An Orange Ripening Mutant Links Plastid NAD(P)H Dehydrogenase Complex Activity to Central and Specialized Metabolism during Tomato Fruit Maturation

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In higher plants, the plastidial NADH dehydrogenase (Ndh) complex supports nonphotochemical electron fluxes from stromal electron donors to plastoquinones. Ndh functions in chloroplasts are not clearly established; however, its activity was linked to the prevention of the overreduction of stroma, especially under stress conditions. Here, we show by the characterization of OrrDs, a dominant transposon-tagged tomato (Solanum lycopersicum) mutant deficient in the NDH-M subunit, that this complex is also essential for the fruit ripening process. Alteration to the NDH complex in fruit changed the climacteric, ripening-associated metabolites and transcripts as well as fruit shelf life. Metabolic processes in chromoplasts of ripening tomato fruit were affected in OrrDs, as mutant fruit were yellow-orange and accumulated substantially less total carotenoids, mainly β-carotene and lutein. The changes in carotenoids were largely influenced by environmental conditions and accompanied by modifications in levels of other fruit antioxidants, namely, flavonoids and tocopherols. In contrast with the pigmentation phenotype in mature mutant fruit, OrrDs leaves and green fruits did not display a visible phenotype but exhibited reduced Ndh complex quantity and activity. This study therefore paves the way for further studies on the role of electron transport and redox reactions in the regulation of fruit ripening and its associated metabolism.

INTRODUCTION

The shift to ripening in fleshy fruit involves multiple metabolic changes, including the accumulation and degradation of pigments such as carotenoids and chlorophyll in tomato (Solanum lycopersicum) (Adams-Phillips et al., 2004). The pathway of carotenoid biosynthesis has been intensively studied, particularly in tomato fruit (Fraser et al., 2009). While most structural genes of the pathway have been characterized (Hirschberg, 2001), very little is known regarding the regulatory network that controls carotenogenesis. The control of carotenoid biosynthesis via the redox status of components of the photosynthetic electron transport has been established during etioplast-to-chloroplast transition (Woitsch and Romer, 2003). In earlier studies, the involvement of quinones in carotenoid desaturation was demonstrated biochemically (Mayer et al., 1990) and genetically (Norris et al., 1995). It was further discovered that phytoene desaturase activity responded to the redox state of the plastoquinone (PQ) pool (Nievelstein et al., 1995). In chloroplasts, PQ oxidoreduction status can be modulated by linear electron transfer and also by one of the two alternative electron pathways operating around photosystem I (PSI; Bukhov and Carpenter, 2004; Johnson, 2005). Moreover, it was proposed that chlororespiration, a respiratory electron transfer chain in the thylakoids, whose functioning has been clearly established in Chlamydomonas reinhardtii (Nixon, 2000; Pettier and Cournac, 2002) could also modulate PQ pool redox status in chloroplasts of higher plants. Although it has been demonstrated that the oxidative reaction of chlororespiration involving a plastid-located terminal oxidase (PTOX; Wu et al., 1999; Carol and Kuntz, 2001) deeply affected carotenogenesis in leaves (Wu et al., 1999; Josse et al., 2000), the participation of the other reactions in modulating the redox state of PQ has not been investigated.

For the last 10 years, many efforts have been pursued to identify the molecular entities underlying the cyclic electron transport.
Several Arabidopsis mutants affected in these pathways have been studied (Munekage et al., 2002; Rumeau et al., 2005; Ishihara et al., 2007; Shikanaı̂, 2007a; DalCorso et al., 2008; Peng et al., 2008; Sirpiö et al., 2009; Takabayashi et al., 2009) and showed no change in their carotenoid content. However, participation of PQ redox state in carotenogenesis in other tissues than leaves cannot be excluded. Indeed, plant genetic modification has shown that in some cases carotenogenesis was differentially affected in fruit and in leaves, suggesting that the metabolic pathways and/or their regulation are different in both organs (Bramley et al., 1992).

The NADH dehydrogenase (Ndh) complex, likely originating from the respiratory electron transfer chain of a cyanobacterial ancestor, catalyzes PQ reduction using soluble electron donors (Burrows et al., 1998; Endo et al., 2008). It participates in one of the two electron pathways operating around PSI. The other pathway likely involves a still uncharacterized ferredoxin-plastoquinone reductase and the two interacting proteins PGR5 and PGR1 (Munekage et al., 2002; DalCorso et al., 2008). Because Ndh and PTOX colocalize in the stroma lamellae (Lennon et al., 2003), they have been implicated in the chlororespiratory pathway, although direct evidence of electron transfer between Ndh and PTOX is still lacking.

Accumulation of the Ndh complex within etiolated leaf tissue (Lennon et al., 2003) led to the hypothesis that the corresponding electron pathway may participate in the etioplast-to-chloroplast transition process by energizing the plastid membrane and favoring synthesis and/or insertion of the photosynthetic complexes. However, the absence of any obvious phenotype related to greening in Ndh-deficient plants (Ishikawa et al., 2008) appears to contradict this hypothesis. By contrast, PTOX is essential during greening and carotenoid biosynthesis (Wu et al., 1999; Carol and Kuntz, 2001). It was suggested to be a component of a redox chain responsible for the desaturation of phytoene and perhaps ω-carotene (Wu et al., 1999; Carol and Kuntz, 2001). In this chain, electrons would be transferred from phytoene (and ω-carotene) to the PQ pool via phytoene desaturase (PDS) and ω-carotene desaturase (ZDS) and from the PQ pool to molecular oxygen via PTOX. The Ndh complex subunits were also detected in nonphotosynthetic tissues, such as maize (Zea mays) etioplasts and tomato fruit (Guera and Sabater, 2002). In tomato fruit, the same authors found that the Ndh complex is mainly present in the outer pericarp tissue and that its amount and activity in the plastids decreased during the transition from chloroplasts to chromoplasts. These findings imply that the Ndh complex may be important for several processes in plant development, including fruit maturation.

As part of our efforts to understand the regulation of metabolic pathways in tomato fruit, we identified a novel transposon-tagged mutant. It was named Orange Ripening (OrrDz) due to its typical pigmentation of the fruit. The transposon insertion created a dominant mutation that altered carotenoid biosynthesis as well as other metabolic pathways in the fruit, such as those producing flavonoids and tocopherols. The ORR protein showed sequence similarity to the Arabidopsis thaliana NDH-M subunit of the Ndh complex. Detailed characterization of the OrrDz mutant and the corresponding gene implies the association of the Ndh complex in the process of fruit ripening and related metabolism.

RESULTS

Isolation of the OrrDz Tomato Fruit Mutant

To identify mutants altered in metabolic pathways associated with tomato fruit ripening, we screened an Ac/Ds transposon-based mutant population for changes in fruit appearance (Meissner et al., 1997). The fruit of one mutant line (termed OrrDz) exhibited orange and yellow sectors, suggesting an insertion in a gene involved in fruit carotenogenesis (Figure 1A). A transposase-free plant recovered from the mutant offspring lines displayed a yellow-orange color in the entire fruit (Figure 1B), and its selfing segregated as expected from a codominant trait, χ² = 0.9 for 1 (red fruit; wild type)/9; 40); 2 (yellow fruit; heterozygote)/22; 40); 1 (orange fruit, homozygote)/9; 40). In addition, crossing orange fruit plants with the wild type resulted in a population that contained only plants with yellow fruit, whereas crossing plants producing yellow fruit with wild-type plants gave two groups of F1 progeny (total 43 plants): one displaying red fruit (26 plants) and the other with yellow fruit (17 plants). Thus, we concluded that the mutation OrrDz exhibits an overdominant mode of inheritance.

The OrrDz mutants (particularly the heterozygotes) contained reduced levels of the yellow flavonoid pigment that normally accumulates in the peel (Figure 1C). The color of the OrrDz ripe fruit was strongly dependent on the environmental conditions (radiation, shading, and temperature; see Supplemental Figure 1 online). Moreover, compared with the wild type, the levels of total soluble solids (TSSs) in the fruit were reduced by 23 and 15% in OrrDz/Orr and OrrDz/Orr, respectively (see Supplemental Figure 2 online). Detached ripe OrrDz/Orr fruit exhibited a strong delay in shrinking when left to dry at room temperature (see Supplemental Figure 2 online) and were often smaller in size.

