Accumulation of Flavonoids in an ntra ntrb Mutant Leads to Tolerance to UV-C

Talaat Bashandy*, Ludivine Taconnatb, Jean-Pierre Renoub, Yves Meyera and Jean-Philippe Reichhelda,1,2

a Laboratoire Génome et Développement des Plantes, Université de Perpignan, UMR CNRS 5096, 52 avenue Paul Alduy, 66860 Perpignan, France
b Unité de Recherche en Génomique Végétale, 91057 Evry cedex, France

ABSTRACT NADPH-dependent thioredoxin reductases (NTRs) are key regulatory enzymes determining the redox state of thioredoxins. There are two genes encoding NTRs (NTRA and NTRB) in the Arabidopsis genome, each encoding a cytosolic and a mitochondrial isoform. A double ntra ntrb mutant has recently been characterized and shows slower plant growth, slightly wrinkled seeds and a remarkable hypersensitivity to buthionine sulfoximine (BSO), a specific inhibitor of glutathione biosynthesis. In this paper, we demonstrate that this mutant also accumulates higher levels of flavonoids. Analysis of transcriptome data showed that several genes of the flavonoid pathway are overexpressed in the ntra ntrb mutant. Accumulation of flavonoids is generally considered a hallmark of plant stress. Nevertheless, no elevation of the expression of genes encoding ROS-detoxification enzymes was observed, suggesting that the ntra ntrb plants do not suffer from oxidative disease. Another hypothesis suggests that flavonoids are specifically synthesized in the ntra ntrb mutant in order to rescue the inactivation of NTR. To test this, the ntra ntrb mutant was crossed with transparent testa 4 (tt4) plants with a mutation in the gene encoding the first enzyme in flavonoid biosynthesis. As ntra ntrb plants are more resistant to UV-C treatment than wild-type plants, this higher resistance was abolished in the ntra ntrb tt4 mutant, suggesting that accumulation of flavonoids in the ntra ntrb mutant protects plants against UV-light.

Key words: abiotic/environmental stress; oxidative and photo-oxidative stress; secondary metabolism - phenylpropanoids and phenolics; gene expression; Arabidopsis.

INTRODUCTION

Exposure of eukaryotic cells to abiotic stress induces various responses in living organisms. Many cellular responses can be triggered by a change in intracellular redox state, resulting in cytotoxicity or stress. Genotoxic and oxidative stresses caused by exposure to UV light, hydrogen peroxide, heavy metals, or high and low temperature cause severe damage to critical cellular macromolecules including nucleic acids, proteins, and lipids (Imlay and Linn, 1988; Halliwell and Gutteridge, 1989). To cope with these toxic effects, cells use a number of defence mechanisms to sense and respond appropriately to oxidative stress. These defence mechanisms involve both low molecular weight antioxidants (glutathione, ascorbic acid, α-tocopherol) and antioxidant enzymes (superoxide dismutases, catalases, reductases, peroxidases) (for reviews, see Van Breusegem et al., 2008; Foyer and Noctor, 2005). The disulfide reductase thioredoxins (TRX) are known to be such intracellular redox regulators. They are small, ubiquitous proteins with two redox-active half-cystine residues in their catalytic active center, having the consensus amino acid sequence -Cys-Gly/Pro-Pro-Cys. All have overlapping and distinct properties and targets, although they function in a similar way. They transfer the reducing equivalents of NADPH to numerous substrates via the reversible oxidoreduction of their thiols. Generally, oxidized thioredoxins are reduced by NADPH-dependent thioredoxin reductases (NTR), forming the NTR/TRX system (Holmgren, 1985). Typically, in plant chloroplasts, thioredoxins are reduced in the light by a ferredoxin-dependent heterodimeric thioredoxin reductase (FTR) (Schürmann and Jacquot, 2000). Thioredoxins maintain cellular redox homeostasis and constitute major defences against oxidative stress, acting directly by reducing oxidized compounds and proteins like peroxiredoxins (PRX) and glutathione peroxidases (GPX) or providing reducing power to several reductases of the cell, namely hydrogen peroxide reductases, MetSO reductases, PAPS reductase, etc. (Carmel-Harel and Storz, 2000; Nordberg and Arner, 2001).

