Running head: Mitochondrial SfGalLDH silencing and ascorbate in tomato

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Silencing of the mitochondrial ascorbate synthesizing enzyme L-galactono-1,4-lactone dehydrogenase (L-GalLDH) affects plant and fruit development in tomato.


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Abstract
L-galactono-1,4-lactone dehydrogenase (L-GalLDH, EC 1.3.2.3) catalyses the last step in the main pathway of vitamin C (L-ascorbic acid) biosynthesis in higher plants. In this study, we first characterized the spatial and temporal expression of \textit{SlGalLDH} in several organs of tomato (\textit{Solanum lycopersicum} L.) plants in parallel with the ascorbate content. \textit{P}35S:\textit{Slgalldh}\textsuperscript{RNAi} silenced-transgenic tomato lines were then generated using an RNAi strategy, in order to evaluate the effect of any resulting modification of the ascorbate pool on plant and fruit development. In all \textit{P}35S:\textit{Slgalldh}\textsuperscript{RNAi} plants with reduced \textit{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 studied exhibited up to an 80\% reduction in \textit{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 TCA cycle, as well as secondary metabolic pathways related to stress response were modified in leaves and fruit of \textit{P}35S:\textit{Slgalldh}\textsuperscript{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, GalLDH could play an important role in the regulation of cell growth related processes in plants.
Introduction:

Vitamin C (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 α-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 references therein).

Micro-organisms, 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 1960’s, the animal pathway has been completely characterized (Burns, 1960). It involves D-glucose as the initial precursor and the last step is catalysed by a microsomal L-gulono-1,4-lactone oxidase, which oxidizes 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 and co-workers 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 L-galactono-1,4-lactone (L-GalL) to ascorbic acid, a reaction catalysed by the L-galactono-1,4-lactone dehydrogenase (here referred to as L-GalLDH), 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 (Ôba 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 fruit, ascorbate is produced via the reduction of D-galacturonic acid 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-mannose-3,5-epimerase (GME) is capable of catalysing two distinct epimerization reactions that produce either GDP-L-galactose 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 myo-inositol as a precursor. By overexpressing myo-inositol oxygenase in Arabidopsis, they observed a 2- to 3-fold increase in ascorbate content in the leaf and discuss the possible contribution of myo-inositol 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-fold and 7-fold increase of ascorbate content in lettuce and tobacco plants, respectively. Whilst, L-GulL oxidising activity has been reported in Arabidopsis by Davey et al. (1999) who measured L-GulL oxidising 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 (Noctor and Foyer, 1998; Smirnoff, 1996), but also for controlling plant development as recently shown in root and other organs (Olmos et al., 2006). In order to investigate the physiological function of L-GalLDH in plants, we analyzed the effect of L-GaLDH 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, 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 P35S:SlgalldhRNAi transgenic lines further suggest that phenotypic changes can be attributed to the resulting modifications in secondary and primary metabolism.
Results

Ascorbate and L-galactono-1,4-lactone dehydrogenase (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 (Figure 1 a-b). In vegetative organs, 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 to 2.3 µmol per g FW) compared to the leaf (6.8 to 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-galactono-1,4-lactone dehydrogenase (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; Tokunaga et al., 2005; Bartoli et al., 2005). Screening of tomato EST databases (SGN and TIGR tomato EST databases; http://www.sgn.cornell.edu and http://www.tigr.org/) allowed the identification of a single cDNA encoding L-GalLDH (named SIGalLDH for Solanum lycopersicum L-GalLDH), which corresponds to a unique gene in tomato, as further confirmed by Southern blot analysis (data not shown). Examination of SIGalLDH expression showed that SIGalLDH transcripts (Figure 1a) and immunodetected SIGalLDH protein (data not shown) were ubiquitously detected in the various tomato organs analyzed. The high SIGalLDH mRNA abundance in the leaf was in agreement with the high ascorbate content in this tissue (Figure 1b) while other organs such as root or fruit did not display this relationship. Close examination of the spatial distribution of SIGalLDH transcripts in developing organs by mRNA in situ hybridization (Figure 1c) further indicated that SIGalLDH 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.

\textbf{P35S:SlgalldhRNAi} 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 \textit{SlGalLDH} activity in tomato, we generated transgenic plants expressing a \textit{SlGalLDH}–specific RNA interference sequence fragment under the control of the CaMV35S promoter (hence named \textit{P35S:SlgalldhRNAi} transformants). Among 12 independent primary \textit{P35S:SlgalldhRNAi} transformants showing the presence of a single copy of the transgene, we selected four transgenic lines showing a range of phenotypic changes varying from severe (line-8) to moderate (line-5) and slight (line-38 and -2) for further analyses on the next generation (T1 plants). To verify that the morphological changes observed in primary transformants were linked to the RNAi-mediated reduction in \textit{SlGalLDH} mRNA, the detailed analysis of \textit{SlGalLDH} mRNA and protein abundance and activity was performed in leaf and/or 20 and 42 DPA fruit from the corresponding T1 homozygous \textit{P35S:SlgalldhRNAi} transgenic lines-2, -5, -8 and -38 and from controls. In all transgenic lines, \textit{SlGalLDH} mRNA, protein abundance and residual \textit{SlGalLDH} activity were highly reduced by comparison with the control plants (Figure 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 (Figure 3). Leaves from the transgenic lines and controls accumulate ascorbate at similar rates when incubated with the
ascorbate precursor, L-galactono-1,4-lactone. Further investigations indicated that no viable plant with \( SlGalLDH \) activity below the threshold level of 15% was ever recovered among the \( P_{35S}:Slgalldh^{RNAi} \) transformants suggesting the essentiality of this activity.