ORR Corresponds to the Subunit M of the Tomato Ndh Complex

Inverse PCR analysis identified a Ds insertion in the 5’ end of a putative tomato homolog (SGN-U579052) of the Arabidopsis gene for subunit M of the Ndh complex (Figure 2). The ORR gene was mapped in the center of tomato chromosome 4 and was found to contain a single intron. Based on the sequence of the entire tomato genome, ORR is a unique locus (see Supplemental Figure 3 online). It putatively encodes a 211-amino acid protein that is 62% identical (75% similar) to the characterized Arabidopsis NDH-M protein (At4g37925; Rumeau et al., 2005) and shows the highest homology (72% identity and 85% similarity) to an olive (Olea europaea) protein (see Supplemental Figure 5 online).

As shown in Figure 2, we discovered a complex insertion with one intact element (Ds-2) flanked by the double 3SS cauliflower mosaic virus (CaMV) promoter (2X 3SS) and another truncated element (Ds-4). Such composite element structures have been previously described (Huang and Dooner, 2008); the most likely explanation for its formation is described in Supplemental Figure 3 online.
A single line that was recovered following a transposon excision event in the \(Orr^{Ds}\) mutant exhibited red pigmented fruit (a revertant phenotype; \(ex-3SS-Orr\); Figure 1D), in which the \(Ds-2\) element had excised and the \(ORR\) was consequently driven by the \(35S\) CaMV promoter (see Supplemental Figure 4 and Supplemental Table 1 online). This excision event generated a typical excision footprint, leaving most of the host duplication intact with a small deletion (see Supplemental Figure 6 online).

We further demonstrated that the insertion cosegregated with the \(Orr^{Ds}\) mutation and was only present in plants that exhibited the mutant phenotype of orange or yellow fruit (see Supplemental Figure 6 online). This validation was performed by examining plants segregating from the selfing of \(Orr^{Ds}/Orr^{Ds}\) fruit (total 40 plants; see above). Selected plants (20 in total) were chosen randomly for evaluation of cosegregation between the insertion and fruit pigmentation using DNA gel blots and PCR analyses. These experiments as well as others performed in the course of this study involving genotyping and cosegregation assays of hundreds of plants for the different \(Orr^{Ds}\) genotypes showed complete linkage between the genotype and the phenotype.
Examination of the ORR full-length transcript revealed that it contained three in-frame AUG codons at its 5' end (AUG-I, II, and III, amino acids 1, 8, and 15 of the putatively encoded protein, respectively, counting from the 5' end). The transposon insertion in the OrrDs mutants was located in between AUG-I and AUG-II (Figure 2). To examine which ORR transcripts are formed in the case of each genotype (wild type; OrrDs/OrrDs and ex-35S-Orr), rapid amplification of cDNA ends–PCR (RACE-PCR) experiments were performed in which the ORR gene 5' upstream region was isolated (Figure 2; see Supplemental Figure 5 online).

The wild-type ORR transcript untranslated region contained AUG-I, II, and III as described above. The homozygous genotype produced a shorter transcript that initiated (i.e., the mRNA) exactly at AUG-III, suggesting that either the transcript will be degraded or a protein starting from the next, downstream AUG, will be generated (termed AUG-IV; amino acid position 64; Figure 2). In the case of the ex-35S-Orr genotype, we identified a long transcript (ex-35S-Orr_L) that contained a small part of the 35S promoter region and a region of the wild-type untranslated region directly upstream of AUG-II (AUG-I was not present; Figure 2; see Supplemental Figure 4 online). A second transcript detected in the ex-35S-Orr genotype was shorter (ex-35S-Orr_S) and started downstream of AUG-III, suggesting that if a protein is produced in this case, its translation would start from AUG-IV (similar to the homozygous transcript; see above).

The capability to genetically complement the OrrDs mutation was tested by generating transgenic plants, which overexpress two different ORR transcripts, starting either from AUG-I or AUG-II,
that were driven by the 35S CaMV promoter (35S-AUGI-ORR and 35S-AUGII-ORR, respectively). Transformation of 35S: AUGI-ORR in the OrrDs/OrrDs background resulted in plants with red fruit that accumulated the typical orange color of the peel (Figure 1E). Complementation of the phenotype was also observed in F1 fruit obtained through a cross between 35S: AUGII-ORR transgenic lines (#1919) and a OrrDs/OrrDs plant (Figure 1F) and a T1 plant obtained after transforming OrrDs/OrrDs with the 35S:AUGII-ORR construct (Figure 1G).

To further exclude the possibility that a gene flanking the insertion site was affected in the mutant, we examined the expression of a putative zinc-finger protein (the closest gene to ORR on its 5’ end; see Figure 2) in the mutant and the wild-type fruit using quantitative RT-PCR (qRT-PCR) and detected no difference. In addition, we annotated a large region flanking ORR on chromosome 4 and found a unigene (SGN-U576377) located 12.2 kb downstream from ORR in the same orientation. It encodes a protein that is 56% identical and 73% similar to the peroxisome biogenesis protein PEX1 from Arabidopsis. Another unigene located 9.6 kb upstream to ORR in the opposite orientation is SGN-U580948, which encodes a putative RPS15A (ribosomal protein S15A). While we cannot completely exclude effects of the Ds insertion on expression of the latter two genes, this possibility is unlikely because of the significant distance between them and the Ds element. Moreover, the nature of the products of these genes does not conform to the unique phenotypes seen in heterozygous and homozygous plants.

This set of results provided strong evidence that the insertion in the putative tomato NDH-M-like ORR gene is responsible for the OrrDs phenotype.

**ORR Gene Expression and Subcellular Localization of Its Corresponding Protein Variants**

qRT-PCR analysis showed that ORR is preferentially expressed in green, chlorophyll-containing tissues, with highest levels detected in mature leaves (Figure 3A). ORR expression was detected in young leaves, immature and mature green fruit (either peel, flesh, or seeds), stems (data not shown), and in flower buds and open flowers (including sepals), while no or traces levels of transcripts were evident in mature fruit tissues (i.e., peel, flesh, and seeds), roots, and pollen. Higher ORR expression in the early green stages of fruit development and subsequent decline in the maturation phase (mature green and breaker stages) was further supported by microarray data (Mintz-Oron et al., 2008; see Supplemental Figure 7 online).

In silico prediction tools (i.e., iPsort and TargetP) suggested that the putative ORR protein (starting from AUG-I) is targeted to the plastids. To experimentally determine the subcellular localization of the ORR protein, a construct was generated in which the entire ORR open reading frame, starting from the AUG-I codon, was fused to the 5’ end of a green fluorescent protein (GFP) reporter. Subcellular localization was investigated using confocal microscopy after transient expression of the ORR (AUG-I)-GFP fusion in tobacco (Nicotiana tabacum) leaves (Figure 3B). The natural chlorophyll red fluorescence signal was used to visualize chloroplasts and was overlaid with the GFP signal. The pattern of ORR (AUG-I)-GFP coincided with that of chlorophyll, confirming its chloroplast localization.

Since the Ds transposon insertion was located in between the ATG-I and ATG-II codons, we examined if the putative proteins starting at either AUG-II or AUG-III are still directed to the plastids as detected for the entire ORR protein (starting from AUG-I). In silico analysis predicted low likelihood of targeting to plastids for a protein starting at either AUG-II or from AUG-III. The subcellular localization of the putative ORR proteins starting from AUG-II and AUG-III was further investigated by transient assays (see Supplemental Figure 8 online). The results showed that ORR proteins initiating at either AUG-II or AUG-III are directed to the nucleus and cytoplasm.

**The OrrDs Mutation Results in Reduced ORR Gene Expression and Ndh Complex Quantity and Activity**

We further examined ORR gene expression in green fruit derived from plants of the OrrDs mutants compared with the wild type (Figure 4A). The results showed that ORR expression is reduced to 13 and 50% in the OrrDs/OrrDs and OrrDs/Orr genotype, respectively (compared with the wild type). By contrast, ORR expression was strongly induced (15-fold compared with expression in the wild type) in green fruit derived from the ex-35S-Orr genotype (Figure 4A).