1 To whom correspondence should be addressed. E-mail jpr@univ-perp.fr, fax (+33) 4 68 66 84 99.
2 The author responsible for distribution of materials integral to the findings presented in this article.
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Plants are distinguishable from other organisms by their very complex thioredoxin system as revealed by sequencing of the *Arabidopsis thaliana* genome (Alonso et al., 2003). About 40 genes coding for TRX and TRX-related proteins have been identified. Among them, at least 11 belong to the cytosolic TRXh group but additional TRX-like proteins are predicted to have a cytosolic localization (Meyer et al., 2006). Genetic approaches aiming to identify functions of cytosolic thioredoxins in knockout plants have largely been limited by the absence of phenotypes of single mutants, presumably due to functional redundancies among members of the multi-gene families of TRX. In order to overcome this redundancy, we previously characterized a double mutant in which both NTRA and NTRB genes encoding cytosolic and mitochondrial thioredoxin reductase isoforms are inactivated. Surprisingly, this mutant does not show a pronounced phenotype (slightly wrinkled seed phenotype, slower plant growth, and pollen with reduced fitness). We suggest that mechanisms are active in the mutant to compensate inactivation of the thioredoxin pathway. Glutathione was found to play a key role in this compensation (Reichheld et al., 2007).

Flavonoids are antioxidant molecules serving as free radical scavengers by locating and neutralizing radicals before they damage the cell. Flavonoids are non-photosynthetic pigments produced by plants and are classified into flavonols, flavones, isoflavones, and anthocyanins, according to their structure. Although the most visible function of the flavonoids is the formation of red and purple anthocyanin pigments, non-pigmented flavonoids play central roles in protection against harmful UV-light and excess visible light, as well as acting in growth and reproduction (Jansen et al., 1998; Winkel-Shirley, 2002). Production of flavonoids from phenylalanine and acetyl CoA is stimulated by stress factors, such as increased UV light (Lois, 1994; Dixon and Paiva, 1995). Mutant plants (tt4, deficient in chalcone synthase), and tt5, deficient in chalcone isomerase) that are unable to accumulate flavonoids are more sensitive to UV-B and UV-C light (Li et al., 1993; Filkowski et al., 2004). In particular, the colorless flavonoids are among the most abundant flavonoids in plants (Böhm et al., 1998). They accumulate in their glycosylated form after an inductive light treatment and absorb UV-B light in the 280–320-nm region and are therefore thought to be effective UV filters (Ormrod et al., 1995; Solovchenko and Schmitz-Eiberger, 2003).

In the present study, we show that the *ntra ntrb* mutant accumulates higher levels of flavonoids than wild-type plants, resulting in higher tolerance of the mutant to UV-C treatment. Our data suggest that induced flavonoids synthesis may constitute an adaptation of the *ntra ntrb* to UV damaging effects.

**RESULTS**

**Transcriptome Analysis of the *ntra ntrb* Mutant**

In order to isolate compensatory genes whose transcription is induced in the *ntra ntrb* mutant, we performed transcriptome-profiling on the almost complete *Arabidopsis* microarray (CATMA) chips (Crowe et al., 2003). Transcript levels of the wild-type (WT) and *ntra ntrb* mutant were analyzed in plants grown on soil and collected 17 d after germination (stage 1.06 according to Boyes et al., 2001). At this developmental stage, growth retardation of the *ntra ntrb* mutant is visible but its overall development is not affected (see Supplemental Figure 1). Therefore, we did not expect to observe modifications of gene expression due to indirect developmental effects. Statistical analysis of comparisons revealed that 3.0% of the 25 238 *Arabidopsis* genes represented on the chip display modifications in mRNA levels (Bonferroni p-value, 5%) between the *ntra ntrb* mutant and WT plants (Supplemental Table 1). Remarkably, this analysis led to the identification of several genes involved in the flavonoid biosynthesis pathway, whose mRNA levels are modified in the mutant (Table 1). Transcript levels of most of these genes increase in the mutant and correspond to both early and late genes from the flavonoid biosynthesis pathway. Among these genes are those corresponding to the TT3, TT4, TT5, TT6, and TT7 loci, whose products are responsible for synthesis of leucoanthocyanidins from 3-malonyl-CoA and 4-coumaroyl-CoA (Figure 1). Another gene induced in the *ntra ntrb* mutant is FL51, whose product is involved in the synthesis of flavonols.