\( P_{35S}:Slgalldh^{RNAi} \) 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, \( P_{35S}:Slgalldh^{RNAi} \) transgenic lines -5 and -8 and controls were grown in a phytotronic chamber in order to avoid any daily climate changes. Measurements of the total ascorbate content (AsA + DHA) as well as of the reduced-ascorbate (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 1). 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, ~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 1). This effect, which was already noticeable in 21 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 1) was similar to that observed in control plants in the greenhouse (Figure 1b). In the control fruit, 80% of total ascorbate was in the form of oxidised ascorbate during the cell division and expansion phases (10 to 20 DPA) while more than 90% of the total ascorbate was in the form of reduced ascorbate (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 which exhibited a slightly higher ascorbate content. In contrast with the leaf, transgenic fruit from lines -5 and -8 exhibited a
significant increase in ascorbate reduced form (AsA) (Table 1), mainly during
the cell division and cell expansion stages of the fruit (10 to 30 DPA).

Because of the putative role of apoplastic ascorbate in the signalling
processes modulating cell growth and development (Pignochi and Foyer, 2003),
we investigated the total and reduced ascorbate content in the apoplast of fully
expanded leaves of 6-week-old P_{35S:SlgalldhRNAi} 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 10-15% of the apoplastic ascorbate
was present as the reduced form. In the P_{35S:SlgalldhRNAi} transgenic line-8 and
line-5, the reduced ascorbate pool remained very low (85 to 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 1).

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 in order to
investigate respiratory parameters in the P_{35S:SlgalldhRNAi} transgenics. When
tricarboxylic acid 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 2). However, when
external NADH dehydrogenases were engaged by addition of exogenous
NADH, the respiration rate in P_{35S:SlgalldhRNAi} transgenic line-8 was increased
by 1.6 fold whereas that in P_{35S:SlgalldhRNAi} transgenic line-5 was similar to the
rate of control plant mitochondria. In the same order, a significant two fold
activation of cyanide-insensitive (alternative oxidase) respiration which was
inhibited by SHAM, was measured in mitochondria from P_{35S:SlgalldhRNAi} line-8.
When no substrate, other than GL, 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 characterisation of this effect revealed that the
germination rate, the plant growth rate, the fruit weight and the diameter were
all reduced (Figure 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 P 35S: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
transformant and control plants at incident irradiance of between 200 to 1000
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 transformants 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 P 35S:SlgalldhRNAi
transgenics, which resulted from a 25% reduction in leaflet area in the most
strongly inhibited lines (Figure 5a-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 (Figure 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 days). 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 (Figure 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 (Figure 5d).

**Functional classification of genes expressed in leaf and ripening fruit of P35S::SlgalldhRNAi transgenic lines.**

In order to investigate the possible contribution of changes in transcript expression triggered by the reduction in SlGaLDH activity and/or variations in ascorbate redox state to the morphological and cellular alterations of transgenic plants (Figure 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, 1269 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 supplementary data online at http://cbi.labri.fr/outils/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 supplementary data online). 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, 7 genes in the fruit and 19 in the leaf encoded proteins with unknown functions or, alternatively, presented no
homology with known genes (Table 3). Most of the genes identified were upregulated 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 MIPS (http://mips.gsf.de/projects/funcat) 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 geranylgeranyl reductase (Giannino et al., 2004), and glutamate decarboxylase (Bouché 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 xyloglucan endotransglucosylase hydrolase, the PS-II (or PS-I) proteins and the glyceraldehyde-3-phosphate dehydrogenase. A third group (III) of 10 transcripts included several genes possibly involved in regulatory processes, for example a PP2C phosphatase, a MADS box protein TDR3 and a 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 photosystem-II protein and Rubisco. Group III contained 12 transcripts, including a serine/threonine protein kinase, a PP2C phosphatase, a WRKY-type and the agamous TAG1 transcription factors and a gibberellin 20-oxidase. Remarkably, the MBF1 (Multiprotein Bridging factor 1) gene, a transcriptional co-activator known to induce the expression of stress-related genes involved in plant defence (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.
We next analysed the relationship between the phenotypes of three
P₃₅S:SlgalldhRNAi lines (-2, -8 and -38) and the changes in the redox state of
ascorbate via a metabolomic approach. We analysed the major pathways of
primary plant metabolism by using an established gas chromatography-mass
spectroscopy (GS-MS) 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 (Table S2). Furthermore, expanding leaves
and orange fruits behaved inversely at the metabolic level (Figure 6 and 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 exception of Val and Met that increased and of Pro that was reduced by
half, significantly in the case of line-8 (Figure 6). In leaves, the level of TCA
cycle intermediates was significantly reduced with the exception of succinate
that displayed a slight but significant increase (Figure 6). Among other organic
acids, threonic acid, which corresponds to a degradation product of the
ascorbate turn-over pathway, displayed an increase in all transformants,
whereas the opposite was observed for the γ-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 P₃₅S:SlgalldhRNAi transgenic lines were
characterised by increases in Fru and Suc, as well as other sugars linked to the
cell wall metabolism like arabinose, galactose, mannose, rhamnose, and
raffinose which increased by up to 5-fold in line-8. Myo-inositol 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 myo-
inositol were significantly reduced, whereas sorbitol and mannitol increased. Interestingly, galacturonic acid, 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 analysed, it is interesting to note that intermediates involved in membrane biogenesis like C16:0 and C18:0 fatty acids were increased (Table S2).
Discussion

The RNAi-reduced expression of SiGalLDH in tomato leads to plants with a residual L-GalLDH activity showing several defects in growth rate and organ size (Figures 4 and 5). All these observations are consistent with the preferential localization of SiGalLDH 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 (Figure 1). Among the primary transformants, two P_{3SS}:SiGalldh^{RNAi} transgenic lines showed the most extreme reduction in total ascorbate content (85 to 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 (Tables 1 & S2). This discrepancy may result from the different functions of these organs, source (leave) 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, giving thus emphasis to the importance of the role of GalLDH in plants and data presented here allow us to draw the following conclusions.

