al., 2000). This reaction requires cytochrome c as a second substrate that is reduced as L-GalL is converted into ascorbate (Oba et al., 1995).

In addition to this pathway, alternative ascorbate biosynthesis pathways have been proposed in plants. Agius et al. (2003) showed that during the ripening phase of strawberry (Fragaria spp.) fruit, ascorbate is produced via the reduction of D-GalUA to L-galactonic acid, which in turn is spontaneously converted to L-galactono-lactone, the immediate precursor of ascorbate. However, this pathway shares the requirement of L-GalLDH activity as the terminal step in ascorbate synthesis. In 2003, Wolucka and Van Montagu suggested an alternative pathway involving L-gulose as an intermediate for the de novo biosynthesis of vitamin C in plants. They showed that in vitro, GDP-D-Man-3,5-epimerase is capable of catalyzing two distinct epimerization reactions that produce either GDP-L-Gal or the novel intermediate, GDP-L-gulose. Nevertheless, the existence of all the enzyme machinery of this putative pathway has yet to be confirmed in vivo. Recently, Lorence et al. (2004) presented molecular and biochemical evidence proposing another possible biosynthetic route that utilizes myoinositol as a precursor. By overexpressing myoinositol oxygenase in Arabidopsis (Arabidopsis thaliana), they observed a 2- to 3-fold increase in ascorbate content in the leaf and discuss the possible contribution of myoinositol in ascorbate biosynthesis in plants. Following constitutive expression of the rat gene encoding L-gulono-lactone (L-GulL) oxidase, Jain and Nessler (2000) achieved a 4- and 7-fold increase of ascorbate content in lettuce (Lactuca sativa) and tobacco (Nicotiana tabacum) plants, respectively. L-GulL oxidizing activity has been reported in Arabidopsis by Davey et al. (1999) who measured L-GulL oxidizing activity and by Wolucka and Van Montagu (2003) who measured L-GulLDH-like activity.

Ascorbate is known to be a vital physiological metabolite in plants for major plant biochemical processes such as photosynthesis (Smirnoff, 1996; Noctor and Foyer, 1998), but also for controlling plant development as recently shown in root and other organs (Olmos et al., 2006). To investigate the physiological function of L-GalLDH in plants, we analyzed the effect of L-GalLDH silencing on ascorbate metabolism and its consequences for plant development. Since fruit is one of the major sources of ascorbate for the human diet, this study was carried out in tomato (Solanum lycopersicum), the model plant for fleshy fruit development. We report here that SlGalLDH silencing profoundly affects plant and fruit growth, probably through the alteration of the mitochondrial function and related changes in ascorbate redox state, particularly in the apoplast. Transcriptome and metabolome analyses of SlGalLDH RNAi transgenic lines further suggest that phenotypic changes can be attributed to the resulting modifications in secondary and primary metabolism.

RESULTS

Ascorbate and L-GalLDH Distribution in Tomato Plant

To extend our understanding of the role of ascorbate in plant growth, we analyzed its distribution in a wide range of tomato organs, including vegetative tissues such as young and mature leaf, stem and root, and reproductive tissues such as flower and fruit at several stages of development (Fig. 1, A and B). In vegetative

![Figure 1. SlGalLDH expression and ascorbate content in cherry tomato plants and fruit. A, Relative SlGalLDH transcript levels in young leaves (Yl), mature leaves (Ml), root (Rt), stem (St), flower (Fl) and in fruit at 10 DPA, 20 DPA, mature green (MG), orange (Or), and red ripe (RR) stages. Data obtained by semiquantitative RT-PCR were normalized against Actin1 mRNA and are expressed as a ratio of arbitrary units. B, Total ascorbate content in the various tomato organs. Data represent mean ± SD of measurements of 10 organs per plant with six individual plants per line (n = 60). C, Detection of SlGalLDH transcripts in developing tomato organs by in situ hybridization. Longitudinal sections of shoot apical meristem (A), root apical meristem (B), young leaf (C), 9 mm flower bud (D), and cross section of fruit at 7 DPA (E) and 20 DPA (F) were prepared and analyzed as described in the experimental procedures. Hybridization signal appears as dark staining. Inserts are negative control corresponding to sense riboprobe. Scale bar = 500 μm.](image-url)
organisms, ascorbate content varied according to the tissue, with high levels in the leaf (young and mature) and comparatively low levels in the root. In flower and fruit, the ascorbate content remained very low (1.7–2.3 μmol per g fresh weight [FW]) compared to the leaf (6.8–7.9 μmol per g FW). The ascorbate concentration was constant throughout fruit development and comparable with the levels reported by Andrews et al. (2004).

Since L-GalLDH catalyzes the terminal step of the major pathway of ascorbate biosynthesis in plants (Wheeler et al., 1998), this enzyme is a good candidate for controlling the variations in ascorbate content in plants (Tamaoki et al., 2003; Bartoli et al., 2005; Tokunaga et al., 2005). Screening of tomato EST databases (SOL Genomics Network [SGN] and The Institute for Genomic Research tomato EST databases; http://www.sgn.cornell.edu and http://www.tigr.org/) allowed the identification of a single cDNA encoding L-GalLDH (named SlGalLDH for tomato L-GalLDH), which corresponds to a unique gene in tomato, as further confirmed by Southern-blot analysis (data not shown). Examination of SlGalLDH expression showed that SlGalLDH transcripts (Fig. 1A) and immunodetected SlGalLDH protein (data not shown) were ubiquitously detected in the various tomato organs analyzed. The high SlGalLDH mRNA abundance in the leaf was in agreement with the high ascorbate content in this tissue (Fig. 1B), while other organs such as root or fruit did not display this relationship. Close examination of the spatial distribution of SlGalLDH transcripts in developing organs by mRNA in situ hybridization (Fig. 1C) further indicated that SlGalLDH transcripts were particularly abundant in tissues displaying a high cell proliferation activity such as the shoot apical meristem, the root apical meristem, young leaves, ovules, and stamen of flowers and young fruit at 10 DPA.

**P**_{**PS5**:**Slgalldh**_{RNAi}}** Transgenic Plants Do Not Display a Reduction of Ascorbate Content as Shown by the Capability to Maintain a Normal Ascorbate Synthesis**

To further investigate the relationship between plant development and SlGalLDH activity in tomato, we generated transgenic plants expressing a SlGalLDH-specific RNA interference sequence fragment under the control of the cauliflower mosaic virus 35S promoter (hence named **P**_{**PS5**:**Slgalldh**_{RNAi}} transgenic plants). Among 12 independent primary **P**_{**PS5**:**Slgalldh**_{RNAi}} transformants showing the presence of a single copy of the transgene, we selected four transgenic lines showing a high SlGalLDH mRNA relative abundance (Fig. 1A) and immunodetected SlGalLDH protein abundance, and residual SlGalLDH activity were highly reduced by comparison with the control plants (Fig. 2), and no significant difference could distinguish the four transgenic lines on the basis of the expression/protein/activity data when statistical analysis was performed. Despite these strong effects, no remarkable variation could be observed in the total ascorbate content in young fully expanded leaves (6.75 ± 0.04 μmol per g FW) and 45 DPA red ripe fruits (1.82 ± 0.03 μmol per g FW) in the lines 2, 5, 8, and 38. The capacity of the transgenic plants to maintain the pool of total ascorbate constant was confirmed for the leaves of the lines 5 and 8 (Fig. 3). Leaves from the transgenic lines and controls accumulate ascrobate at similar rates when incubated with the ascorbate