Since it has been well established that the presence of all NDH subunits is required for the assembly and functioning of the complex (Rumeau et al., 2005; Ishihara et al., 2007; Shikanaï, 2007a, 2007b; Sirpiö et al., 2009; Takabayashi et al., 2009), the presence of the Ndh complex in the different genotypes was investigated using an anti-NDH-H (a different subunit of the Ndh complex) antiserum (Figure 4). Compared with the levels of the wild-type protein, the homoyzogous line retained <10 and 50% of the protein (NDH-H) in leaves and the immature green fruit, respectively (Figures 4B and 4D). The fact that levels of the NDH-H subunit were reduced signifies that the integrity of the entire complex was disrupted in the homogyzous line. The levels of the NDH-H protein was slightly reduced in the heterozygous line compared with the wild type in the leaves but was not significantly different in the immature green fruit (Figures 4B and 4D).

Subsequently, the Ndh complex activity was probed in vivo using chlorophyll fluorescence measurements (Figure 4E). The transient increase in the basal level of chlorophyll fluorescence after a light-to-dark transition has been specifically attributed to PQ and QA reduction via the Ndh complex activity. Accordingly, the postillumination fluorescence rise could not be detected in the homogyzous mutant line leaves and green fruit, demonstrating the absence of a functional Ndh complex (Figure 4E). The heterozygous mutant line showed very slight reduction in the levels of the NDH-H in leaves and no difference in the green fruit, and it was not altered in chlorophyll fluorescence in either tissue (Figure 4E). Thus, the chlorophyll fluorescence measurement corroborated the results obtained by the examination of the NDH-H subunit (see above).

In the case of the ex-35S-Orr genotype, the analyses of both NDH-H subunit levels (in leaves) and chlorophyll fluorescence (in leaves and immature green fruit) showed that these two parameters were similar to the ones observed in wild-type plants.
Thus, the excision event resulted in the induction of ORR transcript levels and restoration of the wild-type phenotype.

Analysis of the Redox Status in the Different *OrrDs* Genotypes

Levels of the reduced and oxidized forms of pyridine nucleotides (NAD\(^+\), NADH, NADP\(^+\), and NADPH) were measured in leaves of wild-type, *OrrD\(^{as}$/Orr*, and *OrrD\(^{as}$/OrrD\(^{as}\) mutant genotypes (Table 1). A significant decrease in the reduced forms was detected in both the homozygous and heterozygous *OrrD\(^{as}\)* mutants.

Having established that the mutant genotypes exhibited altered cellular levels of the pyridine nucleotides, we next determined the activation states of the reactions catalyzed by the plastidial malate dehydrogenase and ADP glucose pyrophosphorylase (AGPase). Both reactions have been suggested previously as markers for shifts in the plastidial redox status (Scheible and Stitt, 1988; Tiessen et al., 2002). Thus, we extracted leaves and assayed both total and initial enzyme activities and subsequently deduced their activation states (Table 2). Compared with the wild type, neither the heterozygote nor the complemented *ex-35S-Orr* line displayed any changes in activities or activation state. By contrast, the homozygote *OrrD\(^{as}$/OrrD\(^{as}\) line was characterized by an elevated total enzyme activity and a decreased activation state of AGPase. This was coupled to increased total and initial activities and decreased activation state of the plastidial malate dehydrogenase. Taken together, these changes are strongly indicative of an altered redox status of the plastids, suggesting that the plastids of the *OrrD\(^{as}$/OrrD\(^{as}\) mutant constitute a more reductive environment.

Primary Metabolism Is Only Moderately Altered in Fruits of the *OrrD\(^{as}\) Mutants

To assess the broad-range consequences of a mutation in the ORR gene on metabolism, we measured the levels of primary metabolites in fruits of the *OrrD\(^{as}\) genotypes using an established gas chromatography–mass spectrometry (GC-MS) method (Fernie et al., 2004). The results revealed that primary (or central) metabolism was essentially unaltered in the *OrrD\(^{as}\) genotypes (Table 3; see Supplemental Table 5 online). Although levels of several of the metabolites were significantly different from those observed in the wild type (of 46 metabolites measured, 16 were different in the heterozygote mutant, 9 in the homozygote, and 9 in the *ex-35S-Orr* line), most of these changes were relatively small (Table 3). An exception was tyramine, whose levels in the heterozygote and homozygote were threefold and twofold, respectively.
higher than in the wild type. Tyramine was reduced to 0.6 of the wild-type level in the complemented line. Intriguingly, significant decrease was observed in the levels of Ala, Lys, and Pro (in both \( \text{OrrDs/ORR} \) and \( \text{OrrDs/OrrDs} \)), and in Asp, Trp, succinate, and glucose (in \( \text{OrrDs/ORR} \)); and in glutamate and maltose (in \( \text{OrrDs/OrrDs} \)). Given that these metabolites normally increase during ripening (Roessner-Tunali et al., 2003; Carrari et al., 2006), this result suggests an alteration in the nature of ripening of the \( \text{OrrDs} \) fruit.

The \( \text{OrrDs} \) Mutants Are Altered in the Levels of Isoprenoids and Flavonoids

The levels of isoprenoids, including carotenoids, that typically accumulate in tomato fruit were measured in green and ripe fruit of the wild type and \( \text{OrrDs} \) mutants (Figure 5; see Supplemental Figure 9 online). Compared with wild-type plants, a reduction of 90 and 60% in total carotenoid level was observed in ripe fruit of \( \text{OrrDs/ORR} \) and \( \text{OrrDs/OrrDs} \), respectively. This severe phenotype was mainly due to a decrease in phytoene, phytofluene, \( \zeta \)-carotene, a lycopene derivative, and lycopene that was reduced dramatically (to 2 and 33% of its wild-type levels in \( \text{OrrDs/ORR} \) and \( \text{OrrDs/OrrDs} \), respectively). By contrast, an increase in both \( \beta \)-carotene (40%) and lutein (30%) was detected in the pericarp of the \( \text{OrrDs/ORR} \) and \( \text{OrrDs/OrrDs} \), respectively (Figure 5; see Supplemental Figure 9 online). In most of the cases, the effect in fruit of the heterozygote plants (\( \text{OrrDs/ORR} \)) was more severe compared with fruit of the homozygote plants (\( \text{OrrDs/OrrDs} \)). Carotenoid levels in genotypes producing red fruit, including
the wild type, wild-typeDs (siblings of the OrrDs/ORR mutant that contain the wild-type ORR alleles), and ex-3SS-Orr plants were not altered. No difference in carotenoid levels was observed between either one of the OrrDs mutants in the green stage of development (data not shown). Apart from the various carotenoids, we also detected altered levels of other isoprenoid pathway compounds, including chlorophylls and tocopherols (Figure 5). Compared with red fruit of the wild type, in which chlorophylls could not be detected, chlorophyll-a accumulated in both the homozygote and heterozygote mutants, while chlorophyll-b accumulated only in the OrrDs/ORR genotype. Levels of α-tocopherol were doubled in the mutant fruit compared with fruit of the wild type.

Fruit peels of the OrrDs mutants plants showed a strong reduction in the content of flavonoids compared with the wild type, and levels of the yellow pigment naringenin chalcone dropped by 85% (Figure 5). Levels of the flavonol rutin were reduced to 33 and 60% in the OrrDs/ORR and OrrDs/OrrDs peels, respectively. The same trend was also observed in the case of several other flavonoids, including various kaempferol derivatives, quercetin trisaccharides, and quercetin derivatives. The more severe phenotype of the heterozygote genotype compared with that of the homozygote in terms of carotenoid and flavonoid levels raises the possibility of posttranslational regulation. However, this is not surprising when taking into consideration that ORR represents one of the numerous Ndh complex subunits and thus changes in its quantity alters protein–protein interactions. A possible explanation for this phenomenon is detailed below (see Discussion).