Further downstream in the pathway, GSTF12 (the *Arabidopsis* Bronze 2 homolog) (Alfenito et al., 1998), involved in vacuolar sequestration of anthocyanins, is also induced. Interestingly, three transcription factors known to regulate most of these genes were also found to have an increased level of mRNA in the *ntra ntrb* mutant. These genes encode R2R3/ MYB transcription factors (PFG3/MYB111, PAP1/MYB75, and PAP2/MYB90) known to regulate expression of genes of the flavonoid pathway (Borevitz et al., 2000; Stracke et al., 2007; Gonzalez et al., 2008). We do not know whether the R2R3/MYB factor MYB113 and MYB114 genes (Stracke et al., 2001) are also overexpressed in the *ntra ntrb* mutant, as they are not represented on the CATMA microarrays.

Further upstream in the pathway, genes encoding one of the four genes encoding for 4-coumarate-CoA ligase isoenzymes (4-CL3), which act in the general phenylpropanoid pathway, and two acetyl-CoA carboxylases (ACC1 and ACC2) are also induced. One single gene (At5g24530) encoding a flavonone 3-hydroxylase-like protein showed a decreased mRNA level in the mutant. This gene is distinct from the TT6 gene and no data are available on the function of its product.

In order to validate microarray data, we confirmed the modifications of mRNA levels for some individual genes by semi-quantitative RT–PCR experiments. Both biological repetitions used for microarray experiments gave similar RT–PCR results (Figure 2A). As expected, in the *ntra ntrb* mutant, no full-length cDNA were found for either NTRA or NTRB. We showed a pronounced increase in mRNA levels in the *ntra ntrb* plants for ACC1, DFR (TT3), F3H (TT6), F3’H (TT7), FL51, and PAP1/MYB75. The induction was much less pronounced for CHI (TT5) in this experiment but more obvious
in another independent experiment (Figure 2B). However, no apparent modification of mRNA level was observed for CHS (TT4) in any experiments (Figure 2A and 2B).

As accumulation of flavonoids is generally considered a hallmark of stressed plants, we checked whether genes encoding antioxidant systems are up-regulated in ntra ntrb plants. mRNA levels of superoxide dismutase, catalase, peroxidase, and glutathione S-transferase (with the exception of GST12) genes were not significantly modified in the ntra ntrb mutant, suggesting that the mutant plants are not subjected to stress conditions (Supplemental Table 1). These data confirm our previous observations showing that the level and redox state of glutathione, a marker of stress in plants, is not modified in the ntra ntrb mutant (Reichheld et al., 2007).

The ntra ntrb Mutant Accumulates More Soluble Flavonoids than Wild-Type Plants

In order to know whether the ntra ntrb mutant accumulates more flavonoids than wild-type plants, pigments were extracted from wild-type and ntra ntrb plants and subjected to absorption spectra analysis (Figure 3A). Two major peaks are observed between 400 and 500 nm and between 630 and 680 nm and have been shown to correspond to carotenoids and chlorophyll (Gross, 1991). The amounts of these two types of pigment appear to be very similar in ntra ntrb and wild-type plants. Two additional peaks are observed: one sharp peak at 265 nm and a broader peak between 300 and 360 nm. Interestingly, these peaks are significantly higher in the ntra ntrb mutant than in wild-type plants. The increase of the 265 nm peak is more pronounced than the one at 300–360 nm (Figure 3A).