![Figure 2. SlGalLDH expression, protein, and activity in P_{PS5:SlgalldhRNAi} transgenic and control plants. A, SlGalLDH mRNA abundance was determined in young leaves (Yl) and fruit at 20 and 42 DPA (orange stage) in P_{PS5:SlgalldhRNAi} plants (line 2, 5, 8, and 38) and compared to control plants. Data obtained by semiquantitative RT-PCR were normalized against Actin1 mRNA and are expressed as percentage of control. Data represent mean ± SD of six individual plants per line. B, Immunodetection of SlGalLDH protein in young leaves from P_{PS5:SlgalldhRNAi} line 2, 5, 8, and 38 and control plants. C, SlGalLDH activity in young leaves from P_{PS5:SlgalldhRNAi} line 2, 5, 8, and 38 compared to control plants. Data represent mean ± SD of six individual plants per line. Asterisks above bars indicate values that were determined by the t test to be significantly different (P < 0.05) from control.](image)
Figure 3. Ascorbate accumulation in tomato leaves. Leaf stripes from P35S:SlgalldhRNAi transgenic line 5 (△), line 8 (□), and control (○) plants were incubated in Murashige and Skoog (white symbols) or Murashige and Skoog containing 25 mM L-GalL (black symbols) in the light. Total ascorbate was assayed as described in “Materials and Methods.” The error bars indicate SEs (n = 3).

The fully expanded fourth leaf of 6-week-old plants. The reduced/total ascorbate ratio was determined for all the samples. Data represent mean from seedlings at the indicated DAS and in developing fruits at the indicated days postanthesis (DPA). Apoplastic ascorbate content was measured in the leaf and Skoog containing 25 mM L-GalL (black symbols) in the light. Total ascorbate was assayed as described in “Materials and Methods.” The error bars indicate SEs (n = 3).

precursor L-GalL. Further investigations indicated that no viable plant with SlGalLDH activity below the threshold level of 15% was recovered among the P35S:SlgalldhRNAi transgenic lines, suggesting the essentiality of this activity.

Table 1. Ascorbate in tomato leaf and fruit of P35S:SlgalldhRNAi transgenic and control plants

| Plant Stage (DAS) | 0Seed | 15  | 21  | 28  | 42  |
|------------------|-------|-----|-----|-----|-----|
| Leaf             |       |     |     |     |     |
| AsA              |       |     |     |     |     |
| Control          | 2.4 ± 0.2 | 1.5 ± 0.1 | 2.5 ± 0.3 | 2.6 ± 0.3 | 1.8 ± 0.1 |
| Line 8           | 2.5 ± 0.3 | 1.2 ± 0.1a | 1.1 ± 0.1a | 1.3 ± 0.1a | 1.4 ± 0.2a |
| Line 5           | 2.5 ± 0.2 | 1.1 ± 0.2a | 1.2 ± 0.1a | 1.4 ± 0.1a | 1.3 ± 0.2a |
| Tot Asc          |       |     |     |     |     |
| Control          | 2.5 ± 0.3 | 2.9 ± 0.2 | 4.5 ± 0.5 | 8.7 ± 0.7 | 8.5 ± 0.3 |
| Line 8           | 2.6 ± 0.2 | 2.9 ± 0.3 | 4.9 ± 0.5 | 6.1 ± 0.4 | 8.1 ± 0.3 |
| Line 5           | 2.6 ± 0.2 | 2.8 ± 0.3 | 5.5 ± 0.5 | 7.7 ± 0.1 | 8.1 ± 0.4 |
| Ratio            |       |     |     |     |     |
| Control          | 0.9   | 0.5 | 0.5 | 0.3 | 0.2 |
| Line 8           | 1     | 0.4 | 0.2 | 0.2 | 0.2 |
| Line 5           | 1     | 0.4 | 0.2 | 0.2 | 0.2 |
| Fruit Stage (DPA)|     |     |     |     |     |
| AsA              |       |     |     |     |     |
| Control          | 0.3 ± 0.1 | 0.4 ± 0.1 | 1.3 ± 0.1 | 1.7 ± 0.1 | 1.7 ± 0.2 |
| Line 8           | 0.5 ± 0.1a | 0.9 ± 0.1a | 1.6 ± 0.2a | 1.6 ± 0.7 | 2.0 ± 0.2 |
| Line 5           | 0.6 ± 0.1a | 0.7 ± 0.1a | 1.6 ± 0.2a | 1.8 ± 0.2 | 1.9 ± 0.2 |
| Tot Asc          |       |     |     |     |     |
| Control          | 1.6 ± 0.1 | 1.6 ± 0.1 | 1.6 ± 0.2 | 1.7 ± 0.1 | 2.0 ± 0.1 |
| Line 8           | 2.0 ± 0.1a | 1.8 ± 0.3 | 1.7 ± 0.2 | 1.6 ± 0.7 | 2.2 ± 0.2 |
| Line 5           | 1.8 ± 0.1a | 1.6 ± 0.1 | 1.6 ± 0.2 | 2.0 ± 0.2 | 2.1 ± 0.2 |
| Ratio            |       |     |     |     |     |
| Control          | 0.2   | 0.2 | 0.8 | 1   | 0.9 |
| Line 8           | 0.2   | 0.5 | 0.9 | 0.9 | 0.9 |
| Line 5           | 0.3   | 0.4 | 0.9 | 0.9 | 0.9 |
| Leaf Apoplast    |       |     |     |     |     |
| Control          |       |     |     |     |     |
| Line 8           |       |     |     |     |     |
| Line 5           |       |     |     |     |     |

P35S:SlgalldhRNAi Transgenic Lines Exhibit a Change in the Redox State of Ascorbate Associated with Alteration of the Mitochondrial Function in the Most Severely Affected Plants

Given the well established fact that ascorbate content of the leaf and ascorbate redox state depend on many environmental parameters, P35S:SlgalldhRNAi transgenic lines 5 and 8 and controls were grown in a phytotronic chamber to avoid any daily climate changes. Measurements of the total ascorbate content (reduced ascorbate [AsA] + dehydroascorbate) as well as of the AsA content were carried out during the development of these plants, from the seed up to the appearance of the first inflorescence (6 weeks after sowing), and within the developing fruit (Table I). Total ascorbate content in the seed and the young leaves of the apex was comparable in both transgenic and control plants. In the germinating seed of transgenic lines and the controls, approximately 90% of ascorbate was as the reduced form AsA. The leaf ratio of AsA to total ascorbate declined, both in the controls and the transgenic lines, throughout plant development (Table I). This effect, which was already noticeable in 21 d after sowing (DAS) transgenic plants, was not due to variations in total ascorbate content but to...
variations in AsA content. The total ascorbate content of control fruit grown in a controlled environment chamber (Table I) was similar to that observed in control plants in the greenhouse (Fig. 1B). In the control fruit, 80% of total ascorbate was in the form of oxidized ascorbate during the cell division and expansion phases (10–20 DPA), while more than 90% of the total ascorbate was in the form of AsA in the ripening fruit. Total ascorbate content from transgenic lines 5 and 8 fruit were not significantly different from fruit of the control, except for the 10 DPA stage that exhibited a slightly higher ascorbate content. In contrast with the leaf, transgenic fruit from lines 5 and 8 exhibited a significant increase in AsA (Table I), mainly during the cell division and expansion stages of the fruit (10–30 DPA).