**Gene Expression Changes in the OrrDs Mutant Fruit**

Microarray gene expression analysis was performed to evaluate the transcriptional changes in the OrrDs mutants (breaker stage fruit). Compared with the wild type, 1048 and 800 genes were altered in the OrrDs heterozygous and homozygous mutants, respectively. A total of 549 and 424 genes were upregulated, while 499 and 376 genes were downregulated in the OrrDs heterozygous and homozygous lines, respectively (Figure 6; see Supplemental Table 2 online). Out of the 602 and 546 genes that were up- or downregulated in the two mutant genotypes, respectively, 371 genes were upregulated in both genotypes, whereas 328 genes were downregulated in both. Conversely, when comparing the ex-3SS-Orr line to the wild type, only 26 genes were upregulated and 30 downregulated when compared with the wild type. Furthermore, most of these changes were restricted to the ex-3SS-Orr line and were not shared with either of the OrrDs insertion lines, indicating that the ex-3SS-Orr is more similar to the wild type. This was also supported by principal component analysis (see Supplemental Figure 10 online).

The changes in expression of genes involved in carotenoid and flavonoid biosynthesis was of particular interest since these two pathways had a major contribution to the OrrDs mutant phenotype (Figure 1). Out of 13 carotenoid biosynthesis genes evaluated by the array analysis, the expression of 1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE (DXS) and of β-CAROTENE HYDROXYLASE (CRTR-B2) was statistically significantly different between the mutants and the wild type (DXS increased and CRTR-B2 decreased; Figure 7A). The results showed that the effect on gene expression in the carotenoid pathway was mild and possibly posttranscriptional. qRT-PCR was subsequently performed to further evaluate the expression of eleven carotenoid-related genes (Figure 7B; see Supplemental Figure 11 online). As detected in the array analysis, expression of DXS was strongly upregulated, while CRTR-B2 expression showed a trend of decreasing in the OrrDs heterozygous mutant fruit. One major difference was in PSY1 expression, which was downregulated in the qRT-PCR analysis but not in the array data.

The flavonoid pathway was also affected at the transcriptional level as expression of CHALCONE SYNTHASE2 (CHS2) and

### Table 1. Analysis of Pyridine Nucleotides in Leaves of the OrrDs Mutant Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NAD+ (nmol g⁻¹ FW)</th>
<th>NADH (nmol g⁻¹ FW)</th>
<th>NADP⁺ (nmol g⁻¹ FW)</th>
<th>NADPH (nmol g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>16.50 ± 1.80</td>
<td>1.40 ± 0.11</td>
<td>11.90 ± 3.70</td>
<td>2.06 ± 0.25</td>
</tr>
<tr>
<td>OrrDs/OrrDs</td>
<td>16.95 ± 2.30</td>
<td>0.92 ± 0.05</td>
<td>10.34 ± 1.70</td>
<td>1.89 ± 0.10</td>
</tr>
<tr>
<td>OrrDs/ORR</td>
<td>18.08 ± 0.73</td>
<td>1.08 ± 0.17</td>
<td>11.80 ± 2.50</td>
<td>1.70 ± 0.30</td>
</tr>
</tbody>
</table>

The values are the mean ± SD of three replicates. Values of the mutant lines that differ significantly (P < 0.05) from the wild type are depicted in bold. FW, fresh weight.

### Table 2. Enzyme Activities in Wild-Type and OrrDs Mutant Genotypes in Leaves

<table>
<thead>
<tr>
<th>Activities (nmol min⁻¹ g⁻¹ FW)</th>
<th>ORR/ORR (Wild Type)</th>
<th>OrrDs/ORS</th>
<th>OrrDs/OrrDs</th>
<th>ex-3SS-Orr</th>
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<tbody>
<tr>
<td>AGPase initial</td>
<td>85.96 ± 2.800</td>
<td>85.96 ± 5.45</td>
<td>71.15 ± 6.36</td>
<td>73.32 ± 7.29</td>
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<tr>
<td>AGPase total</td>
<td>139.19 ± 11.19</td>
<td>147.28 ± 22.11</td>
<td>375.09 ± 44.22</td>
<td>248.24 ± 23.32</td>
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<tr>
<td>AGPase % of activation</td>
<td>69.40 ± 9.46</td>
<td>77.30 ± 12.17</td>
<td>21.10 ± 5.20</td>
<td>30.20 ± 6.86</td>
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<tr>
<td>NADP-MDH initial</td>
<td>45.83 ± 4.12</td>
<td>43.82 ± 2.36</td>
<td>72.30 ± 1.52</td>
<td>67.89 ± 7.16</td>
</tr>
<tr>
<td>NADP-MDH total</td>
<td>106.73 ± 19.66</td>
<td>101.96 ± 8.32</td>
<td>205.61 ± 15.74</td>
<td>179.24 ± 11.51</td>
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<tr>
<td>NADP-MDH % of activation</td>
<td>53.40 ± 6.09</td>
<td>46.00 ± 4.35</td>
<td>35.80 ± 2.17</td>
<td>38.00 ± 3.47</td>
</tr>
</tbody>
</table>

Data presented are means ± SE of measurements from four independent plants per genotype; values set in bold were determined by the t test to be significantly different (P < 0.05) from the wild type. MDH, malate dehydrogenase; FW, fresh weight.

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are mean Metabolite levels were determined in immature green fruit. Data are normalized to the mean response calculated for the wild type. Values presented

Miscellaneous Sugars and sugar alcohols Organic acids

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Metabolite levels were determined in immature green fruit. Data are normalized to the mean response calculated for the wild type. Values presented are mean ± SE of four replicates. Values in bold denote significant differences that were determined by t test analysis (P < 0.05). For details on the entire set of metabolites detected in this experiment, see Supplemental Table 5 online.

PREPHENATE DEHYDROGENASE (PDM) upstream of Phe was downregulated compared with the wild type (Figure 7C). It is important to note that although most of the flavonoid pathway genes displayed a reduction in expression, this was relatively mild and for most genes not statistically significant. qRT-PCR analysis confirmed that expression of CHS and PDH was reduced in the OrrDx heterozygous mutant fruit (Figure 7D).

Apart from the targeted analysis of transcripts involved in the carotenoid and flavonoid pathways described above, an unbiased investigation of the differences in the two mutant genotypes and the wild type was performed. To this aim, a pathway enrichment analysis of differentially expressed genes and a Wilcoxon test on the magnitude of changes within a pathway were performed to identify grossly changed categories (Usadel et al., 2006). Both analyses identified that the light reactions of photosynthetic processes as well as tetratylroll biosynthesis were upregulated, whereas transcripts encoding for enzymes involved in the Calvin cycle reactions were unchanged in both mutants, which is in line with the apparent upregulation of chlorophyll in the mutant (Figure 5). Interestingly, both analyses consistently highlighted changes in cell wall metabolism. Here, a general downregulation of cell wall related processes, such as nucleotide sugar metabolism, providing precursors for cell wall metabolism, as well as many pectin methyl and acetyl esterases, was observed. However, the potentially pectin derived rhamnose that was measured by means of GC-MS was unchanged (see Supplemental Table 2 online), and galacturonic acid, which is a major constituent of pectin, was not detected. Nevertheless, several enzymes acting on xyloglucan were changed; indeed, three of these were amongst the genes most strongly downregulated in the heterozygous mutant (Table 4). This might be in line with the observed changes of free xylose that was identified by means of GC-MS.

Another interesting observation revealed by the global pathway-based tests was changes in protein synthesis and degradation. In the case of protein synthesis, this was largely attributable to a strong (more than fourfold) upregulation of Elongation factor 1α (Les.48761.1.s1_at, SGN-U580191). However, in the case of protein degradation, a strong coordinated upregulation of many transcripts, which encode either proteasomal subunits or regulatory particles, could be observed (data not shown).

Further inspection of changed metabolites revealed that consistent changes in both mutant lines were largely confined to amino acid metabolism (Table 3; see Supplemental Table 5 online). These were primarily restricted to a downregulation in levels of the branched chain amino acids Ile and Lys. However, inspection of biosynthesis and degradation pathways for these amino acids did not reveal marked, significant changes in the respective genes. One potential exception was the probe set
LesAffx.9260.1.S1_at (SGN-U578917) being similar to dihydrodipicolinate reductase. Interestingly, this unigene was most similar to the Arabidopsis gene chlororespiration reduction 1, which has been shown not to be involved in Lys biosynthesis but rather plays a role in accumulation of the chloroplast Ndh complex (Shimizu and Shikanai, 2007) consistent with the changes in the ORR gene.