**SiGalLDH activity is essential for plant and fruit growth in tomato.**

How the plant compensates for SiGalLDH reduction in the P_{3SS}:SiGalldh^{RNAi} transgenic lines studied (Table 1, Figure 2), in order to maintain a pool of ascorbate similar to the controls, remains an open question.
The most plausible explanation is that residual SlGalLDH 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-galactono-1,4-lactone (Figure 3) showing that even in line-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 \( P_{3SS}:Slgalldh^{RNAi} \) lines gives some support to this hypothesis in that a significant decline in the content of threonate (Figure 5), the end-product of one of the known pathways of ascorbate degradation (DeBolt et al., 2006; Green and Fry, 2004). However, it should be noted that in leaves from the transgenic plants, threonate and its precursor DHA strongly accumulated (Figure 6, 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; Lorence et al., 2004; Wolucka and Van Montagu, 2003). The pathway described by Agius et al. (2003) also requires L-GalLDH to catalyse ascorbate biosynthesis. While the gulose pathway proposed by Wolucka and Van Montagu (2003) still remains hypothetical, another plant pathway involving myo-inositol has been proposed by Lorence et al. (2004). Elevated myo-inositol levels are usually associated with stress conditions (Nelson et al., 1998). In \( P_{3SS}:Slgalldh^{RNAi} \) lines, the significant increase in myo-inositol level in the leaf (Figure 6, Table S2) could, thus, be indicative of the function of this alternative pathway in vegetative organs.

\( Reduced \ SlGalLDH \ expression \ may \ affect \ cell \ growth \ by \ modifying \ mitochondrial-related \ energy \ metabolism \)

Given the accepted role of ascorbate in photosynthesis, the hypothesis of an alteration in the photosynthetic capacity could have been anticipated in the \( P_{3SS}:Slgalldh^{RNAi} \) lines. However, our results clearly indicate that the reduced
organ growth in the P_{35S}:Slgalldh^{RNAi} lines does not result from an impaired photosynthesis, which was not affected in these plants despite the fact that more than ten of the significantly up-regulated transcripts were directly related to photosynthesis (Table 3).

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 Krebs cycle (Figure 6) and respiration rate observed in the most affected line-8 (Table 2). 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 (Bartoli et al., 2006; Smirnoff et al., 2000) since the activity of the mitochondrial electron transport chain also varies with light intensity (Raghavendra and Padmasee, 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 AOX activity associated in particular with high alternative NAD(P)H dehydrogenase activity (Dutilleul et al., 2005). However, some divergences exist since CMSII mutant was able to permanently adjust the cell redox homeostasis, which was not the case in the P_{35S}:Slgalldh^{RNAi} 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 Krebs 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 (Noctor and Foyer, 1998; Smirnoff, 1996). 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 1). Organ growth in plants depends on the processes of cell division and cell expansion which 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 were clearly related to changes in cell size and not in cell number (Figure 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 cell lines 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 Inzé, 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 1). 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 signalling pathways known as the mitochondrial retrograde regulation (MRR), and participate in the response to oxidative stress (reviewed by 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 cross-talk between several defence and growth regulating pathways in order 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 signalling pathways, inducing a set of stress and defence-related genes. Hence, the transcriptome analysis revealed that a high proportion of the genes differentially expressed in the leaf and fruit from $P_{35S}\text{:Slgalldh}^{RNAi}$ 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 signalling for plant-defence and stress-response pathways (Table 3). This is particularly obvious in the fruit from $P_{35S}\text{:Slgalldh}^{RNAi}$ plants where the transcriptional coactivator Multiprotein Bridging Factor 1 (MBF1) previously shown to be ethylene-regulated in tomato (Alba et al., 2005), is highly expressed (>4.5 fold change). Constitutive expression of MBF1c gene in Arabidopsis confers to the plants 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 apolastic ascorbate redox state and hormonal signalling for plant defence was recently pinpointed in studies on the Arabidopsis ascorbate mutant vtc1, which is affected in ABA synthesis and response (Pastori et al., 2003) and on the ascorbate oxidase over-expressing 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. proline (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 (signalling) features with respect to ascorbate.
Materials and Methods

Plant material and growth conditions

Cherry tomato \( [\text{Solanum lycopersicum} \text{ L. cv West Virginia 106 (WVa106)}] \) plants were grown in a greenhouse with supplemental lighting when needed with lightperiod 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 oligoelements plus 3.5 mM KNO\(_3\), 1 mM K\(_2\)SO\(_4\), 2 mM KH\(_2\)PO\(_4\), 6 mM Ca(NO\(_3\))\(_2\), 2 mM MgSO\(_4\) until the stage fruit set of the first truss and then 4 mM KNO\(_3\), 1.5 mM K\(_2\)SO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 4 mM Ca(NO\(_3\))\(_2\), 1.5 mM MgSO\(_4\). For \emph{in vitro} culture, plants were grown on ¼ MS basal Murashige & Skoog medium (Kalys-Duchefa, St Ismier, 38330-France) containing 3% sucrose under 14/10 hr 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 days post-anthesis (DPA) and fruit diameter. Prior to all biochemical and molecular analysis, 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 CO\(_2\) analyzer using infrared detection (LCA3 – Analytical Development Corporation).