Because of the putative role of apoplastic ascorbate in the signaling processes modulating cell growth and development (Pignocchi and Foyer, 2003), we investigated the total and reduced ascorbate content in the apoplast of fully expanded leaves of 6-week-old P35S-SgalldhRNAi plants of transgenic lines 8 and 5. In the leaves of control plants, only 10% of the total ascorbate was found in the apoplast (648 ± 49 nmol per g FW) in agreement with previous findings of Noctor and Foyer (1998), and 10% to 15% of the apoplastic ascorbate was present as the reduced form. In the P35S-SgalldhRNAi transgenic line 8 and line 5, the reduced ascorbate pool remained very low (85–121 nmol per g FW), as in the controls. This occurred despite the fact that the apoplastic total ascorbate content was decreased by about 50%, due to a reduction of the oxidized ascorbate pool (Table I).

Table II. Respiration and SlGalLDH activity in isolated mitochondria from leaves

| Respiration Electron Donor | Control          | Line 5 | Line 8     |
|---------------------------|-----------------|--------|-----------|
|                           | nmol O2 min⁻¹ mg⁻¹ protein |
| 2 mM NADH                 | 48.7 ± 5.5      | 46 ± 4.5 | 73.6 ± 3.7a |
| + 2.5 mM ADP              | 86.9 ± 8.4      | 75.8 ± 7.1 | 139.9 ± 4.1a |
| + 2 mM KCN                | 10.4 ± 1.8      | 10.9 ± 3.8 | 20.6 ± 3.2a |
| + 1 mM SHAM               | 8.4 ± 2.5       | 9.6 ± 1.9   | 12.5 ± 2.1a |
| Respiratory control       | 1.78            | 1.65     | 1.90      |
| 5 mM succinate            | 39.7 ± 7.8      | 48.4 ± 7.3 | 42.6 ± 10.9 |
| + 2.5 mM ADP              | 86.9 ± 13.8     | 97.1 ± 12.8 | 95.2 ± 20.7 |
| Respiratory control       | 2.19            | 2.00     | 2.23      |
| 5 mM Pyr/malate           | 29.5 ± 4.5      | 34 ± 4.4   | 30.6 ± 3.4 |
| + 2.5 mM ADP              | 42.8 ± 8.3      | 47.6 ± 8.8 | 46.8 ± 4.6 |
| Respiratory control       | 1.45            | 1.40     | 1.53      |
| 5 mM A3A                  | 39.9 ± 6.4      | 42.1 ± 5.1 | 39.7 ± 3.9 |
| 2.5 mM TMPD               | 409.5 ± 32.5    | 393.7 ± 44.3 | 380.1 ± 45.4 |
| 4.2 mM l-GalL             | 9.7 ± 0.9       | 5 ± 0.9a   | 5.4 ± 0.6a |
| + 10 mM cytochrome c      | 13.4 ± 1.6      | 6.9 ± 0.7a | 6.5 ± 0.8a |

SlGalLDH Activity

| Control          | Line 5 | Line 8     |
|-----------------|--------|-----------|
| nmol cytochrome c min⁻¹ mg⁻¹ protein |
| 96 ± 15.1        | 49.6 ± 10.5a | 20 ± 5a   |

Given that Millar et al. (2003) demonstrated that GalLDH activity is associated with the complex I of the mitochondrial electron transport chain and that it has been documented that respiration can control ascorbate synthesis in plants (Bartoli et al., 2006), intact leaf mitochondria were isolated to investigate respiratory parameters in the P35S-SgalldhRNAi transgenics. When tricarboxylic acid (TCA) cycle substrates pyruvate and malate were used to drive the electron chain, as well as when electrons entered the chain via succinate dehydrogenase by the addition of succinate, the respiration rate in the transgenic line 5 and 8 was identical to the controls (Table II). However, when external NADH dehydrogenases were engaged by addition of exogenous NADH, the respiration rate in P35S-SgalldhRNAi transgenic line 8 was increased by 1.6-fold whereas that in P35S-SgalldhRNAi transgenic line 5 was similar to the rate of control plant mitochondria. In the same order, a significant 2-fold activation of cyanide-insensitive (alternative oxidase) respiration that was inhibited by salicylhydroxamic acid (SHAM), was measured in mitochondria from P35S-SgalldhRNAi line 8. When no substrate, other than l-GalL, was used to introduce electrons flow to cytochrome c, the respiration rate in both transgenic lines was reduced to about 50% of that observed in mitochondria isolated from the control.
The difference in the respiratory activities in the different transgenic lines demonstrates that a threshold level of 50% must be exceeded for the reduction of GalLDH abundance to impact the mitochondrial electron transport chain.

Transgenic Plants with Reduced L-GalLDH Activity Display a Reduced Plant Growth Rate and Fruit Size

When T1 transgenic plants were grown in the greenhouse, it soon became apparent that the growth of the aerial parts was reduced compared with that of the controls. Detailed characterization of this effect revealed that the germination rate, the plant growth rate, the fruit weight, and the diameter were all reduced (Fig. 4). When this experiment was replicated in a growth chamber with tightly controlled growth conditions similar results were obtained. To investigate how the reduction in SlGalLDH mRNA abundance, protein, and activity could affect plant growth and morphology in the transgenic plants, we conducted more detailed analyses of lines 2, 5, 8, and 38. Given the reduction in aerial yield in the P35S:SlgalldhRNAi transformants and the importance of ascorbate for the photosynthetic process, we next assessed to what extent the transgenic plants exhibited altered photosynthetic rates. The rate of CO2 assimilation was measured on fully expanded leaves of the transgenic and control plants at incident irradiance of between 200 to 1,000 μmol m⁻² s⁻¹. At this light intensity range, the CO2 assimilation curve was linear and the calculated photosynthetic rate, 16.2 ± 0.8 nmol CO2 per μmol photon, was invariant in transgenic plants with respect to the controls. It is noteworthy that chlorophyll content was also unaltered (1.4 ± 0.2 mg chl per g FW).

In contrast, leaf size was strongly affected in the P35S:SlgalldhRNAi transgenics, which resulted from a 25% reduction in leaflet area in the most strongly inhibited lines (Fig. 5, A and B). Analysis of epidermal cell size indicated that this result does not stem from a reduction in cell number but rather from a reduction in cell size, which was also reduced by about 25% compared to that of the control (Fig. 5B). Similar observations were made in fruit, which exhibited a cell size reduction of 15% to 22% in the transgenic lines 8 and 5, despite exhibiting an unaltered ripening period (45 d). The analysis of a cross section taken from the equatorial area of a red ripe fruit revealed that the transgenic lines produced fruits with a thinner pericarp. Given that it is very difficult to obtain suitable samples from red ripe fruit for histological analysis (Cheniclet et al., personal communication), we compared the thickness of the pericarp in fruits at 20 DPA, at which stage the fruits reached 85% of their final size. Cytological analysis revealed that the fruit pericarp thickness was significantly decreased in all lines (Fig. 5C). Detailed comparisons indicated that this was a result of a cell size reduction in the pericarp rather than an alteration in the number of cell layers (Fig. 5D).

Figure 4. Phenotypic comparison between P35S:SlgalldhRNAi transgenic and control plants. A, Germination and plant growth. Left and middle section, seedlings from P35S:SlgalldhRNAi line 2, 5, 8, and 38 and from control at 10 DAS; right section, 6-week-old plants from severely affected P35S:SlgalldhRNAi line 5 and from control. B, Growth kinetic. Plant height from P35S:SlgalldhRNAi lines 5 and 8 and from control were measured every 4 d starting from 6 d after germination. Data represent mean ± SD of 10 individual plants. C, Fruit size. Top section, pictures of ripe fruit from P35S:SlgalldhRNAi lines 2, 5, 8, and 38 and from control at 10 DAS; right section, 6-week-old plants from severely affected P35S:SlgalldhRNAi line 5 and from control. B, Growth kinetic. Plant height from P35S:SlgalldhRNAi lines 5 and 8 and from control were measured every 4 d starting from 6 d after germination. Data represent mean ± SD of 10 individual plants. C, Fruit size. Top section, pictures of ripe fruit from P35S:SlgalldhRNAi lines 2, 5, 8, and 38 and from control; bottom section, fruit diameter measured on 42 DPA fruit. Data represent mean ± SD of 10 fruits per plant with six individual plants per line (n = 60 fruits). Asterisks above bars indicate values that were determined by the t test to be significantly different (P < 0.05) from control.