Further investigation of the genes that were most strongly changed in the heterozygous mutant (Table 4) revealed that apart from genes involved in cell wall modification (mainly ones encoding for enzymes acting on xyloglucan), a relatively large number of genes putatively encoding transcription factors were changed, many of them belong to the ethylene responsive factor gene family.

### The Effect on Ripening-Related Gene Expression and Ethylene Levels in the OrrDs Mutant Fruit

To examine the effect on the ripening process in the OrrDs mutant fruit, genes known or previously suggested to be involved in...
ripening were checked for differential expression (see Supplementary Data Set 1 online). Here, Les.4450.1.S1_at, which potentially queries a MADS box gene similar to the RIPENING INHIBITOR (RIN) and Les.132.1.S1_at potentially reporting the expression of a gene similar to tomato ACO1, both playing a role in fruit ripening (Nakatsu et al., 1998; Vrebalov et al., 2002), were more than fivefold upregulated (data not shown). However, comparing the individual oligonucleotide sequences present in these probe sets with the deposited nucleotide sequences for RIN and ACO1 revealed that these were unlikely to reflect their expression. This is in accordance with the fact that other probe sets interrogating these genes seemed unchanged (see Supplementary Table 4 online). Furthermore, the probe sets reporting the expression of ERT1b and ERT10 also failed to detect any changes (Picton et al., 1993). However, three probe sets reporting the expression of the E8 ripening-related gene (Deikman and Fischer, 1998) were consistently upregulated in the OrrDs mutants and downregulated in the ex-3SS-Orr line, albeit these findings were not significant. Genes short-listed by Alba et al. (2005) as good candidates for genes involved in ripening regulation were also tested for differential expression (see Supplementary Table 3 online, bottom part). However, none of these genes were significantly changed, and with the exception of MBF1 and bZIP1, no trend was visible either (see Supplementary Table 3 online).

To obtain a more conclusive picture regarding the effect on ripening-associated gene expression, genes shown by Alba et al. (2005) to change during ripening or fruit developmental were also examined. Interestingly, of the 336 probe sets that could be mapped to the original TOM1-based list of genes changing during ripening or development, 54 and 47 were flagged as differentially expressed in the OrrD/OrR and OrrD/OrD mutants, respectively, indicating a mild enrichment (P < 0.05) of potentially ripening-related genes (see Supplemental Data Set 1 online).

We also performed measurements of ethylene, which marks the typical climacteric during fruit ripening in tomato (Figure 8). Ethylene emission levels were measured using GC coupled to a flame ionization detector 11 times starting from day 1 after the harvest of green fruit. The results showed that the peak in ethylene emission was delayed and reduced in fruit of both OrrD/OrR and OrrD/OrD (stronger in the former), although not diminished, compared with its peak in wild-type fruit 6 d after harvest in the wild type compared with 13 to 16 d after harvest of the mutant fruit. The ethylene emission profile of ex-3SS-Orr fruit was very similar to the one observed in wild-type fruit.

Examining the correspondence in fruit development (from flowering to ripening and from the breaker to ripening) between the wild-type and the OrrDs genotypes showed that the mutants were slightly delayed in their development, although this was not statistically significant.

DISCUSSION

Altered NDH Activity in Plastids of Mature Green Fruit Impinges on Subsequent Metabolism in Chromoplasts

Characterization of the OrrD mutant and its corresponding gene encoding the NDH-M subunit described in this study indicate that Ndh complex and redox control play essential roles during tomato fruit maturation in ripening-related metabolism, most notably carotenoid biosynthesis. Interestingly, although other Ndh subunit mutations have been isolated, unlike OrrD, these mutants do not exhibit a clear visual phenotype (Burrows et al., 1998; Sazanov et al., 1998a; Shikanai et al., 1998; Horváth et al., 1998; Nakatsu et al., 1998; Vrebalov et al., 2002).

Figure 6. Gene Expression Changes in the OrrDs Mutants.

Venn diagrams of genes changed in expression in fruit (breaker stage) of the three OrrDs genotypes compared with the wild type (WT). The Venn diagrams show significantly up- and downregulated genes in fruit of the OrrD/OrR (red), OrrD/OrD (green) and the ex-3SS-Orr (blue) lines when compared with the wild type. Genes being significantly up- or downregulated in several of the genotypes are shown in the respective overlap regions. [See online article for color version of this figure.]
Figure 7. The Effect of the OrrDs Mutation on Gene Expression in the Isoprenoid and Phenylpropanoid/Flavonoid Biosynthesis Pathways.

(A) Representation of gene expression and metabolite levels changes in fruit derived from the OrrDs/ORR genotype compared with that of wild-type plants. Microarray probe sets reporting the gene expression of likely pathway genes are depicted by color-coded boxes indicating the log 2 fold change of the respective transcripts according to a false-color scale reproduced in the figure, where white represents no change, red an upregulation, and green a downregulation. Each individual box represents a unique probe set present on the chip hybridizing to isoforms of the respective gene indicated next to it. Nonsignificantly changed probe sets are depicted in gray. Metabolites where quantitative data were available are represented as black text on colored boxes, where the box color indicates log 2 fold changes following the same color scheme as transcripts. Metabolites not detectable in the mutant are painted on a blue box, whereas the ones not detectable in the wild type are painted on a yellow box. The electron transport pathway involving the Ndh complex and PTOX (adapted from Carol and Kuntz, 2001) is represented in a box on the left. GGPP, geranylgeranylpyrophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; PSY, phytoene synthase; CrtISO, carotene isomerase; CRTL-E, LCY-E, lycopene e-cyclase; LCY-B, lycopene β-cyclase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; CAO, chlorophyll a oxidase.

(B) Expression of genes in the isoprenoid pathway (including carotenoid-related) in the heterozygous OrrDs mutant and wild-type breaker stage fruit as detected by qRT-PCR. Full names of genes are given in (C). Expression data were normalized to the expression of the CLATHRIN ADAPTOR COMPLEXES SUBUNIT (CAC) gene; values are means ± SE (n = 3). Asterisks indicate values that are significantly different from the wild type at *P < 0.05 and **P < 0.01.

(C) Representation of gene expression and metabolite levels changes in fruit derived from the OrrDs/ORR genotype compared with wild-type plants. The array and metabolite analysis data are presented in the same way as Figure 7A. PAL, Phe-ammonia lyase; 4CL, 4-coumaroyl-CoA synthase 2; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; 3GT, anthocyanidin 3-O-glucosyltransferase; RT, rhamnosyltransferase; CAD, cinnamyl-alcohol dehydrogenase; F3′5′H, flavanone 3′5′ hydroxylase.

(D) Expression of genes related with phenylpropanoids/flavonoids pathway in the heterozygous OrrDs mutant and wild-type breaker stage fruit as detected by qRT-PCR. Full names of genes are given in (B). Expression data were normalized to the expression of the CAC gene; values are means ± SE (n = 3).
2000). The specific expression of ORR in chlorophyll-containing tissues (i.e., stems, leaves, young flowers, and immature green fruits) is consistent with the involvement of the Ndh complex in alternative electron pathways around PSI (Nixon, 2000; Pellier and Cournac, 2002; Bukhov and Carpenter, 2004; Johnson, 2005). At the onset of fruit maturation and degreening, Ndh-M expression is downregulated, in agreement with the data of Guera and Sabater (2002) that demonstrated reduction in the Ndh complex quantity and activity during fruit maturation. Interestingly, genomics database; http://ted.bti.cornell.edu/) indicate that the expression of Ndh-N and Ndh-O does not change during fruit maturation; see Supplemental Table 7 online).

The visual and biochemical phenotypes of the OrrDs mutant implied that in addition to its importance during the early green stages of fruit development (Guera and Sabater, 2002), the Ndh complex also influences the mature fruit once the transition of chloroplasts to chromoplasts takes place. These observations in OrrDs resemble the effects seen in fruits of the tomato ghost (gh) mutant when averted from light (Shahbazi et al., 2007). This mutant is impaired in the plastid terminal oxidase gene (PTOX). When green stage gh fruit are covered and mature in the dark, they remain green and do not exhibit the typical white gh phenotype. Similar to OrrDs, covered fruit of the gh mutant are severely affected in their carotenoid composition (Barr et al., 2004). Authors of this article suggested that the gh mutation reversibly changes the plastid ultrastructure in green fruits exposed to light. Consequently, carotenogenesis, which is performed by membrane-associated enzymes, is severely affected in mature fruits. Although the green-stage fruits of the OrrDs mutant visually showed a wild-type phenotype, we cannot rule out a possible damage to plastids that slows the conversion of chloroplasts to chromoplasts.