### Table 1. Modified Expression of Flavonoid Biosynthesis Genes.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>AGI code</th>
<th>Fold change ntra ntrb/Col-0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA carboxylase 1 (ACC1)</td>
<td>At1g36160</td>
<td>2.14</td>
</tr>
<tr>
<td>Flavonoid 3′-hydroxylase (F3′H)/transparent testa 7 protein (TT7)</td>
<td>At5g07990</td>
<td>1.83</td>
</tr>
<tr>
<td>Flavanone 3-hydroxylase (F3H)/transparent testa 6 protein (TT6)</td>
<td>At3g51240</td>
<td>1.80</td>
</tr>
<tr>
<td>Glutathione S-transferase GSTF12/TT19</td>
<td>At5g17220</td>
<td>1.55</td>
</tr>
<tr>
<td>Flavonol synthase 1 (FS1)</td>
<td>At5g08640</td>
<td>1.30</td>
</tr>
<tr>
<td>myb family transcription factor (MYB111)/production of flavonol glycosides (PFG3)</td>
<td>At5g49330</td>
<td>1.17</td>
</tr>
<tr>
<td>4-coumarate–CoA ligase 3 (4CL3)</td>
<td>At1g65060</td>
<td>1.02</td>
</tr>
<tr>
<td>Chalcone isomerase (CHI)/transparent testa 5 protein (TT5)</td>
<td>At3g55120</td>
<td>0.99</td>
</tr>
<tr>
<td>myb family transcription factor (MYB75/PAP1)</td>
<td>At1g56650</td>
<td>0.88</td>
</tr>
<tr>
<td>Chalcone synthase/transparent testa 4 protein (TT4)</td>
<td>At5g13930</td>
<td>0.86</td>
</tr>
<tr>
<td>Dihydroflavonol 4-reductase (DFR)/transparent testa 3 protein (TT3)</td>
<td>At5g42800</td>
<td>0.79</td>
</tr>
<tr>
<td>Acetyl-CoA carboxylase 2 (ACC2)</td>
<td>At1g36180</td>
<td>0.75</td>
</tr>
<tr>
<td>Chalcone isomerase-like (CHI-like)</td>
<td>At5g05270</td>
<td>0.72</td>
</tr>
<tr>
<td>Flavonol synthase, putative</td>
<td>At5g63600</td>
<td>0.71</td>
</tr>
<tr>
<td>myb family transcription factor (MYB90/PAP2)</td>
<td>At1g66390</td>
<td>0.60</td>
</tr>
<tr>
<td>Flavanone 3-hydroxylase-like protein</td>
<td>At5g24530</td>
<td>−1.50</td>
</tr>
</tbody>
</table>

The differentially expressed genes (Bonferroni p-value < 0.05) are sorted by their log2 ratio, which is the difference between the log2 intensity for the ntra ntrb sample and the log2 intensity for the Col-0 sample.

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**Figure 1.** Schematic Representation of the Flavonoid Biosynthetic Pathway.

Bracketed indicate the early and late divisions of the flavonoid pathway in Arabidopsis. Enzymes corresponding to genes induced in the transcriptomic data are indicated in bold. ACC, acetyl-CoA carboxylase; 4CL, 4-coumarate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; GST, glutathione S-transferase. Note that the action of FLS is required for the biosynthesis of flavonols while DFR is necessary for the formation of anthocyanidins.
These peaks were shown to correspond to UV-absorbing pigments, mainly flavonoids (Lois and Buchanan, 1994). To confirm this statement, we analyzed absorption spectra of pigments extracted from \textit{tt4\_583} mutant plants—a mutant in which flavonoid biosynthesis is impaired (see below). The peaks located between 265 and 360 nm are much lower in the \textit{tt4\_583} mutant than in wild-type plants, suggesting that these peaks represent flavonoid pigments.

Anthocyanins were quantified in the same batch of plants. Absorption at 530 nm is higher in \textit{ntra \ ntrb} than in wild-type plants (Figure 3B). Nevertheless, the relative absorption values were low in comparison with other pigments, suggesting that anthocyanins do not accumulate to high levels in the different types of plants. Accordingly, the residual absorption in the \textit{tt4\_583} mutant in which the flavonoid pathway is impaired is about 25% of the absorption of wild-type plants.

**Isolation of a \textit{ntra \ ntrb tt4} Mutant in Col-0**

In order to determine whether accumulation of flavonoids in the \textit{ntra \ ntrb} mutant is able to compensate the absence of NTR, we wished to inactivate flavonoid biosynthesis in the \textit{ntra \ ntrb} mutant. We decided to inactivate all types of flavonoids by inactivating chalcone synthase (CHS), an enzyme controlling an early step in flavonoid biosynthesis. Several mutants in which chalcone synthase is inactivated have been isolated previously but, to our knowledge, no T-DNA mutant has yet been characterized in the Col-0 ecotype, that of the \textit{ntra \ ntrb} mutant. Therefore, in order to avoid any heterosis effects while crossing mutants, we isolated the SALK\_520583 mutant line (SALK library, Alonso et al., 2003, www.arabidopsis.org/Blast/) harbouring a T-DNA insertion in the second exon of the CHS gene (Figure 4). PCR mapping of the insertion showed that two T-DNAs have been inserted head-to-tail in the CHS locus. In order to assess whether the insertion impairs gene function, steady state mRNA levels for the CHS gene were measured by RT–PCR in the mutant using gene-specific oligonucleotides. As expected, CHS mRNAs are undetectable in the mutant (Figure 2B), suggesting that the CHS gene is inactivated. The \textit{tt4} phenotype of the mutant is confirmed by the phenotype of mutant seeds that show a yellow color (Supplemental Figure 2), a common trait of several transparent testa (\textit{tt}) mutants (Shirley et al., 1995). Spectrophotometric measurements also show very low levels of anthocyanin accumulation in the \textit{tt4} mutant (Figure 3B). Therefore, we propose that the SALK\_520583 mutant can be considered as a \textit{tt4} mutant allele and will be called \textit{tt4\_583}.