Functional Classification of Genes Expressed in Leaf and Ripening Fruit of P35S:SlgalldhRNAi Transgenic Lines

To investigate the possible contribution of changes in transcript expression triggered by the reduction in
SlGalLDH activity and/or variations in ascorbate redox state to the morphological and cellular alterations of transgenic plants (Figs. 4 and 5), we compared the transcript expression profiles of a fully expanded fourth leaf and of 42 DPA (orange) fruit in the severely affected line 8 and in control plants. Among the 13,400 cDNAs corresponding to 8,700 transcripts present on the TOM1 tomato microarray analyzed, 1,269 in the leaf and 92 in the fruit were significantly different (P-value < 0.02, four independent slide hybridizations, including two dye swaps and two replicates per slide) in line 8 versus control plants (see http://cbi.labri.fr/outsils/data/Tomato/VitC/sup.html). A previously reported and recently further enhanced annotation of the genes represented on the TOM1 array combined with the MapMan ontology (Thimm et al., 2004; Urbanczyk-Wochniak et al., 2006) was used for analyses (see http://cbi.labri.fr/outsils/data/Tomato/VitC/sup.html). More detailed analyses were performed on the most differentially expressed genes selected using a mean ratio threshold >1.6, representing 35 genes in the fruit and 83 genes in the leaf; of these, seven genes in the fruit and 19 in the leaf encoded proteins with unknown functions or, alternatively, presented no homology with known genes (Table III). Most of the genes identified were up-regulated in the leaf from the transgenic lines, only 5% of the differentially expressed genes were repressed in either leaf or fruit.

Classification of the known genes into the different functional categories defined by the Munich Information Center for Protein Sequences (http://mpis.gsf.de/projects/funct) indicated the following distribution in leaf and fruit tissues: In the leaf, a first group (I) contained 18 stress-related transcripts known to be induced by various stresses (wounding, cold, light, etc.), such as geranylgeranyld reductase (Giannino et al., 2004) and Glu decarboxylase (Bouche and Fromm, 2004). A second group (II) contained 21 sugar metabolism-related genes involved in photosynthesis, carbohydrate, and cell wall metabolism, e.g., the Rubisco activase, the xylanogluvac endotransglucosylase hydrolase, the PSI (or PSII) proteins, and the glyceraldehyde-3-P dehydrogenase. A third group (III) of 10 transcripts included several genes possibly involved in regulatory processes, for example a protein phosphatase 2C (PP2C) phosphatase, a MADS-box protein TDR3, and an F-box protein. A last group (IV) contained 10 genes encoding miscellaneous proteins related to transport and various metabolisms.

In the fruit, group I contained six stress-related transcripts including three heat shock proteins. Group II contained six transcripts, two of them encoding a PSII protein and Rubisco. Group III contained 12 transcripts, including a Ser/Thr protein kinase, a PP2C phosphatase, a WRKY type, and the agamous TAG1 transcription factors and a GA 20-oxidase. Remarkably, the MULTIPROTEIN BRIDGING FACTOR1 (MBF1) gene, a transcriptional coactivator known to induce the expression of stress-related genes involved in plant defense (Suzuki et al., 2005) and expressed in tomato (Zegzouti et al., 1999), displayed a high expression in the transgenic line 8 (ratio >4.5) compared to control plants. In addition, several genes involved in ethylene signal transduction such as the ethylene receptor ETR2 or in ethylene response, including the MBF1 factor, the ethylene-responsive protein ERF1, and the ripening-regulated protein DDTFR8 (Zegzouti et al., 1999) were strongly induced in the ripening fruit. Group IV contained four genes encoding proteins related to miscellaneous metabolisms.

**Figure 5.** Microscopic analysis of leaf and fruit pericarp of P35S SlagalldhRNAi transgenic and control plants. A, Micrograph of collodion imprint of adaxial epidermal cells of fully expanded fourth leaf from P35S SlagalldhRNAi line 8 and control plant. Scale bar = 100 µm. B, Leaflet area and adaxial epidermal cell size of fully expanded fourth and fifth leaf from P35S SlagalldhRNAi lines 5 and 8 and control plant. The region examined was located between two midveins in the first 5 cm of the leaflet. Data represent mean ± SD of four individual leaves per plant with six plants per line (n = 24). C, Micrograph of pericarp section of 20 DPA fruit from P35S SlagalldhRNAi line 5, 8, and control. Scale bar = 200 µm. D, Pericarp thickness, number of cell layers, and cell size of 20 DPA fruit from P35S SlagalldhRNAi lines 2, 5, 8, and 38 and control. Measurements were done by in situ observations of a region of interest located between the vessels in transverse pericarp sections from the equatorial region of the fruit. Data represent the mean ± SD of pericarp sections from 10 fruits per plant with six individual plants per line (n = 60). a, Values that were determined by the t test to be significantly different (P < 0.05) from control.
Table III. Genes differentially expressed in leaf and orange fruit of P15S:SlgalldhRNAi line 8 versus control plants