Respiration determinations of isolated mitochondria

To measure mitochondrial respiratory parameters, mitochondria were prepared from young leaf (50-100g) following the method of Holtzapffel \textit{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 sucrose, 0.1% (w/v) BSA, 5 mM Kh\(_2\)PO\(_4\), 5 mM MgCl\(_2\) and 10 mM KCl to a concentration of 10-50 mg protein mL\(^{-1}\). Respiration measurement was performed using O\(_2\)-electrode (782 Oxygen meter, Strathkelvin Instruments,
Glasgow, UK) in the presence of several electron donors and respiratory inhibitors like KCN and SHAM (salicylhydroxamic acid). Mitochondrial integrity was determined according to Holtzapffel et al. (2002) and was generally between 75-85%.

**Cytological analyses**

Pericarp cell number and size were measured in fruits as described by Cheniclet et al. (2005). The cell size of leaflet epidermis was measured by applying a thin layer of collodion 4% resin (Merck, Darmstadt, Germany) of about 2-3 cm² on the adaxial surface of the fourth and fifth leaves on the plant. After evaporation of the solvent, the epidermis imprint was visualised under a Zeiss Axiophot microscope with a Spot digital camera (Diagnostic instruments, Sterling Heights, MI). Cell size of leaflet epidermis was determined using the Image Pro-Plus software (Media Cybernetics, Silver Spring, MA). Leaflet surface was determined by scanning the same leaves used for epidermis imprint and the resulting image was analyzed using the Image Pro-Plus software.

**RNA extraction, RT-PCR analysis and DNA construct preparation**

Total RNA extraction from tomato fruit and semi-quantitative RT-PCR analysis using *SlGalLDH* gene-specific primers (Table S1), were performed as described in Lemaire-Chamley et al. (2005). In order to obtain an *SlGalLDH* specific cDNA fragment, reverse transcription (SuperScript™ II Reverse transcriptase) was performed using 2 µg of total RNA (Invitrogen, life technologies). A 133 bp *SlGalLDH* DNA fragment located in the 3' UTR of the cDNA (GenBank accession no. AB080690) was then amplified by PCR using Ex Taq™ DNA polymerase (TaKaRa Bio INC, Japan) and the gene-specific primers GLDF4 and GLDR4. 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)). This construct was checked by sequencing and introduced into cherry tomato (WVa106) plants by *Agrobacterium tumefaciens* strain GV3101 according to Hamza and Chupeau (1993). The first generation plants (T0) were characterized and screened. Of the 12 lines studied, 4 lines named 2, 5, 8, and 38 were selected for further detailed analyses in the T1 generation. Control plants correspond to plants transformed with an empty vector following the same protocol.

**Experimental design for transcriptome and metabolome analyses**

Plant materials used for these studies were the leaflet of the fourth leaf and the outer pericarp of the orange fruit. One centimetre length young leaves were tagged at the plant apex in order to allow comparison of leaflets at the same age and position on the plant. For fruit studies, flowers were tagged at anthesis (fully opened flower) and fruit were further selected according to their colour measured on the external surface of fruit using a chromameter. The Lab values corresponding to orange stage were respectively: L45, a~20, b~30. Once harvested, the leaflets and the outer pericarp were combined in pools of 10-15 samples, frozen in N2 and stored at -80°C.

For transcript expression profiling of *P*$_{35S}$:*SlgalldhRNAi* silenced lines versus control plants, microarray experiments were performed using two biological repeats for each tissue (4 slides per experiment) and technical duplicates with the dyes reversed (dye swap), as described in Lemaire-Chamley *et al.* (2005).

**Microarray analysis**

*Hybridization and data acquisition*

The TOM1 cDNA microarrays (Center for Gene expression at the Boyce Thompson Institute; [http://bti.cornell.edu/CGEP/CGEP.html](http://bti.cornell.edu/CGEP/CGEP.html)) contain 13400 printed elements corresponding to approximatively 8700 unigenes (Alba *et al.*, 2004). The "Cyscribe Post Labelling kit" from Amersham (catalog #RPN5660X,
Amersham biosciences corp, NJ USA) was used according to the manufacturer’s recommendations, with 30 µg of total RNA per condition. The pooled cy3- and cy5-labelled 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; 5 X Denhardt’s solution; 1 µg mL⁻¹ denatured salmon sperm DNA (Stratagene)). Slides were hybridized in an automatic hybridization station HS 4800 (Tecan), with a washing pre-run 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 16h, with medium agitation and then washed sequentially at 30°C in 1X SSC; 0.1% SDS for 1 min, this step was repeated 3 times, in 0.1X SSC; 0.1% SDS for 1 min, 3 times 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 in order 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://bioinf.wehi.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 substraction 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 \((P_{35S:SlgalldhRNAi}^{transgenic\ line\ vs\ WT})\) and for each gene. The calculated \(P\)-values for this contrast were adjusted for multiple testing with the
FDR method. All analyses were performed using the default parameter setting of LIMMA.

**In situ hybridization**

In order 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 (Table S1). The sense and antisense digoxygenin-labelled riboprobes were generated by run off transcription using T7 and SP6 RNA polymerases according to the manufacturer’s protocol (Roche Diagnostics, Meylan, France). 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 Bereterbide *et al.* (2002).

**Production of a rabbit polyclonal antibody anti-SlGalLDH and IgG purification**

For polyclonal antibody preparation, two synthetic polypeptides corresponding to the SlGalLDH protein were prepared by the Eurogentec Company (Herstal, Belgique). Their sequences which were deduced from the SlGalLDH tomato gene (accession no AB080690) were: H2N-MSKEKGPPKNKPYTC-CONH2 (aa 321-335) and H2N-AYNQARKELDPNRILC-CONH2 (aa 559-573). The “+C” corresponds to a cysteine residue needed for the coupling with the KLH protein carrier. The antibodies were produced by rabbit immunized with a mix of the two peptides. Total G-type immunoglobulins (IgG) were purified on affinity column (AF-amino TOYOPEARL 650M) by Eurogentec.