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*Figure 2. RT–PCR Experiments Comparing Flavonoid Biosynthesis Gene Expression in Col-0, \textit{ntra \ ntrb}, \textit{tt4\_583}, and \textit{ntra \ ntrb tt4} Mutant Plants.*

(A) Two different batches of 17-day-old plants of wild-type (Col-0) and \textit{ntra \ ntrb} mutants grown in soil under a long day regime (16 h day/8 h dark) were collected for cDNA preparation. 25 PCR cycles were performed except for the \textit{F3\_H, MYB75, NTRA, and NTRB} genes, for which 28 cycles were performed.

(B) 17-day-old plants of wild-type (Col-0), \textit{ntra \ ntrb}, \textit{tt4\_583}, and \textit{ntra \ ntrb tt4} mutants grown in soil under a 16 h day/8 h dark regime were collected for cDNA preparation. 25 and 28 PCR cycles were performed.
We crossed the tt4_583 homozygote mutant with our ntra ntrb homozygote mutant. Triple ntra ntrb tt4 homozygote mutants were isolated in the F2 generation. No full-length mRNAs for NTRA, NTRB, and CHS were detected in the ntra ntrb tt4 mutant (Figure 2B). As for the tt4_583 mutant, the ntra ntrb tt4 mutant has yellow seeds (Supplemental Figure 2). ntra ntrb tt4 mutant plants accumulate very low levels of anthocyanin, confirming that the flavonoid pathway is inactivated (data not shown).

Inactivation of the Flavonoid Pathway in ntra ntrb Does Not Impair the Activation of Flavonoid Biosynthesis Genes

In order to know whether the level of expression of certain flavonoid biosynthesis genes is influenced by the tt4 mutation, we performed semi-quantitative RT–PCR experiments on cDNAs synthetized from mRNAs from Col-0, ntra ntrb, tt4_583, and ntra ntrb tt4 plants (Figure 2B). In this experiment, we confirmed the increase of mRNA levels in the ntra ntrb mutant for ACC1, CHI (TT5), DFR (TT3), F3H (TT6), F3’ H (TT7), FLS1, and PAP1/MYB75. Compared with wild-type plants, in the tt4_583 mutant, the mRNA level of some of the genes is not affected (CHI, PAP1/MYB75) or, more surprisingly, decreased (ACC1, F3H, F3’ H, FLS1), suggesting that some feedback regulation mechanisms occur in this mutant (Figure 2B). However, compared with tt4_583 plants, in the ntra ntrb tt4 mutant, a marked increase of mRNA levels for all the studied genes is observed, suggesting that the tt4_583 mutation does not impair the activation of flavonoid biosynthesis genes occurring in the ntra ntrb mutant (Figure 2B).

Inactivation of the Flavonoid Pathway in ntra ntrb Does Not Impair the Growth Retardation Phenotype of the ntra ntrb Mutant

Accumulation of flavonoids has been shown to result in reduced size phenotype (Besseau et al., 2007). Recently, we described a retarded growth phenotype in the ntra ntrb mutant (Reichheld et al., 2007 and Supplemental Figure 1). In order to know whether accumulation of flavonoid in the ntra ntrb mutant is responsible for its retarded growth, we compared growth of the ntra ntrb mutant with the ntra ntrb tt4 mutant (Supplemental Figure 3). Inactivation of the flavonoid pathway did not rescue the retarded growth of the ntra ntrb mutant, suggesting that accumulation of flavonoids is not responsible for the growth retardation in the ntra ntrb mutant.