| SGN  | Description                          | P Value    | Ratio |
|------|--------------------------------------|------------|-------|
| Leaf | Group I: Stress                      |            |       |
| U212549 | Glu decarboxylase                   | 0.008      | 2.4   |
| U212565 | Ripening-associated membrane protein | 0.013      | 1.7   |
| U212578 | Alcoholacyltransferase               | 0.009      | 1.6   |
| U212870 | Polyphenol oxidase chloroplastic     | 0.010      | 1.6   |
| U212989 | TSI-1 protein                       | 0.016      | 1.7   |
| U213021 | Wound-induced proteinase inhibitor  | 0.013      | 1.6   |
| U213031 | Wound-induced proteinase inhibitor  | 0.008      | 2.3   |
| U213190 | Cold-induced glucosyltransferase   | 0.015      | 1.7   |
| U213588 | Protease inhibitor                  | 0.010      | 2.1   |
| U213613 | Ethylene-responsive proteinase inhibitor | 0.012 | 1.9   |
| U214777 | Pro-rich protein                     | 0.010      | 2.3   |
| U216788 | Pro synthetase associated protein   | 0.008      | 2.5   |
| U215124 | Ultraviolet-B repressible protein   | 0.014      | 1.6   |
| U224685 | Glucan-1,3-β-glucosidase            | 0.010      | 2.1   |
| U226439 | UDP-glucuronosyltransferase         | 0.013      | 1.7   |
| U218272 | Ser protease                        | 0.017      | 1.8   |
| U214067 | Geranylgeranyl reductase            | 0.008      | 1.8   |
| Group II: Photosynthesis; Carbon and Cell Wall Metabolism | | |
| U212564 | Plasticid aldolase                  | 0.011      | 1.7   |
| U212700 | Plasticid aldolase                  | 0.011      | 1.8   |
| U212863 | Chlorophyll a/b-binding protein precursor | 0.011 | 1.6   |
| U212865 | Chlorophyll a/b-binding protein precursor | 0.008 | 2.3   |
| U212939 | Rubisco activase                    | 0.009      | 1.9   |
| U212940 | Rubisco activase                    | 0.014      | 1.8   |
| U212941 | Rubisco activase                    | 0.010      | 2.3   |
| U213031 | Plastocyanin chloroplast precursor  | 0.014      | 1.9   |
| U213044 | Xyloglucane endotransglucosylase    | 0.009      | 1.9   |
| U213080 | Carbonic anhydrase precursor        | 0.012      | 1.7   |
| U213214 | PSII core complex proteins          | 0.011      | 1.8   |
| U213287 | PSII core complex proteins          | 0.010      | 1.9   |
| U213381 | PSI reaction center subunit         | 0.012      | 1.9   |
| U213390 | PSI reaction center subunit         | 0.011      | 1.7   |
| U213493 | Pectine esterase                    | 0.008      | 1.7   |
| U213559 | Pectine esterase                    | 0.010      | 1.7   |
| U214487 | Extensin                            | 0.011      | 1.6   |
| U216405 | Xyloglucane endotransglycosylase    | 0.014      | 1.7   |
| U216855 | Thioredoxin                         | 0.008      | 1.6   |
| U220664 | Glycerol-3-P acyltransferase        | 0.008      | 1.9   |
| U225515 | Glyceraldehyde-3-P dehydrogenase    | 0.011      | 1.8   |
| Group III: Signal Transduction and Hormones | | |
| U212854 | Calmodulin                          | 0.009      | 1.6   |
| U214384 | Apetala2-like protein               | 0.015      | 1.6   |
| U214458 | RNA-binding protein                 | 0.011      | 1.7   |
| U220213 | MADS-box protein TDR3               | 0.011      | 1.9   |
| U221524 | Ser/Thr protein kinase              | 0.010      | 2.0   |
| U222333 | Ethylene response binding protein   | 0.011      | 1.6   |
| U223492 | F-box protein                       | 0.017      | 2.1   |
| U225548 | PP2C                                 | 0.011      | 1.7   |
| U213123 | Arg decarboxylase                   | 0.011      | 1.6   |
| U213755 | Auxin-binding protein               | 0.012      | 2.3   |
| Group IV: Miscellaneous | | |
| U213050 | Glycolate oxidase                   | 0.010      | 1.8   |
| U213604 | Inorganic pyrophosphatase           | 0.014      | 1.7   |
| U213912 | Aminomethyltransferase              | 0.010      | 1.8   |
| U214617 | Acetyl-CoA C-acyltransferase        | 0.013      | 1.9   |

(Table continues on following page.)
Changes in Metabolite Profiles in the $P_{35S}:Slgalldh^{RNAi}$ Transgenic Lines

We next analyzed the relationship between the phenotypes of three $P_{35S}:Slgalldh^{RNAi}$ lines (2, 8, and 38) and the changes in the redox state of ascorbate via a metabolomic approach. We analyzed the major pathways of primary plant metabolism by using an established gas chromatography-mass spectroscopy method (Fernie et al., 2004). As would be expected, the metabolomic analyses confirmed the rank order of the lines, 5 and 8 being the most affected at the metabolic level (Supplemental Table S2). Furthermore, expanding leaves and orange fruits behaved inversely at the metabolic level (Fig. 6; Supplemental Table S2). In leaves, metabolite analyses revealed a decrease in the levels of the major amino acids whereas some of them, Cys, Pro, Thr, and Val increased. In contrast, in orange fruit, the levels of amino acids were largely unaffected, with the

| SGN$^a$ | Description | $P$ Value$^b$ | Ratio$^c$ |
|---------|-------------|---------------|----------|
| U215017 | Sterol C-methyl transferase | 0.010 | 1.7 |
| U216066 | Vacuolar ATP-synthase subunit | 0.010 | 1.8 |
| U216256 | Cytochrome c oxidase | 0.012 | 1.6 |
| U216323 | Translation initiation factor | 0.011 | 1.6 |
| U216736 | Ca$^{2+}$-ATPase | 0.015 | 1.6 |
| U216923 | Lipid transfer protein | 0.010 | 1.7 |
| U217914 | Metal-transporting P-type ATPase | 0.012 | 1.8 |
| U217993 | Adapin | 0.012 | 1.6 |
| U218299 | Ribosomal protein | 0.011 | 1.6 |
| U218653 | Sterol C-methyl transferase | 0.011 | 1.6 |
| U240467 | Developmental protein related | 0.008 | 1.8 |

### Orange Fruit

**Group I: Stress**

| U212932 | Heat shock protein | 0.012 | 2.1 |
| U213790 | Acidic endochitinase | 0.011 | 1.9 |
| U216459 | Gly-rich protein | 0.012 | 2.0 |
| U216468 | Cytosolic heat shock protein | 0.011 | 4.2 |
| U218323 | Heat shock protein | 0.011 | 2.6 |
| U214174 | Coumarate-CoA ligase | 0.012 | 2.2 |

**Group II: Photosynthesis; Carbon and Cell Wall Metabolism**

| U213287 | PSII | 0.013 | 2.9 |
| U213624 | Pyruvate decarboxylase | 0.012 | 2.3 |
| U216001 | Extensin class I | 0.016 | 1.6 |
| U216086 | Carbohydrate kinase | 0.014 | 2.5 |
| U225512 | Rubisco | 0.011 | 2.1 |
| U225539 | Glc-1-P adenyl transferase | 0.016 | 1.7 |

**Group III: Signal Transduction and Hormones**

| U213126 | Calreticulin | 0.018 | 1.6 |
| U214610 | WRKY protein | 0.012 | −1.6 |
| U215004 | AGAMOUS protein | 0.011 | 1.6 |
| U216554 | Hydroxysteroid dehydrogenase | 0.018 | 2.3 |
| U216991 | Ser/Thr protein kinase | 0.011 | 2.0 |
| U217359 | MBF1 | 0.013 | 4.5 |
| U219135 | Ethylene responsive factor JERF2 | 0.018 | 1.6 |
| U219631 | GA 20-oxidase | 0.017 | 1.9 |
| U229641 | Ethylene-receptor 2 ETR2 | 0.016 | 1.6 |
| U221533 | PP2C | 0.011 | 1.8 |
| U223066 | AP2/ERF-type protein | 0.018 | −1.8 |
| U225322 | Ser/Thr protein kinase | 0.016 | 1.7 |

**Group IV: Miscellaneous**

| U213332 | Peptidyl prolyl isomerase | 0.017 | 2.0 |
| U216689 | Threonyl-tRNA synthetase | 0.019 | 1.8 |
| U217998 | Metal transporter | 0.011 | 1.8 |
| U219908 | Ripening regulated protein DDTFR8 | 0.013 | 4.5 |

$^a$SGN tomato unigenes identification number of cDNA spotted on TOM1 microarray (http://www.sgn.cornell.edu/search/direct_search.pl?search=unigene). $^b$Probability of the $t$ test. $^c$Mean ratio of the normalized data between $P_{35S}:Slgalldh^{RNAi}$ line 8 and control plants.
exception of Val and Met that increased and of Pro that was reduced by half, significantly in the case of line 8 (Fig. 6). In leaves, the level of TCA cycle intermediates was significantly reduced, with the exception of succinate that displayed a slight but significant increase (Fig. 6). Among other organic acids, threonic acid, which corresponds to a degradation product of the ascorbate turnover pathway, displayed an increase in all transformants, whereas the opposite was observed for the \( \gamma \)-aminobutyric acid (GABA) content. In orange fruits, the content of the TCA cycle intermediates was unchanged; however, the succinate content displayed an increase similar to that observed in the leaf. The most important change was for malate, which increased by up to 4.6-fold in line 8. Among the other organic acids, GABA accumulated up to 2.8-fold in fruit of line 8 whereas threonate was slightly reduced.