**Enzyme activity, SDS-PAGE and Immuno-detection of SlGalLDH.**

Fresh young leaves (~ 0.5 g) were homogenized with a mortar and pestle in 1mL of 0.1 M Tris-HCl buffer (pH 7.5) that contained 0.3M sucrose, 1% (w/v)
Polyvinylpolypyrrolidone, 0.2% (w/v) bovine serum albumin (BSA), 1 mM DTT and 50 mM EDTA. SlGalLDH activity was assayed spectrophotometrically at room temperature by measuring the increase in absorbance at 550 nm accompanied by the reduction of Cyt c as described by Ôba 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 SlGalLDH antibody at 1/300 dilution. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated IgG diluted 1/10000 (Chemicon, Temecula, CA) with the BM 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 to 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 10000 g 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 homocysteine (0.1%) to measure the total ascorbate. After the incubation, 500 µl of citrate-phosphate buffer (2M, pH 2.3) were added. The absorbance at 524 nm was measured immediately after addition of 500 µl DCIP (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 Turcsanyi et al. (2000). Leaflets of the fourth leaf (about 1 g) were washed in distilled water and were twice vacuum infiltrated (-70kPa) for 3 min with 50 mL of 10 mM citrate buffer (pH 3.0) containing 100 mM KCl to reach maximum infiltration of cellular air spaces. Leaflets were then carefully blotted dry, rolled and inserted into a 5 mL tip placed over a preweighed centrifuge tube containing 50 µl of 5% (w/v) cold metaphosphoric acid. The IWF (about 100 µl.g⁻¹ FW) was subsequently collected by centrifugation at 1200 g for 10 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 glucose-6-phosphate dehydrogenase (EC 1.1.1.49), an enzyme located in the cytoplasm and the chloroplast stroma, was absent in the IWF, indicating that IWF was not contaminated by intracellular sap. In order to measure de novo ascorbate biosynthesis in leaves, 10-20 g of leaflet were rapidly washed in distilled water, sliced in fine stripes and incubated at 25°C under continuous light (200 µmol photons m−2 s−1) in Murashide-Skoog medium buffered with 100 mM Tris-HCl, (pH 8.0) in the presence of 25 mM gulono-1,4-lactone or galactono-1,4-lactone (~200mg tissue in 5 mL medium). In time course of the incubation, the tissues were wringed on paper and snap frozen in liquid N2, and stored at -80°C prior to ascorbate assay.

**Determination of levels of other metabolites**

Samples of leaflets and orange fruit pericarp prepared as described above were used for metabolite extraction as described by Nunes-Nesi et al. (2005). The level of all metabolites was quantified by GC-MS exactly following the protocol described by Roessner et al. (2001), with the exception that the peak identification was optimized to tomato tissues (Roessner-Tunali et al., 2003).
Supplemental Material

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

Table S2. Central metabolism of fully expanded leaf and orange fruit of $P_{35S:SlgalldhRNAi}$ transgenic and control plants.

Analysis of the transcript expression profiling using TOM1 tomato microarray in the $P_{35S:SlgalldhRNAi}$ transgenic and control plants is accessible at the following URL: http://cbi.labri.fr/outils/data/Tomato/VitC/sup.html.
Acknowledgements

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Literature Cited


Figure legends.

**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 (days post anthesis), 20 DPA, mature green (MG), orange (Or) and red ripe (RR) stages. Data obtained by semi-quantitative 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 6 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.

**Figure 2.** *SlGalLDH* expression, protein and activity in *P35S:SlgalldhRNAi* transgenic and control plants.

(a) *SlGalLDH* mRNA relative abundance were determined in young leaves (Yl) and fruit at 20 and 42 DPA (orange stage) in *P35S:SlgalldhRNAi* plants (line-2, -5, -8 and -38) and compared to control plants. Data obtained by semi-quantitative 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 *P35S:SlgalldhRNAi* line-2, -5, -8 and -38 and control plants. (c) *SlGalLDH* activity in young leaves from *P35S:SlgalldhRNAi* line-2, -5, -8 and -38 compared to control plants. Data represent mean ± SD of six individual plants.
per line. Star symbols above bars indicate values that were determined by the t test to be significantly different (P<0.05) from control.

**Figure 3.** Ascorbate accumulation in tomato leaves.

Leaf stripes from *P35S:SlgalldhRNAi* transgenic line-5 (△), line-8 (□) and control (○) plants were incubated in MS (open symbols) or MS containing 25 mM L-galactono-1,4-lactone (filled symbols) in the light. Total ascorbate was assayed as described in the Material and Methods. The error bars indicate standard errors (n=3).

**Figure 4.** Phenotypic comparison between *P35:SlgalldhRNAi* transgenic and control plants.

(a) Germination and plant growth. Left and middle panel, seedlings from *P35:SlgalldhRNAi* line-2, -5, -8 and -38 and from control at 10 days after sowing (DAS); right panel, 6-week-old plants from severely affected *P35:SlgalldhRNAi* line-5 and from control. (b) Growth kinetic. Plant height from *P35:SlgalldhRNAi* line-5, -8 and from control were measured every 4 days starting from 6 days after germination. Data represent mean ± SD of 10 individual plants. (c) Fruit size. Top panel, pictures of ripe fruit from *P35:SlgalldhRNAi* line-2, -5, -8 and -38 and from control; bottom panel, fruit diameter measured on 42 DPA fruit. Data represent mean ± SD of 10 fruits per plant with 6 individual plants per line (n = 60 fruits). Star symbols above bars indicate values that were determined by the t test to be significantly different (P<0.05) from control.