In the same way, we wished to know whether accumulation of flavonoid could be responsible for the pronounced growth inhibition of ntra ntrb triggered by the glutathione biosynthesis inhibitor buthionine sulfoximine (BSO), a particular phenotypic trait of the ntra ntrb mutation (Reichheld et al.,...
We compared plant growth of both ntra ntrb and ntra ntrb tt4 mutants on media supplemented with BSO. Here again, growth of both mutants is equally affected by BSO, showing that flavonoids are not involved in the BSO-dependent growth inhibition in the ntra ntrb mutant (Supplemental Figure 4).

Accumulation of Flavonoids in the ntra ntrb Mutant Protects Plants against UV Radiation

It has been shown that flavonoids are the major photoprotective pigments protecting plants from UV radiation (Li et al., 1993; Lois and Buchanan, 1994; Jin et al., 2000; Filkowski et al., 2004). To further determine the biological function of flavonoid accumulation in the ntra ntrb mutant, we subjected the mutant to UV-C treatment (Figure 5A). When 17 d old, wild-type, ntra ntrb, and the ntra ntrb mutant complemented by a genomic construct containing the NTRB gene (C10) (Reichheld et al., 2007) were exposed to UV-C (1.5 kJ m⁻²) and then incubated in a growth chamber for 3 d, wild-type plants bleached and died. However, the ntra ntrb plants mostly survived this treatment, suggesting that the mutant is more tolerant to the lethal effects of the UV radiation. Complementation of the mutant with the NTRB gene restores the UV sensitivity, demonstrating that the UV-tolerance of the mutant is linked to the inactivation of NTR genes.

In order to know whether flavonoids are involved in the tolerance, we subjected the ntra ntrb tt4 mutants to the same UV conditions (Figure 5B). Interestingly, inactivation of the flavonoid pathway in the ntra ntrb mutant restored UV sensitivity, strongly suggesting that the UV tolerance of the ntra ntrb is due to accumulation of flavonoids.

NTR Genes Are Induced by UV-C Treatment

In order to characterize further the NTR-dependent UV tolerance, we wished to determine whether the NTR genes are under UV dependence. We subjected ProNTR:GUS plants to UV-C treatment. Both ProNTRA:GUS and ProNTRB:GUS are activated by irradiation with UV-C light (1.5 kJ m⁻²) and the activation of both promoters is even more pronounced using a higher dose of UV-C (5 kJ m⁻²) (Figure 6A). To confirm promoter/GUS fusion data, we performed RT-PCR on cDNA from plants subjected to UV-C treatment. mRNA levels of both NTRA and NTRB genes are greatly induced after 24 h treatment. Similarly, the mRNA level of the CHS gene is also induced under UV-C treatment (Figure 6B).

DISCUSSION

Flavonoid Biosynthesis Is Induced in the ntra ntrb Mutant

We previously characterized the ntra ntrb mutant as a mutant inactivated in both mitochondrial and cytosolic thioredoxin reductases. Surprisingly, this mutant does not show a pronounced phenotype. We suggest that mechanisms are active in the mutant to compensate inactivation of the thioredoxin reductases. Glutathione plays a key role in this compensation (Reichheld et al., 2007) allowing partial reduction of cytosolic thioredoxins. Our microarray experiments were initially aimed at finding genes implicated in this alternative glutathione dependent pathway. In fact, the genes implicated in glutathione synthesis and glutathione reduction are expressed at the same level in Col0 and the ntra ntrb mutant. The expression of glutaredoxin genes is also similar in the wild-type and the ntra ntrb double mutant. Glutaredoxins are reduced by glutathione and are most probably the direct reductant of thioredoxins in the alternative pathway. Thus, it appears that the reduction of cytosolic thioredoxins through the glutathione pathway takes place without change in gene expression.
stress-induced genes (superoxide dismutases, peroxidases, catalase, etc.) are not significantly affected in the ntra ntrb mutant, suggesting that the mutant does not suffer from endogenous generation of oxygen radicals. In particular, we did not observe any modification of mRNA levels of direct target proteins of the NTR (TRXh, TRXo) or in known targets of those TRX. Most of these targets are indeed involved in antioxidant mechanisms (MSR, Prx-II, Gpx). Therefore, the selective accumulation of flavonoids in the mutant might have biological significance. Flavonoids are a remarkably diverse group of secondary products with a vast array of biological functions, including roles in stress protection, pigmentation for recruitment of pollinators and seed dispersers.