Analysis of the leaf carbohydrate content revealed that the \( \text{P35S:SlgalldhRNAi} \) transgenic lines were characterized by increases in Fru and Suc, as well as other sugars linked to the cell wall metabolism like Ara, Gal, Man, Rha, and raffinose, which increased by up to 5-fold in line 8. Myoinositol increased significantly in both SlGalLDH lines. In fruit, the level of sugars was largely unaffected; however, the levels of some cell wall-related sugars and myoinositol were significantly reduced, whereas sorbitol and mannitol increased. Interestingly, GalUA, an intermediate of the alternative ascorbate biosynthesis pathway and a degradation product of the cell wall pectins during fruit ripening (Agius et al., 2003) decreased significantly in all transgenic plants. Among the other compounds analyzed, it is interesting to note that intermediates involved in membrane biogenesis like C16:0 and C18:0 fatty acids were increased (Supplemental Table S2).

**DISCUSSION**

The RNAi-reduced expression of SlGalLDH in tomato leads to plants with a residual \( \text{L-GalLDH} \) showing several defects in growth rate and organ size (Figs. 4 and 5). All these observations are consistent with the preferential localization of SlGalLDH transcripts in tissues actively engaged in cell division and/or expansion such as root tips or floral meristem and young fruit, as well as the photosynthetic leaves (Fig. 1). Among the primary transformants, two \( \text{P35S:SlgalldhRNAi} \) transgenic lines showed the most extreme reduction in
total ascorbate content (85–170 nmol ascorbate per g FW) and displayed very severe growth defects such as stunted plants with deformed leaves. These plants remained unable to set flowers and fruits and could not be propagated by cuttings, which prevented their use in subsequent studies. This result suggests the existence of a threshold level of L-GalLDH activity, below which ascorbate content and consequently plant growth are so severely impaired that reduction in L-GalLDH activity can be lethal to the plant. Another intriguing result to emphasize is the apparent complexity of ascorbate regulation in different plant tissues. Silencing of GalLDH had opposite effects in leaves and fruits on metabolic profiles and ascorbate redox state (Supplemental Tables S1 and S2). This discrepancy may result from the different functions of these organs, source (leaves), or sink (fruit). Most probably, it may result from the close relationship between photosynthetic electron transport in chloroplasts and ascorbate pool size in leaves, suggested by Yabuta et al. (2007). Silencing GalLDH has a significant effect on both the transcriptome and metabolome, thus giving emphasis to the importance of the role of GalLDH in plants, and data presented here allow us to draw the following conclusions.

**SGalLDH Activity Is Essential for Plant and Fruit Growth in Tomato**

How the plant compensates for SGalLDH reduction in the P35S:SlgalldhRNAi transgenic lines studied (Table I; Fig. 2), to maintain a pool of ascorbate similar to the controls, remains an open question. The most plausible explanation is that residual SGalLDH activity is high enough in the transgenic lines we tested to sustain sufficient ascorbate biosynthesis. This is supported by the experiment of incubation with L-GalL (Fig. 3) showing that even in lines 5 and 8, in which the activity was severely affected, the ascorbate accumulation rate was maintained at the same level as that of control plants. However, we cannot rule out that the maintenance of the ascorbate pool is associated with a reduction of its turnover. The metabolic analysis of fruit from P35S:SlgalldhRNAi lines gives some support to this hypothesis in that a significant decline in the content of threonate (Fig. 5), the end product of one of the known pathways of ascorbate degradation (Green and Fry, 2005; DeBolt et al., 2006), exists. However, it should be noted that in leaves from the transgenic plants, threonate and its precursor dehydroascorbate strongly accumulated (Fig. 6; Supplemental Table S2), raising the possibility of different regulation of ascorbate degradation in vegetative and reproductive organs. Another possible way for these plants to maintain the vital pool of ascorbate is the activation of alternative pathways of ascorbate biosynthesis (Agius et al., 2003; Wolucka and Van Montagu, 2003; Lorence et al., 2004). The pathway described by Agius et al. (2003) also requires L-GalLDH to catalyze ascorbate biosynthesis. While the

gulose pathway proposed by Wolucka and Van Montagu (2003) still remains hypothetical, another plant pathway involving myoinositol has been proposed by Lorence et al. (2004). Elevated myoinositol levels are usually associated with stress conditions (Nelson et al., 1998). In P35S:SlgalldhRNAi lines, the significant increase in myoinositol level in the leaf (Fig. 6; Supplemental Table S2) could, thus, be indicative of the function of this alternative pathway in vegetative organs.

**Reduced SGalLDH Expression May Affect Cell Growth by Modifying Mitochondrial-Related Energy Metabolism**

Given the accepted role of ascorbate in photosynthesis, the hypothesis of an alteration in the photosynthetic capacity could have been anticipated in the P35S:SlgalldhRNAi lines. However, our results clearly indicate that the reduced organ growth in the P35S:SlgalldhRNAi lines does not result from an impaired photosynthesis, which was not affected in these plants despite the fact that more than 10 of the significantly up-regulated transcripts were directly related to photosynthesis (Table III).

One of the most important metabolic consequences that could explain cell growth impairment in the leaf is the change of mitochondrial function, as shown by the alteration of the Kreb’s cycle (Fig. 6) and respiration rate observed in the most affected line 8 (Table II). This finding is interesting regarding the results of a recent study that supports a strong functional link between respiration and ascorbate synthesis and suggests that L-GalLDH is associated with complex I of the mitochondrial electron transport chain (Millar et al., 2003). This functional association may explain why the capacity of L-GalLDH is variable dependent on growth light intensity (Smirnoff, 2000; Bartoli et al., 2006) since the activity of the mitochondrial electron transport chain also varies with light intensity (Raghavendra and Padmasree, 2003). It is thus conceivable that the removal of most of the L-GalLDH protein from complex I affects electron transport. In support of this theory, levels of organic acids of the TCA cycle are decreased in leaf and fruit and some changes in metabolite and transcript profiles are similar to those observed in illuminated leaves of tomato deficient in TCA cycle enzyme expression (Carrari et al., 2003; Urbanczyk-Wochniak et al., 2006). Furthermore, the results presented here are comparable to those observed in the CMSII mutant of tobacco, which is deficient in complex I function, has a severely inhibited growth phenotype, and displayed constitutively high alternative oxidase activity associated in particular with high alternative NAD(P)H dehydrogenase activity (Dutillleul et al., 2003). However, some divergences exist since the CMSII mutant was able to permanently adjust the cell redox homeostasis, which was not the case in the P35S:SlgalldhRNAi lines, at least in the case of ascorbate. Interestingly, our results corroborate the
work of Millar et al. (2003) who showed that respiration controls ascorbate synthesis in plants, particularly through a link between complex I and GalLDH protein. In the transgenic tomato plants described here, the reduction in SlGalLDH activity appears to directly affect the efficiency of the alternative respiratory pathway and consequently the mitochondrial function, with the combination of changes in ascorbate and respiration resulting in a consequent alteration of the cell growth processes. Since several enzymes of the Kreb’s cycle are redox regulated (Balmer et al., 2004), it is conceivable that any variation in the redox state of the plant may have additional effects on plant growth by modulating energy pathways.