**Figure 5.** Microscopic analysis of leaf and fruit pericarp of *P35S:SlgalldhRNAi* transgenic and control plants.
(a) Micrograph of collodion imprint of adaxial epidermal cells of fully expanded fourth leaf from P35S:SlgalldhRNAi line 8 and control plant. Scale bar = 100µm. (b) Leaflet area and adaxial epidermal cell size of fully expanded fourth and fifth leaf from P35S:SlgalldhRNAi line-5, -8 and control plant. The region examined was located between two mid-veins in the first 5 cm of the leaflet. Data represent mean ± SD of 4 individual leaves per plant with 6 plants per line \( (n = 24) \). (c) Micrograph of pericarp section of 20 DPA fruit from P35S:SlgalldhRNAi 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:SlgalldhRNAi line-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 6 individual plants per line \( (n = 60) \). a indicates values that were determined by the \( t \) test to be significantly different \( (P<0.05) \) from control.

**Figure 6.** Description of central metabolism of fully expanded leaves and orange fruits from plants of the P35S:SlgldhRNAi line-8.

Metabolite content of leaf and orange fruit were determined as described in “experimental procedures”. Data were normalized with respect to the mean response calculated for the control (to allow statistical assessment, individual sample from this set of plants were normalized in the same way). A colour code indicates that values for metabolite content were determined by the \( t \) test to be significantly different \( (P<0.05) \) from control (empty vector transgenic plant). Metabolites marked in red indicate that their relative content was decreased with respect to the control, and those marked in blue were increased. Examples of metabolite changes are represented by the values (mean ± SE) of determinations of six individuals plants with 10 leaves and fruits of each plant. Abbreviations: G6P, glucose-6-phosphate; 3-PGA, 3-phosphoglycerate; F6P,
fructose-6-phosphate; FA16:0, palmitate; FA18:0, stearate; PEP, phosphoenolpyruvate.
Table 1. Ascorbate in tomato leaf and fruit of P<sub>35S::SlgalldhRNAi</sub> transgenic and control plants.

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<td></td>
<td>Tot Asc</td>
<td>Control</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Tot Asc</td>
<td>Line-8</td>
<td>2.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Tot Asc</td>
<td>Line-5</td>
<td>1.8 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Ratio</td>
<td>Control</td>
<td>0.2</td>
<td>0.2</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ratio</td>
<td>Line-8</td>
<td>0.2</td>
<td>0.5</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Ratio</td>
<td>Line-5</td>
<td>0.3</td>
<td>0.4</td>
<td>0.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leaf Apoplast</th>
<th>Control</th>
<th>Line-8</th>
<th>Line-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsA</td>
<td>(nmol.g⁻¹ FW)</td>
<td>85 ± 60</td>
<td>111 ± 10</td>
</tr>
<tr>
<td>Tot Asc</td>
<td>648 ± 49</td>
<td>402 ± 47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>370 ± 104&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Reduced (AsA) and total ascorbate (Tot Asc) contents of P<sub>35S::SlgalldhRNAi</sub> line-8, line-5 and control plants were measured in young developing leaves from seedlings at the indicated days after sowing (DAS) and in developing fruits at the indicated days post anthesis (DPA). Apoplastic ascorbate content was measured in the fully expanded fourth leaf of 6-week-old plants. The reduced/total ascorbate ratio was determined for all the samples. Data represent mean ± SD of six individual plants with two repeats per plant. a indicates values that were determined by the t test to be significantly different (P<0.05) from control.
Table 2. Respiration and SlGalLDH activity in isolated mitochondria from leaves.

<table>
<thead>
<tr>
<th>Respiration</th>
<th>Control</th>
<th>Line-5</th>
<th>Line-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron donor (nmol O₂ min⁻¹ mg⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM NADH –</td>
<td>48.7 ± 5.5</td>
<td>46 ± 4.5</td>
<td>73.6 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 2.5 mM ADP</td>
<td>86.9 ± 8.4</td>
<td>75.8 ± 7.1</td>
<td>139.9 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 2 mM KCN</td>
<td>10.4 ± 1.8</td>
<td>10.9 ± 3.8</td>
<td>20.6 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 1 mM SHAM</td>
<td>8.4 ± 2.5</td>
<td>9.6 ± 1.9</td>
<td>12.5 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Respiratory Control</td>
<td>1.78</td>
<td>1.65</td>
<td>1.90</td>
</tr>
<tr>
<td>5 mM Succinate –</td>
<td>39.7 ± 7.8</td>
<td>48.4 ± 7.3</td>
<td>42.6 ± 10.9</td>
</tr>
<tr>
<td>+ 2.5 mM ADP</td>
<td>86.9 ± 13</td>
<td>97.1 ± 12.8</td>
<td>95.2 ± 20.7</td>
</tr>
<tr>
<td>Respiratory Control</td>
<td>2.19</td>
<td>2.00</td>
<td>2.23</td>
</tr>
<tr>
<td>5 mM Pyr/Malate –</td>
<td>29.5 ± 4.5</td>
<td>34 ± 4.4</td>
<td>30.6 ± 3.4</td>
</tr>
<tr>
<td>+ 2.5 mM ADP</td>
<td>42.8 ± 8.3</td>
<td>47.6 ± 8.8</td>
<td>46.8 ± 4.6</td>
</tr>
<tr>
<td>Respiratory Control</td>
<td>1.45</td>
<td>1.40</td>
<td>1.53</td>
</tr>
<tr>
<td>5 mM AsA –</td>
<td>39.9 ± 6.4</td>
<td>42.1 ± 5.1</td>
<td>39.7 ± 3.9</td>
</tr>
<tr>
<td>2.5 mM TMPD –</td>
<td>409.5 ± 32.5</td>
<td>393.7 ± 44.3</td>
<td>380.1 ± 45.4</td>
</tr>
<tr>
<td>4.2 mM GL –</td>
<td>9.7 ± 0.9</td>
<td>5 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ 10 µM Cyt c</td>
<td>13.4 ± 1.6</td>
<td>6.9 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