Flavonoid Biosynthesis Genes Are Induced in a Coordinated Way in the ntra ntrb Mutant

The microarray data showed induction of mRNA level in several early genes of the flavonoid biosynthesis pathway (TT5, TT6, TT7, TT3) as well as three MYB transcription factors (PFG3/MYB111, MYB75/PAP1, and MYB90/PAP2). These factors have been shown to co-regulate several early genes of the pathway, suggesting that they might be responsible for flavonoid biosynthesis gene activation in the ntra ntrb mutant. Interestingly, most of the flavonoid biosynthesis genes that we found induced in our transcriptomic analyses were reported as putative PFG3/MYB111 target genes (Stracke et al., 2007). This suggests that induction of this transcription factor is the primary consequence of the ntra ntrb mutation and that this gene controls the induction of the complete panel of flavonoid biosynthesis genes. We also found that the activation of flavonoid biosynthesis genes is maintained in the ntra ntrb tt4 mutant, even when flavonoid biosynthesis is impaired, suggesting the absence of feedback regulation by the internal level of flavonoids.

The enzymes synthesized by the TT5, TT6, TT7, TT3 genes (CHI, F3H, F3′H, DFR) have been shown to be involved in the early steps of the flavonoid pathway, upstream from the branch point of the different flavonoid biosynthesis pathways, suggesting that different types of flavonoids may accumulate in the mutant (see Figure 1). Analyses of pigment absorption spectra support our transcriptomic data. Selective modifications of the absorption spectrum of plant pigments were detected in ntra ntrb mutant plants compared with wild-type plants. Carotenoids and chlorophyll appeared to be unmodified, suggesting that chloroplast functions are not perturbed in the mutant. Accordingly, bleeding has never been observed in this mutant. Our anthocyanin measurements suggest that the pigmented flavonoids accumulate in the ntra ntrb mutant (Figure 3B). This accumulation may be responsible for the slightly darker color of the ntra ntrb plants and seeds. Accordingly, we found induction of some late genes involved in the anthocyanin biosynthesis pathway (DFR, GST12) in the transcriptomic analyses. Nevertheless, compared with non-pigmented flavonoids, anthocyanins do not accumulate to a high level in the mutant (Figure 3B). Therefore, it is unlikely that this slight accumulation has a biological significance in the ntra ntrb mutant.

![Figure 6. NTRA, NTRB and CHS Genes Are Induced by UV-C.](image-url)
More consistently, a marked accumulation of UV-protective pigments was observed in the ntra ntrb mutant. These pigments are probably flavonoids because the corresponding peaks were not detected in the tt4_583 mutant. Different types of non-pigmented flavonoids (flavonols, flavonol glycosides, flavones, isoflavones, etc.) have been shown to have absorption maxima between 260 and 350 nm. HPLC and LC–MS analyses will be needed to determine which types of non-pigmented flavonoids accumulate in the ntra ntrb mutant.

Thioredoxin reductase, like other flavo-proteins, has been shown to reduce quinones, presumably via the FAD co-factor (Bironaite et al., 1998; Miskiniene et al., 1998). Therefore, the quinone residues of flavonoids are potential targets of NTR. Modification of the redox state of flavonoids, rather than an increase of the pool of flavonoids, could influence the absorption spectrum in the ntra ntrb mutant. This hypothesis has been tested by reducing the ntra ntrb pigment extract by NADPH and NTRA. The absorption spectrum was not modified after reduction (data not shown), suggesting that modified redox state of flavonoids in the ntra ntrb mutant does not account for the modification of absorption spectrum in the ntra ntrb mutant.

Accumulation of Non-Pigmented Flavonoids in ntra ntrb Mutant Leads to Tolerance to UV Radiation

UV radiation is known to lead to ROS production in plants (Mittler, 2002). The higher tolerance of the ntra ntrb mutant to UV-C is therefore surprising, given the putative anti-oxidant role of thioredoxins. We have previously shown that the ntra ntrb mutant is not tolerant to oxidative stress conditions (Reichheld et al., 2007), suggesting that UV-C tolerance is uncoupled from ROS tolerance. Remarkably, accumulation of non-pigmented flavonoids was shown to shield plants from UV radiation (Zhao et al., 2007). However, inhibition of flavonoid biosynthesis leads to sensitivity to UV-B (Li et al., 1993; Lois and Buchanan, 1994) and, to a lesser extent, to UV-C (Filkowski et al., 2004). The UV-C tolerance of the ntra ntrb mutant is probably linked to accumulation of flavonoids in leaves. The fact that this tolerance is lost in the ntra ntrb tt4 mutant strongly supports this point. Induction of flavonoid synthesis may constitute an adaptation to UV damage effect. Whether the ntra ntrb mutant is also tolerant to UV-B will have to be determined.