Growth of P35S:SlgalldhRNAi Plants Is Adjusted through Changes in Transcript Expression and Metabolic Profiles

Very little is known about the exact mechanisms by which ascorbate regulates cell growth in plants (Smirnoff, 1996; Noctor and Foyer, 1998). This is all the more complex since in P35S:SlgalldhRNAi plants the vegetative and reproductive tissues behaved oppositely with regard to the ascorbate redox state in comparison to the controls (Table I). Organ growth in plants depends on the processes of cell division and cell expansion that are separately controlled during development (Mizukami, 2001). In the P35S:SlgalldhRNAi transgenic plants, the cell division phase was not affected, since in the most severely affected line 8, the reduction in leaflet and fruit size was clearly related to cell enlargement processes (Fig. 5). Our results are in contrast with the work carried out in BY-2 cells by Tabata et al. (2001) showing that GalLDH antisense transgenic cells displayed a 30% reduction of ascorbate content compared to the wild type, and significant changes of division and growth processes. This discrepancy can be attributed to the fact that BY-2 cells are undifferentiated plant material, in contrast to whole plants organs (Geelen and Inze, 2001). A putative mechanism by which ascorbate could influence cell enlargement has been proposed by Smirnoff (2000). This hypothesis is based on the existence of an ascorbate redox cycle that could stimulate cell expansion through the extrusion of protons in the cell wall, in agreement with the acid growth theory (Rayle and Cleland, 1992). Accordingly, it is tempting to suggest that the reduction in SlGalLDH activity in P35S:SlgalldhRNAi transgenic plants has led to the reduction in plant cell size and this causes a change in apoplastic ascorbate (Table I). Recent genetic evidence from tobacco plants expressing sense and antisense ascorbate oxidase indicates that alteration of ascorbate oxidase activity and ascorbate redox state of the apoplast may to some extent affect plant growth (Pignocchi et al., 2006).

Several recent reports have suggested that plant mitochondria are part of signaling pathways known as the mitochondrial retrograde regulation (MRR), and participate in the response to oxidative stress (for review, see Rhoads and Subbaiah, 2007). According to the MRR process, changes in the mitochondrial function trigger altered nuclear transcript expression. Much of what is known about plant MRR turns around the response to a dysfunctional mitochondrial electron transport chain and induction of genes encoding enzymes involved in the recovery of mitochondrial function, such as AOX and alternative NAD(P)H dehydrogenases as well as genes encoding proteins involved in the maintenance of the redox homeostasis, such as glutathion reductase, catalases, ascorbate peroxidases, and superoxide dismutases. Variations in ascorbate redox state could in turn modulate the crosstalk between several defense and growth regulating pathways to adapt the plant to its challenging environment. Overall our data are consistent with the hypothesis, suggesting that the changes in ascorbate redox state in the plant induced by the silencing of SlGalLDH mimic stress-related MRR signaling pathways, inducing a set of stress- and defense-related genes. Hence, the transcriptome analysis revealed that a high proportion of the genes differentially expressed in the leaf and fruit from P35S:SlgalldhRNAi lines are known to be induced by a wide range of biotic or abiotic stresses. Most of them are hormone-responsive genes with functions in signaling for plant-defense and stress-response pathways (Table III). This is particularly obvious in the fruit from P35S:SlgalldhRNAi plants where the transcriptional coactivator MBF1, previously shown to be ethylene regulated in tomato (Alba et al., 2004), is highly expressed (>4.5-fold change). Constitutive expression of MBF1 gene in Arabidopsis confers to the plant an enhanced tolerance to environmental stresses and to bacterial infection, possibly by modulating the ethylene-response signal transduction pathway (Suzuki et al., 2005). Besides, a wide range of genes up- or down-regulated in the transgenic plants are also involved in signal transduction pathways and may participate in the regulation of developmental processes (e.g. the MADS- box gene Agamous; Ferrario et al., 2006). The tight connection between ascorbate content and/or apoplastic ascorbate redox state and hormonal signaling for plant defense was recently pinpointed in studies on the Arabidopsis ascorbate mutant vtc1, which is affected in ascorbic acid synthesis and response (Pastori et al., 2003) and on the ascorbate oxidase overexpressing tobacco lines showing altered sensitivity to auxin (Pignocchi et al., 2006). Similarly, the metabolic analysis revealed significant changes for several stress-related compounds, e.g. Pro (Parre et al., 2007) and GABA (Lancien and Roberts, 2006). Taken all together, the extensive analysis of P35S:SlgalldhRNAi plants further highlights the complexity of ascorbate regulation and its relation with different aspects of plant metabolism and also plant tissues, e.g. the photosynthetic leaf and the developing fruit, since they display both distinct (metabolism) and common (signaling) features with respect to ascorbate.
MATERIALS AND METHODS

Plant Material and Growth Conditions
Cherry tomato (Solanum lycopersicum ‘West Virginia 106′) plants were grown in a greenhouse with supplemental lighting when needed with a light period of 14/10 h, under a thermoperiod of 25°C/20°C, and watered daily three times with a solution at pH 5.8 containing oligolements plus 3.5 mM KNO₃, 1 mM K₂SO₄, 2 mM KH₂PO₄, 6 mM Ca(NO₃)₂, and 2 mM MgSO₄ until the stage fruit set of the first truss, and then 4 mM KNO₃, 1.5 mM K₂SO₄, 1.5 mM KH₂PO₄, 4 mM Ca(NO₃)₂, and 1.5 mM MgSO₄. For in vitro culture, plants were grown on one-fourth Murashige and Skoog basal Murashige and Skoog medium (Kalisys-Duchefa) containing 3% Suc under 14/10 h photoperiod at 400 μmol m⁻² s⁻¹ and at 25°C. Tomato fruits were harvested at various developmental stages according to the number of DPA and fruit diameter. Prior to all biochemical and molecular analyses, samples were quickly frozen in liquid nitrogen, ground to fine powders, and stored at −80°C until use.

Determination of Photosynthetic Activity

Photosynthetic activity of attached leaves was measured with a CO₂ analyzer using infrared detection (LCA3; Analytical Development Corporation).

Respiration Determinations of Isolated Mitochondria

To measure mitochondrial respiratory parameters, mitochondria were prepared from young leaf (50–100 g) following the method of Holtzapffel et al. (2002). After isolation and purifications steps, the mitochondria were washed and resuspended in the incubation medium containing 10 mM TES, pH 7.2, 300 mM Suc, 0.1% (w/v) bovine serum albumin (BSA), 5 mM KH₂PO₄, 5 mM MgCl₂, and 10 mM KCl to a concentration of 10 to 50 mg protein ml⁻¹. Respiration measurement was performed using O₂ electrode (782 Oxygen meter, Strathkelvin Instruments) in the presence of several electron donors (cytochrome c, succinate, fumarate, and glycerol 3-phosphate) and several electron acceptors (O₂, ferricyanide, etc.) until the attainment of steady-state respiration. The oxygen consumption rate (OCR) for each substrate was determined in the presence of each electron acceptor as the difference of the maximal OCR with and without the electron acceptor. This OCR was the measure of oxygen consumption rate (OCR). Experimentally determined respiration rates for isolated mitochondria were consistent with expected respiration rates for the electron acceptors used. In addition, the use of the electron acceptors was validated using the DCMU (dichlorophenyltriazuronitril) treatment to induce the non-cyclic electron transport to provide the steady-state OCR for each acceptor used. In these experiments, the OCRs were measured after the addition of 50 μM DCMU to the mitochondrial suspension. OCRs were determined using the DCMU-induced steady-state OCR for each acceptor used.