SlGalLDH activity (nmol Cyt c min⁻¹ mg⁻¹ protein)

<table>
<thead>
<tr>
<th>SlGalLDH activity</th>
<th>Control</th>
<th>Line-5</th>
<th>Line-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 ± 15.1</td>
<td>49.6 ± 10.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Respiration was measured in intact mitochondria prepared from young developing leaves of P35S:SlgalldhRNAi line-8, line-5 and control plants as described in the Material and Methods. The reaction was conducted with 500 µg of mitochondria protein in 1 mL of respiration buffer in the presence of substrates at concentration as indicated. Prior to measure the respiration in the presence of GL, the mitochondria were incubated with 12.5 µM antimycine A. SlGalLDH activity was measured with 30 µg of mitochondrial protein as described in the Material and Methods. Data represent mean ± SD of seven or eight individual mitochondrial isolations. a indicates values that were determined by the t test to be significantly different (P<0.01) from control.
## Table 3. Genes Differentially Expressed in Leaf and Orange Fruit of P<sub>35S::SlgalldhRNAi</sub> Line-8 vs Control Plants

<table>
<thead>
<tr>
<th>SGN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description</th>
<th>P-Value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SGN&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description</th>
<th>P-Value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LEAF</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>LEAF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group I : Stress</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>Group IV : Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U212569</td>
<td>Glutamate decarboxylase</td>
<td>0.008</td>
<td>2.4</td>
<td>U213050</td>
<td>Glycolate oxidase</td>
<td>0.010</td>
<td>1.8</td>
</tr>
<tr>
<td>U212565</td>
<td>Ripening-associated membrane protein</td>
<td>0.013</td>
<td>1.7</td>
<td>U213604</td>
<td>Inorganic pyrophosphatase</td>
<td>0.014</td>
<td>1.7</td>
</tr>
<tr>
<td>U212578</td>
<td>Alcohololtransferase</td>
<td>0.009</td>
<td>-1.6</td>
<td>U213912</td>
<td>Aminomethyltransferase</td>
<td>0.010</td>
<td>1.8</td>
</tr>
<tr>
<td>U212870</td>
<td>Polyphenol oxidase chloroplastic</td>
<td>0.010</td>
<td>1.6</td>
<td>U214617</td>
<td>Acetyl-CoA C-acyltransferase</td>
<td>0.013</td>
<td>1.9</td>
</tr>
<tr>
<td>U212899</td>
<td>TSI-1 protein</td>
<td>0.016</td>
<td>1.7</td>
<td>U215017</td>
<td>Sterol C-methyl transferase</td>
<td>0.010</td>
<td>1.7</td>
</tr>
<tr>
<td>U213021</td>
<td>Wound-induced proteinase inhibitor</td>
<td>0.013</td>
<td>1.6</td>
<td>U215077</td>
<td>1.013</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>U212700</td>
<td>Plastidic aldolase</td>
<td>0.011</td>
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<td>Protease inhibitor</td>
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<td>2.1</td>
</tr>
<tr>
<td>U212863</td>
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<td>U213559</td>
<td>Pectine esterase</td>
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<td>1.7</td>
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<tr>
<td>U212932</td>
<td>Heat shock protein</td>
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<td>U213332</td>
<td>Peptidyl prolyl isomerase</td>
<td>0.017</td>
<td>-2.1</td>
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<tr>
<td>U212939</td>
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<td>U223333</td>
<td>Ethylene response binding protein</td>
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<td>U223492</td>
<td>F-box protein</td>
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<td>-2.1</td>
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<tr>
<td>U212989</td>
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<td>F-box protein</td>
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<tr>
<td>U213559</td>
<td>Metallothionein-like protein</td>
<td>0.010</td>
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<td>U225515</td>
<td>Glyceraldehyde-3-P dehydrogenase</td>
<td>0.011</td>
<td>1.8</td>
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<tr>
<td>U213564</td>
<td>Polyphosphate transferase</td>
<td>0.011</td>
<td>1.8</td>
<td>U215755</td>
<td>Auxin-binding protein</td>
<td>0.012</td>
<td>2.3</td>
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<tr>
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<td>1.8</td>
<td>U215755</td>
<td>Auxin-binding protein</td>
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<td>2.3</td>
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<tr>
<td>U213564</td>
<td>Polyphosphate transferase</td>
<td>0.011</td>
<td>1.8</td>
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<td>Auxin-binding protein</td>
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<tr>
<td>U213564</td>
<td>Polyphosphate transferase</td>
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<td>U215755</td>
<td>Auxin-binding protein</td>
<td>0.012</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* SGN: Tomato Unigenes identification number of cDNA spotted on TOM1 microarray (http://www.sgn.cornell.edu/search/direct_search.pl?search=unigene).  
* P-Value: Probability of the t-test.  
* Ratio: Mean ratio of the normalized data between P<sub>35S::SlgalldhRNAi</sub> Line-8 and Control Plants.
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 (days post anthesis), 20 DPA, mature green (MG), orange (Or) and red ripe (RR) stages. Data obtained by semi-quantitative 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 6 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 (S), root apical meristem (R), young leaf (L), 9 mm flower bud (F), and cross-section of dividing fruit at 7 DPA (D), and growing at 20 DPA (G) 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.
Figure 2. 