Lack of a significant difference between OrrDs and the wild type in carotenoid content and composition in green fruits indicates that the Ndh complex affects the carotenoid
biosynthesis pathway in fruit chromoplasts and not in chloroplasts. Nevertheless, no alterations in carotenoids levels and content were observed in \( \text{OrrDs} \) flowers that also contain chromoplasts. In an \( \text{ndh-h} \)-defective mutant of tobacco (Zapata et al., 2005), the carotenoid profile was not changed in leaves nor when we examined its chromoplast-containing nectary tissue. Thus, it appears that the effect of reduced Ndh complex activity on carotenoid pigment composition is restricted to the mature tomato fruit tissue. The reduction in levels of the red carotenoid lycopene, combined with the increase in the yellow-orange carotenoids \( \beta \)-carotene and lutein, and the large reduction in the levels of several flavonoids, particularly of the yellow flavonoid pigment naringenin chalcone, explain the pale-yellow/orange appearance of the \( \text{OrrDs} \) mature fruit. The fruit phenotype is similar to the one observed when the tomato \( y \), colorless peel, and the \( r \) mutations are combined (Rick and Butler, 1956).

**\( \text{OrrDs} \) Is a Unique Gain-of-Function Mutation**

The \( \text{OrrDs} \) mutant is not a null mutation, as we detected reduced expression in the \( \text{OrrDs}/\text{OrrDs} \) genotype compared with the wild type. The residual \( \text{ORR} \) expression is most probably due to the fact that the promoter region of \( \text{ORR} \) is located in the 5’ region of the \( Ds \) element. In addition, transposon-induced transcription of the neighboring region (i.e., readout transcription) has been reported previously in transgenic tomato plants (Rudenko et al., 1994).

This mutation results in an overdominant phenotype that is reflected by the enhancement of several but not all of the parameters examined in this study. Most traits that exhibited a more severe phenotype in the \( \text{OrrDs}/\text{ORR} \) heterozygous fruit than in the homozygous ones were observed in fruit that contained chromoplasts. These traits included carotenoid and flavonoid composition, global gene expression changes, levels of chlorophylls, \( \alpha \)-tocopherol, and TSS, and fruit texture (postharvest shrinking). On the other hand, traits that were more affected in the \( \text{OrrDs}/\text{OrrDs} \) fruit than in the heterozygous are those associated with chloroplast-containing tissues (leaves and green fruit), such as chlorophyll fluorescence, Ndh complex levels, and Ndh activity. A possible explanation for the overdominance phenomenon may be that \( \text{ORR} \) is only one out of 14 subunits of the Ndh complex, and changes in \( \text{ORR} \) quantity likely alter the complex activity and levels. The \( \text{ORR} \) proteins that are likely to be incorporated into the Ndh complex in either the homozygous or heterozygous \( \text{OrrDs} \) lines are different in their quantity. In the homozygous lines, either no proteins or alternatively small amounts of much shorter ones in the case of plastidic localization (starting at AUG-IV, from amino acid residue 64) will be incorporated into the complex. However, in the heterozygous line, it is expected that a half of the proper protein dose will be incorporated, while the shorter protein (from AUG-IV) might also be inserted to the complex (if localized to the plastids). It is also likely that the short protein (from AUG-IV) will be localized to a different subcellular compartment (e.g., the cytosol). Another possible explanation is the activity of a compensatory process that reduces the effect on the Ndh complex function in the green tissues (in the case of the heterozygous) and in the mature fruit (in the case of the homozygous). Such a mechanism could be related to other alternative electron pathways, such as those involving PGR5/PGR1 (Munekage et al., 2002; DalCorso et al., 2008), NDH-2 [a type II NAD(P)H dehydrogenase; Corneille et al., 1998; Desplats et al., 2009], and/or ferredoxin NADPH reductase (Zhang et al., 2001).

Since the \( \text{OrrDs} \) mutation exhibits an overdominant phenotype, it could not be as a result of a loss of function (i.e., haploinsufficient), but rather a consequence of either a gain-of-function mutation (whose gene product become constitutively active due to a gain of a novel function normally not found in the wild-type protein phenotype) or dominant-negative (whose gene product adversely affects the normal wild-type gene product) mutations. If \( \text{OrrDs} \) was a dominant-negative mutation, we should have observed the \( \text{OrrDs} \) phenotype by overexpression of the \( \text{ORR} \) gene either in the wild type or in the mutant backgrounds. However, plants overexpressing the \( \text{ORR} \) gene in the wild-type background and the \( \text{ex-3Ss-orr} \) line displayed a wild-type phenotype. In addition, the \( \text{OrrDs} \) phenotype was complemented by overexpression of \( \text{ORR} \) in the mutant background. Thus, \( \text{OrrDs} \) is not a consequence of a dominant-negative mutation but more likely a gain-of-function mutation.

**The Mutation in \( \text{ORR} \) Affects the Redox State of Fruit Plastids**

The role of the Ndh complex in chlororespiration and cyclic electron transport around PSI suggested that alterations in this complex could influence the redox state of its cellular environment. The NDH-M subunit encoded by \( \text{ORR} \) in tomato is most likely directed to the plastids, as indicated by localization assays of transiently expressed full-length transcript (starting from AUG-11) (Figure 3). The consequences of the lack of Ndh activity on the redox status of chloroplasts has not been reported probably since the electron donors in the stroma have not been identified and also because the alternative dark electron
flux proceeds at very low rate. The levels of NADH and NADPH were reduced in the *OrrD* mutant lines. Consistent with these changes in the plastidial redox status was the alteration in the activities and activation state of the AGPase and plastidial malate dehydrogenase (Scheible and Stitt, 1988; Tiessen et al., 2002). Together, these results indicate that in tomato, the lack of the Ndh complex alters the plastid redox status.

**Involvement of ORR in the Carotenoid Biosynthetic Pathway**

The changes observed in the carotenoid pathway do not seem to result from altered transcript levels, as expression of most genes of the pathways were not altered in the *OrrD* mutant. More probably, the effect on carotenoid composition is instigated by obstructing enzymes in the pathway that require redox electron chain for their activity. Desaturation of phytoene to lycopene catalyzed by PDS and ZDS necessitates acceptors for eight hydrogen atoms. It has been demonstrated that PQ serves as an intermediate in transferring electrons between phytoene desaturase and molecular oxygen (Mayer et al., 1990; Norris et al., 1995), a process mediated by a bound flavin (Hugueney et al., 1992). In chloroplasts, oxidized PQ required for PDS activity is regenerated from plastoquinol by electron transfer to PSI. In the dark and in chromoplasts, where photosynthetic electron transport is inactive, oxidation of plastoquinol is performed by PTOX.

The interaction of PQ with the electron transfer chain is unspecific as evidenced by the fact that when expressed in *Escherichia coli* cells both plant enzymes are able to mechanistically link to the bacterial respiratory redox chains (Mayer et al., 1990; Niewelstein et al., 1995). An analogous situation exists in cyanobacteria where plastoquinol can be oxidized in darkness because the electron transport reactions of photosynthesis and respiration are linked in the same membranes (Sandmann and Malkin, 1984). Similar to PDS and ZDS, other enzymes involved in carotenoid biosynthesis also contain a conserved FAD binding motif in their N termini, for example, CRTISO, LCY-E, LCY-B, and ZEP, suggesting the involvement of redox balance in these reactions even though they do not involve net electron transfer. This is the case in lycopene β-cyclase, which was active in vitro only in the presence of NADH or NADPH (Schnurr et al., 1996), and carotenoid isomerase, which was dependent on redox driving force (Isaacson et al., 2004). Involvement of FAD in ZEP activity has also been demonstrated (Buch et al., 1996). It has been recently demonstrated that the bacterial lycopene cyclase (CRTY) requires a reduced FAD as a catalytic cofactor probably for stabilization of a transition state in an acid-base reaction (Yu et al., 2010). A similar phenomenon has also been discovered in the case of the carotene isomerase (CRTISO; Q. Yu, P. Beyer, and J. Hirschberg, unpublished results). The role of the redox state in the case of these enzymes is unknown. However, the above results imply that it is indirect and not associated with net electron transfer.