RNA Extraction, Reverse Transcription-PCR Analysis, and DNA Construct Preparation

Total RNA extraction from tomato fruit and semiquantitative reverse transcription (RT)-PCR analysis using SlGalLDH gene-specific primers (Supplemental Table S1) were performed as described in Lemaire-Chamley et al. (2005). To obtain an SlGalLDH-specific cDNA fragment, RT (SuperScript II Reverse transcriptase) was performed using 2 μl of total RNA (Invitrogen, life technologies). A 133 bp SlGalLDH DNA fragment located in the 3'-untranslated region of the cDNA (GenBank accession no. AB080590) was then amplified by PCR using Ex Taq DNA polymerase (TaKaRa Bio INC) and the gene-specific primers GLDF1 and GLDR1. The purified DNA fragment was cloned as an inverted repeat under the control of the 35S promoter using the Gateway cloning system as described by Karimi et al. (2002), first into the entry vector (pDONR 201), then into the destination vector [pK7GWIWG2(1)]

Microarray Analysis

Hybridization and Data Acquisition

The TOM1 cDNA microarrays (Center for Gene Expression at the Boyce Thompson Institute; http://bit.comell.edu/CGEP/CGEP.html) contain 13,400 printed elements corresponding to approximately 8,700 unigenes (Alba et al., 2004). The Cyscribe Post Labeling kit from Amersham (catalog no. RPN6560X, Amersham Biosciences) was used according to the manufacturer’s recommendations, with 30 μg of total RNA per condition. The pooled cy3- and cy5-labeled cDNAs were then concentrated on Microcon YM-30 columns (Amicon Bioseparations, Millipore) and mixed with 90 μL of hybridization solution containing 1:1 (v:v) formamide (5X SSC, 0.25% SDS), 5X Denhardt’s solution, and 1 μg ml⁻¹ denatured salmon sperm DNA (Stratagene). Slides were hybridized in an automated hybridization station HS 4800 (Tecan) with a washing prerun in 1X SSC, 0.1% SDS, for 1 min. The probe solution was boiled for 1 min and then injected into the hybridization chamber. Slides were incubated at 42°C for 16 h, with medium agitation, and then washed sequentially at 30°C in 1X SSC, 0.1% SDS for 1 min, this step was repeated three times, in 0.1X SSC, 0.1% SDS for 3 min, and finally in 0.1X SSC for 30 s. Slides were dried in the hybridization station for 3 min, with 2.7 bars of nitrogen gas. Microarray slides were scanned with a GenePix 4000 B fluorescence reader (Axon Instruments) using GenePix 4.0 image acquisition software with photomultiplier tube voltage adjusted to 500 V for Cy3 and 600 V for Cy5.

Bioinformatic Analysis

Spot flagging was done first by GenePix (missing spots) and then by visual inspection of the images to exclude the bad spots (saturation and heterogeneity). Raw data files were submitted to LIMMA v2.3.3 Bioconductor package (Smyth et al., 2005; http://bioinr.wihi.edu.au/limma/). Negatively flagged spots were excluded from further analysis by giving them a zero weight value. Data were then normalized by the print-tip lowess method without background subtraction followed by the scale method to adjust the data between the slides. The correlation between the replicated spots was calculated (duplicateCorrelation function) and the linear model was fitted with the lmFit function for each gene, using this correlation. Moderated t statistics and log odds of differential expression were computed (ebayes function) for the contrast of interest (Pm控股股东/Pm控股股东 transgenic line versus wild type) and for each gene. The calculated P values for this contrast were adjusted for multiple testing with the false discovery rate method. All analyses were performed using the default parameter setting of LIMMA.

In Situ Hybridization

To use SlGalLDH as a probe, riboprobes were synthesized from plasmids containing a 506 bp fragment of SlGalLDH cDNA obtained from tomato by PCR using GLDF1 and GLDR1 primers (Supplemental Table S1). The sense and antisense digoxigenin-labeled riboprobes were generated by run off transcription using T7 and SP6 RNA polymerases according to the manufacturer’s protocol (Roche Diagnostics). For in situ hybridization, tomato flower buds, fruits of 7 and 20 DPA, young leaves, and shoot and root tips were sampled and processed as described by Bereteriaide et al. (2002).
Production of a Rabbit Polyclonal Antibody Anti-SiGalLDH and IgG Purification

For polyclonal antibody preparation, two synthetic polypeptides corresponding to the SiGalLDH protein were prepared by the Eurogentec Company. Their sequences that were deduced from the SiGalLDH tomato gene (accession no. AB080900) were: H2N-MSKEGKPPNKPKYIT-C-CONH2 (amino acids 321–335) and H2N-AYNQARKELDPNRL-C-CONH2 (amino acids 559–573). The +C corresponds to a Cys residue needed for the coupling with the keyhole limpet hemocyanin protein carrier. The antibodies were produced by rabbit immunized with a mix of the two peptides. Total IgGs were purified on affinity column (AffinoTOYOPEARL 650 s) by Eurogentec.

Enzyme Activity, SDS-PAGE, and Immunodetection of SiGalLDH

Fresh young leaves (approximately 0.5 g) were homogenized with a mortar and pestle in 1 mL of 0.1 M Tris-HCl buffer (pH 7.5) that contained 0.3 M Suc, 1% (w/v) polyvinylpyrrolidone, 0.2% (w/v) BSA, 1 mM dithiothreitol, and 50 mM EDTA. SiGalLDH activity was assayed spectrophotometrically at room temperature by measuring the increase in A245, accompanied by the reduction of cytochrome c as described by Oba et al. (1995). Protein was quantified using BSA as a standard. Protein samples (10 μg per lane) were separated on 15% SDS-PAGE and transferred to Immobilon-P membrane prior to immunological detection by western-blot analysis using the SiGalLDH antibody at 1:300 dilution. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1:10,000 (Chemicon) with the Bio-Rad Chemiluminescence blotting substrate (POD) system from Roche Applied Science.

Ascorbic Acid Analysis

Ascorbate analyses were performed according to Leipner et al. (1997). Between 0.5 and 1 g of frozen samples were homogenized in 2.5 mL of cold 3% (w/v) metaphosphoric acid and 2.5 mM EDTA. The homogenate was then centrifuged at 10,000g for 10 min at 4°C. An aliquot of 200 μL was incubated for 15 min at room temperature with 100 μL of K2HPO4, 45%, and either 50 μL of distilled water to measure the reduced ascorbate, or 50 μL of homo-Cys (0.1%) to measure the total ascorbate. After the incubation, 500 μL of citrate-P buffer (2 M, pH 2.3) were added. The A265 was measured immediately after addition of 500 μL 2,6-dichlorophenolindophenol (0.008% w/v). For measurement of apoplastic ascorbate content, the intercellular washing fluid (IWF) was prepared using a method similar to that described by Turcsányi et al. (2000). Leaflets of the fourth leaf (about 1 g) were washed in distilled water and were twice vacuum infiltrated (2070 Pa) to measure the total ascorbate content. The A265 was subsequently determined by centrifugation at 12,000g for 20 min at 4°C and immediately used for ascorbate measurement. The time from the harvest of the leaf to the beginning of centrifugation was less than 10 min. Activity of Glc-6-P dehydrogenase (EC 1.1.1.49), an enzyme located in the cytoplasm and the chloroplast stroma, was absent in the IWF, indicating that the harvest of the leaf to the beginning of centrifugation was less than 10 min. The peak activity of Glc-6-P dehydrogenase was determined by maximal L-galactono-1,4-lactone dehydrogenase (GalLDH) activity under drought stress. Plant Cell Environ 23: 1073–1081

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Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB080900.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Sets of PCR primers used to amplify specific regions of genes of interest.

Supplemental Table S2. Central metabolism of fully expanded leaf and orange fruit of P35S/SiGalLDH (XG1) transgenic and control plants.

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