A. SlGalLDH mRNA relative abundance were determined in young leaves (Yl) and fruit at 20 and 42 DPA (orange stage) in P₃₅S:SlgalldhRNAi plants (line-2, -5, -8 and -38) and compared to control plants. Data obtained by semi-quantitative 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 P₃₅S:SlgalldhRNAi line-2, -5, -8 and -38 and control plants. C, SlGalLDH activity in young leaves from P₃₅S:SlgalldhRNAi line-2, -5, -8 and -38 compared to control plants. Data represent mean ± SD of six individual plants per line. Star symbols above bars indicate values that were determined by the t test to be significantly different (P<0.05) from control.
Figure 3. Ascorbate accumulation in tomato leaves.

Leaf stripes from P35S:SlgalldhRNAi transgenic line-5 (△), line-8 (□) and control (○) plants were incubated in MS (open symbols) or MS containing 25 mM L-galactono-1,4-lactone (filled symbols) in the light. Total ascorbate was assayed as described in the Material and Methods. The error bars indicate standard errors (n=3).


Figure 4. Phenotypic comparison between P<sub>35::Slgalldh<sup>RNAi</sup> lines transgenic and control plants.

A, germination and plant growth. Left and middle panel, seedlings from P<sub>35::Slgalldh<sup>RNAi</sup> line-2, -5, -8 and -38 and from control at 10 days after sowing (DAS); right panel, 6-week-old plants from severely affected P<sub>35::Slgalldh<sup>RNAi</sup> line-5 and from control. B, growth kinetic. Plant height from P<sub>35::Slgalldh<sup>RNAi</sup> line-5, -8 and from control were measured every 4 days starting from 6 days after germination. Data represent mean ± SD of 10 individual plants. C, fruit size. Top panel, pictures of ripe fruit from P<sub>35::Slgalldh<sup>RNAi</sup> line-2, -5, -8 and -38 and from control; bottom panel, fruit diameter measured on 42 DPA fruit. Data represent mean ± SD of 10 fruits per plant with 6 individual plants per line (n = 60 fruits). Star symbols above bars indicate values that were determined by the t test to be significantly different (P<0.05) from control.
Figure 5. Microscopic analysis of leaf and fruit pericarp of P35S:SlgalldhRNAi transgenic and control plants.

A. Micrograph of collodion imprint of adaxial epidermal cells of fully expanded fourth leaf from P35S:SlgalldhRNAi line 8 and control plant. Scale bar = 100µm. B. Leaflet area and adaxial epidermal cell size of fully expanded fourth and fifth leaf from P35S:SlgalldhRNAi line-5, -8 and control plant. The region examined was located between two mid-veins in the first 5 cm of the leaflet. Data represent mean ± SD of 4 individual leaves per plant with 6 plants per line (n = 24). C. Micrograph of pericarp section of 20 DPA fruit from P35S:SlgalldhRNAi 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:SlgalldhRNAi line-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 6 individual plants per line (n = 60). * indicates values that were determined by the t test to be significantly different (P<0.05) from control.

<table>
<thead>
<tr>
<th>Line</th>
<th>Leaflet (cm²)</th>
<th>Epidermal cell (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38 ± 6</td>
<td>141 ± 9</td>
</tr>
<tr>
<td>Line-5</td>
<td>23 ± 5</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>Line-8</td>
<td>28 ± 6</td>
<td>98 ± 7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line</th>
<th>Area</th>
<th>Pericarp cell layers</th>
<th>Pericarp thickness</th>
<th>Pericarp cell size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line-2</td>
<td>23 ± 5</td>
<td>97 ± 1</td>
<td>91 ± 1</td>
<td>92 ± 1</td>
</tr>
<tr>
<td>Line-5</td>
<td>28 ± 6</td>
<td>98 ± 1</td>
<td>78 ± 1</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>Line-8</td>
<td>28 ± 6</td>
<td>95 ± 1</td>
<td>85 ± 1</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>Line-38</td>
<td>38 ± 6</td>
<td>96 ± 1</td>
<td>88 ± 1</td>
<td>87 ± 1</td>
</tr>
</tbody>
</table>

% relative to control

<table>
<thead>
<tr>
<th>Line</th>
<th>Pericarp cell layers</th>
<th>Pericarp thickness</th>
<th>Pericarp cell size</th>
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</thead>
<tbody>
<tr>
<td>Line-2</td>
<td>97 ± 1</td>
<td>91 ± 1</td>
<td>92 ± 1</td>
</tr>
<tr>
<td>Line-5</td>
<td>98 ± 1</td>
<td>78 ± 1</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>Line-8</td>
<td>95 ± 1</td>
<td>85 ± 1</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>Line-38</td>
<td>96 ± 1</td>
<td>88 ± 1</td>
<td>87 ± 1</td>
</tr>
</tbody>
</table>
Figure 6. Description of central metabolism of fully expanded leaves and orange fruits from plants of the P35S:SlgldhRNAi line-8.

Metabolite content of leaf and orange fruit were determined as described in “experimental procedures”. Data were normalized with respect to the mean response calculated for the control (to allow statistical assessment, individual sample from this set of plants were normalized in the same way). A colour code indicates that values for metabolite content were determined by the t test to be significantly different (P<0.05) from control (empty vector transgenic plant). Metabolites marked in red indicate that their relative content was decreased with respect to the control, and those marked in blue were increased. Abbreviations: G6P, glucose-6-phosphate; 3-PGA, 3-phosphoglycerate; F6P, fructose-6-phosphate; FA16:0, palmitate; FA18:0, stearate; PEP, phosphoenolpyruvate.