The changes in the carotenoid pathway intermediates we observed in the *OrrD* mutants cannot be explained solely by altered activities of PDS, ZDS, and CRTISO. If this was the case, we should have detected an accumulation of the PDS substrate phytoene, as seen in the gh leaves, flowers, and fruit (Zapata et al., 2005) and reduced levels of metabolites downstream to ζ-carotene. In addition to the expected reduced levels of phytofluene, ζ-carotene, and lycopene, we also measured reduced concentration of phytoene. Interestingly, levels of β-carotene, neoxanthin, and lutein, all derived from lycopene, were increased. It is also important to note that expression of ORR genes encoding PDS, ZDS, and CRTISO are not correlated during development; while expression of ORR is reduced after the green stage, PDS, ZDS, and CRTISO show an increase in their transcript levels (Josse et al., 2000).

It was earlier reported that alteration in PQ redox induces intracellular signaling leading to changes in chloroplast and nuclear genes expression (Rodermel, 2001; Pfannschmidt, 2003). Thus, an alternative explanation to the involvement of ORR in carotenoid biosynthesis might be that Ndh-catalyzed PQ reduction in plastids of fruit triggers the expression of genes encoding enzymes involved in carotenoid biosynthesis. Steinbrenner and Linden (2003) demonstrated that in *Haematococcus pluvialis*, an astaxanthin-accumulating green alga, the photosynthetic PQ pool functions as a redox sensor for the upregulation of carotenoid biosynthesis genes. In the *OrrD* mutant, changes in the expression of carotenoid biosynthetic genes were detected in fruit at breaker stage, although not many genes of the pathway were altered in expression.

The changes in the *OrrD* carotenoid composition together with the previous reports with respect to the chlororespiratory function of the Ndh complex suggest that the PTOX encoded by the tomato *GH* gene and ORR are part of the same pathway. Publicly available microarray data indicated that expression of GHOST gradually increases from early fruit development until ripening before it declines sharply (Tomato Functional Genomics Database; see Supplemental Table 7 online). Thus, expression of *GH* and ORR only partially overlaps in the early stages of fruit development. The interaction between ORR and GH should therefore be examined in subsequent work through the generation of double *OrrD*/*gh* mutant lines.

**A Proposed Link between NDH Complex Activity, Antioxidant Systems, and the Process of Fruit Ripening**

Apart from changes in carotenoid composition, *OrrD* displayed alteration in additional metabolite classes in fruit, including chlorophylls, tocopherols, and flavonoids. Changes in specialized metabolism that increased total antioxidant content have been demonstrated in fruits of high-pigment (*hp*) tomato mutants (Bino et al., 2005). Downregulation of the *DE-ETIOLATED1* gene that corresponds to the *hp2* mutation through an RNA interference approach resulted in similar metabolic changes, including an increase in levels of carotenoids, flavonoids, and chlorogenic acid (Davuluri et al., 2005; Enfissi et al., 2010). An increase in chloroplast size and number in fruits of the high-pigment mutants *hp1* and *hp3* was suggested to trigger the changes in metabolism of antioxidants (Cookson et al., 2003; Galpaz et al., 2008). This combined effect on pathways generating antioxidant or photoprotective activities might represent a compensatory mechanism between the different classes of protecting compounds that is perhaps due to a change in plastid structure or number at early fruit development. In the case of metabolites that showed elevated levels in mature *OrrD* fruits
The Plant Material

Methods

Plant Material

The OrrDs mutant (Solanum lycopersicum cv Micro-Tom) was identified among the transposon insertion population generated by Meissner et al. (1997). For metabolic analysis, peel and flesh were dissected manually as described by Mintz-Oron et al. (2008). Fruit at a particular developmental stage up to breakever were harvested according to their appearance; from the breakever stage and further days were counted following breakever.

Isolation of the OrrDs Insertion and Genotyping

DNA was extracted from leaves as described by Eshed and Zamir (1995). Isolation of the transposon flanking regions was performed by long-range inverse PCR as described previously (Meissner et al., 2000). The wild-type ORR locus and the insertion allele were genotyped by oligonucleotides 1 and 2 (1149-bp amplicon) and 1 and 3 (808 bp), respectively (see Supplemental Table 4 online). DNA gel blot analysis was according to Sambrook et al. (1989) using the kanamycin gene as a probe.

Isoprenoid and Flavonoid Extraction and Analyses

Isoprenoid extraction was performed as described by Bino et al. (2005). Analysis was performed by the use of an HPLC-PDA detector and a YMC C30 column as described by Fraser et al. (2000). Peak areas of the carotenoid compounds were determined at the wavelength providing maximum absorbance (see Supplemental Methods online) using Waters Millennium32 software. Flavonoids were extracted in methanol and profiled with UPLC-PDA as described previously by Mintz-Oron et al. (2008) for the profiling of semipolar compounds. Detailed description of sample preparation and injection and compound quantitation can be found in the supplemental figures and Supplemental Methods online.

RACE and Gene Expression Analyses

The 5′ RACE-PCR was performed with the SMARTer RACE cDNA amplification kit according to the manufacturer’s instructions (Clontech; see Supplemental Table 4 online for oligonucleotides). Fruit RNA was extracted as described by Verwoerd et al. (1989) while from other tissues using the Tri-reagent method (Molecular Research Center). qRT-PCR for the ORR gene (oligonucleotides 7 and 8; see Supplemental Table 3 online) and ASR1 the endogenous control (oligonucleotides 11 and 12) was performed as described by Mintz-Oron et al. (2008). Global gene expression was analyzed in OrrDs homozygote and heterozygote mutants, ex-3SS-Orr, and the wild type (10 pooled breaker stage fruit collected from five plants in three replicates for each genotype). Sample preparation and hybridization to the Affymetrix Tomato GeneChip was as described by Mintz-Oron et al. (2008). Normalization of the raw expression intensity values was performed using RNA analysis (irizarry et al., 2003) using the BioConductor software suite (Gentleman et al., 2004). Subsequently, arrays were quality controlled using standard procedures, such as RNA degradation plots, relative log expression, and others (see Supplemental Figure 7 online). Even though two arrays showed somewhat lower quality in the normalized unscaled errors of the means plots (see Supplemental Figure 7D online), and one of these also showed a somewhat stronger RNA degradation (see Supplemental Figure 7C online), they were included for final analysis. Subsequently, differentially expressed genes were extracted as follows: first, a linear model was fitted to the data using the limma package (Smyth, 2004), which included the single factor genotype. P values for differentially expressed genes were then extracted using the empirical Bayes procedure of the limma package, for the contrasts OrrDs/OrrDs versus the wild type, OrrDs/Orr versus the wild type, and ex-3SS-Orr versus the wild type. The resulting P values were globally corrected using false discovery rate control at a level of 1% (Benjamini and Yekutieli, 2001).

Pathway Analyses

To investigate changes in processes and to identify co-ordinated changes in pathways and processes, we used PageMan software (Usadel et al., 2006) and the according tomato ontology (Urbanczyk-Wochniak et al., 2006) to perform a Wilcoxon test on the log-fold change values and a Fisher’s exact test for an enrichment of categories, harboring significantly up- and downregulated genes.

Comparison with Ripening Genes

To test for ripening genes, SGN unigenes potentially involved in ripening were extracted from Supplemental Table 1 of Alba et al. (2005). These SGN release #1 unigenes were converted to SGN release #4 unigene versions using the SGN resource (Mueller et al., 2005) and finally matched to Affymetrix probe set identifiers (see Supplemental Data Set 1 online). A Fisher’s exact test was performed to test for overrepresentation of ripening related genes differentially expressed in the OrrDs heterozygous mutant.

Chlorophyll Fluorescence

Chlorophyll fluorescence was measured at room temperature on attached leaves using a PAM-2000 modulated fluorometer (Waltz Effeltrich)
as previously described by Rumeau et al. (2005). Postillumination chlorophyll fluorescence rises were recorded under low nonactinic light following a 5-min actinic illumination (250 μmol photons m⁻² s⁻¹).

Preparation of Chloroplasts and Thylakoid Membranes and Immunoblotting

Chloroplasts were extracted from tomato leaves using the protocol described for tobacco (Nicotiana tabacum) by Szaranow et al. (1998b). Briefly, tomato leaves were ground in 50 mM Tricine buffer at pH 7.8 containing 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl₂, 2 mM ascorbate, and 5 mM DTT using a Waring blender. Chloroplasts were collected by three successive low-speed centrifugations to avoid mitochondria contamination. Chloroplasts were osmotically lysed in 10 mM Tricine buffer at pH 7.8 containing 10 mM NaCl and 10 mM MgCl₂. Thylakoid membranes were collected by centrifugation. Chloroplasts were extracted from green tomatoes according to Bathgate et al. (1985). Tomatoes were sliced and the gelatinous material and seeds were discarded. The tomato pericarp tomatoes according to Bathgate et al. (1985). Tomatoes were sliced and the gelatinous material and seeds were discarded. The tomato pericarp was homogenized in buffer containing 0.6 M sucrose and 0.1 M Na-phosphate, pH 7.5 (2 mL/g fresh material), using a Waring blender. The homogenate was filtered twice through three layers of Miracloth and centrifuged 3 min at 1500 g. The pellet was resuspended in buffer containing 0.6 M sucrose and 0.01 M Na-phosphate, pH 7.5, and centrifuged as before. Washed chloroplasts were resuspended in a small volume of resuspension buffer and loaded on a discontinuous sucrose gradient prepared using 0.01 M Na-phosphate, pH 7.5, and consisting of three layers of 30, 45, and 55% sucrose, respectively. Following centrifugation for 15 min at 25000 g, bands formed at the 30/45% sucrose and at the 45/55% sucrose interfaces were collected and pooled. Finally, chloroplasts were recovered by centrifugation for 5 min at 15000 g. Proteins were subjected to SDS-PAGE (15% polyacrylamide) and then electrotransferred onto nitrocellulose membranes (0.45 μm; Biotrace NT). Bound proteins were subsequently probed with the antiserum and visualized with 1:5000 diluted anti-rabbit IgG-IRDye800 antibody (Invitrogen). Detection was enabled by the Odyssey infrared imaging system (LI-COR Biosciences).

Gene Expression Analysis by Real-Time PCR

For two-step, real-time qRT-PCR, a known amount of DNase-treated total tomato fruit RNA was reverse transcribed using Invitrogen’s SuperScript II RTase kit. Platinum SYBR SuperMix (Invitrogen) RT-PCR reactions were tracked on an ABI 7300 instrument (Applied Biosystems). Each sample was PCR amplified using the same amount of cDNA template in triplicate reactions. Gene-specific quantitative PCR primer pairs for the 19 selected genes encoding peel-associated proteins were designed by the Primer Express software (Applied Biosystems). Tomato ASR1 and CAC were used as endogenous controls. Following an initial step in the thermal cycler for 15 min at 95°C, PCR amplification proceeded for 40 cycles of 15 s at 95°C and 30 s at 60°C and was completed by a melting curve analysis to confirm specificity of PCR products. The baseline and threshold values were adjusted according to the manufacturer’s instructions. Similar results were obtained from relative quantification of transcript abundance determined independently by the standard curve method described in Applied Biosystems User Bulletin 2 (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/documents/cms/040980.pdf). Oligonucleotide sequences designed for the amplification of the transcripts are listed in Supplemental Table 4 online.

Accession Numbers

Sequence data from this article can be found in GenBank/EMBL or Sol Genomics Network databases under the following accession numbers: Arabidopsis Ndh-M protein, NP_001031804, and gene, NM_001036727; tomato ORR, SGN-U579052; putative tomato Elongation factor 1α, SGN-U580191; putative tomato dihydropicolinate reductase, SGN-U578917; 1-deoxy-d-xylulose 5-phosphate synthase (DXS), SGN-U567647; phenylalanine synthase (PAL), SGN-U573964; Phe-ammonia lyase (PAL), SGN-U578057; PDS, SGN-U565586; carotene isomerase (CrtISO), SGN-U584987; ZDS, SGN-U568357; Lycopene e-cyclase (CRTL-E and LCY-E), SGN-U567885; Lycopene β-cyclase (LCY-B), SGN-U563340; CRTR-B1, SGN-U568806; CRTR-B2, SGN-U568611; zeaxanthin epoxidase (ZEP), SGN-U569421; violaxanthin deepoxidase (VDE), SGN-U577684; PDH, SGN-U573964; Phe-ammonia lyase (PAL), SGN-U577677; Cinnamic acid 4-hydroxylase (C4H), SGN-U586432; 4-coumaroyl-CoA synthase 2 (4CL), SGN-U573880; CHS1, SGN-U579222; CHS2, SGN-U580856; chalcone isomerase (CHI), SGN-U573550; flavanone 3-hydroxylase (F3H), SGN-U563669; flavonol synthase (FLS),
Supplemental Data

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

Supplemental Figure 1. The Effect of Season and Light on the OrrD4 Phenotype.

Supplemental Figure 2. Examination of Shelf Life, TSS, and Triple Response of the OrrD4 Mutants.

Supplemental Figure 3. The Proposed Mechanism That Led to the Unique OrrD4 Mutation and the Excision Event (ex-3SS-Orr).

Supplemental Figure 4. Orr Gene Sequences and Transcripts.

Supplemental Figure 5. Homology between Orr and Other Plant NDH-M Proteins.

Supplemental Figure 6. Segregation of Genotypes and Phenotypes in an Offspring Derived from the OrrD4 Mutant.

Supplemental Figure 7. Orr Gene Expression in the Peel and Flesh Tissues during Five Stages of Fruit Development.

Supplemental Figure 8. Evaluation of the Subcellular Localization of ORR (Starting from Either AUG-I, AUG-II, or AUG-III).

Supplemental Figure 9. Analysis of Carotenoids in Extracts of Wild-Type and OrrD4 Mutants (Presented as a Percentage of Total Carotenoids).

Supplemental Figure 10. Routine Quality Controls Performed on the Array Data.

Supplemental Figure 11. Quantitative Real-Time PCR Results of Selected Genes.

Supplemental Table 1. Genotype and Phenotypes of Plants Belonging to the Progeny of an ex-3SS-Orr Line.

Supplemental Table 2. The Effect of the OrrD4 Mutation on Primary Metabolite Levels.

Supplemental Table 3. Fold Changes in the OrrD4 Mutant Lines Compared with the Wild Type for Probe Sets That Report Expression of Genes That Are Potentially Implicated or Change in Ripening.

Supplemental Table 4. List of Oligonucleotides Used in This Study.

Supplemental Table 5. Expression of Nuclear Genes Putatively Encoding Ndh Complex Subunits and PTOX during Tomato Fruit Development.

Supplemental Data Set 1. Comparison of Genes That Were Altered in Alba et al. (2005) to Their Alterations in This Experiment.

Supplemental Data Set 2. Detailed Results of the Tomato Array Experiments.

Supplemental Methods.

ACKNOWLEDGEMENTS

We thank Tali Mandel, Michal Mintz, and Lior Tal for their valuable help. We are grateful to members of the Aharoni, Levy, and Wolf laboratories for useful discussions, Ester Feldmesser for assistance with array analysis, Merav Yativ for the carotenoid analysis, and Dani Zamir and Tzili Pieban for their assistance with mapping the ORR locus. A.A. is an incumbent of the Adolfo and Evelyn Blum Career Development Chair. A.A.L. is an incumbent of the Gilbert de Botton chair in plant sciences. Research in the A.A. laboratory was supported by a grant from the Israel Ministry of Science (IMOS Project 3-2552) and the Y. Leon Benzoziyo Institute for Molecular Medicine. Research in the A.A.L. laboratory was supported by the German Federal Ministry of Education and Research within the framework of German-Israeli Project Cooperation (DIP Grant E.3.1). Research in the J.H. laboratory was supported by Israel Science Foundation Grant 548/05.

Received February 14, 2010; revised May 14, 2010; accepted June 7, 2010; published June 22, 2010.

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