What is the actual effector of this gene activation in the ntra ntrb mutant? Mutants in which flavonoid synthesis is induced have already been isolated in plants (Albert et al., 1997; Zhao et al., 2007; Bieza and Lois, 2001; Tanaka et al., 2002). In most cases, they define negative regulators of flavonoid biosynthesis. NTRs may constitute new negative regulators of flavonoid synthesis. On the basis of our knowledge on the NTRs, cytosolic and mitochondrial TRX are the unique targets of NTRs. Thus, it is highly probable that NTRs act on flavonoid biosynthesis through one or several of the 10–20 cytosolic or mitochondrial thioredoxins or thioredoxin-like proteins that are encoded in the Arabidopsis genome. As previously shown, a glutathione pathway is redundant with NTR TRX reduction. Nevertheless, up to now, the reduction state has only been established for TRX h3 (At5g42980), the most abundant cytosolic TRX. It is possible that some of the other TRXs are less efficiently reduced by the glutathione pathway. Thus, looking at the reduction state of cytosolic and mitochondrial TRXs in the ntra ntrb mutant or a survey of the flavonoid synthesis in the available TRX mutants should allow us to go a step further in our knowledge of the NTR regulation of flavonoids. Nevertheless, we cannot exclude that the accumulation of flavonoids in the ntra ntrb mutant results from an indirect consequence of the NTR deficiency.

Our data indicate that NTRA and NTRB gene expression is induced by UV-C, suggesting that NTR may act in response to UV-C. Interestingly, UV-C treatment was shown to induce gene expression of the NTR target thioredoxin TRXh5 (Laloi et al., 2004). This appears to be incoherent with the NTR negative control of flavonoids that constitutes an efficient defence against UV-C. In fact, NTRs, through the multiple TRXs that they reduce, are implicated in various defences and signals that may in some situations be in apparent conflict.

METHODS

Plant Materials, Growth Conditions, and Treatments

Seedlings and plants from Arabidopsis thaliana ecotype Columbia (wild-type, ntra ntrb, and tt4_583 mutant lines) were used for the experiments. T-DNA mutant lines were provided by the SALK laboratory (Alonso et al., 2003) (www.arabidopsis.org). For in-vitro seedlings, seeds were surface sterilized and plated on 0.5 Murashige and Skoog medium including Gamborg B5 vitamins (M0231, Duchefa), 1% (w/v) sucrose, and 0.8% (w/v) plant agar. Supplementation of the growth medium with L-buthionine-(S,R)-sulfoximine (BSO, Sigma-Aldrich) was performed as described previously (Reichheld et al., 2007). For plants grown in soil, seeds were sown in pots containing a mixture of soil and vermiculite (3:1 v/v) and irrigated with water. Both plants and seedlings were grown at 22°C, 70% hygrometry, under a 16 h light (4500 lux)/8 h dark regime. UV-C irradiation was performed using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) fitted with 254-nm UV-C light bulbs. Ten-day-old in-vitro seedlings or 21-day-old plants grown in soil were irradiated. The UV-C energy delivered in each experiment was measured by a UV-C sensor fitted inside the Stratalinker irradiation chamber.

Transcriptome Studies

Microarray analysis was carried out with the CATMA array (Crowe et al., 2003; Hilson et al., 2004) containing 24 576 gene-specific tags from Arabidopsis thaliana. RNA samples from two independent biological replicates were used for each comparison, with dye-swap technical replicates (i.e. four hybridizations per comparison). The labelling, hybridizations, and scanning were performed as previously described (Lurin et al., 2004). Normalization and statistical analysis were based on two dye swaps (Lurin et al., 2004). To determine differentially expressed genes, we performed a paired t-test on the log ratios, assuming that the variance of the log ratios was the
same for all genes. The raw $p$-values were adjusted by the Bonferroni method, which controls the FWER (Ge et al., 2003). We considered as being differentially expressed the genes with a FWER at threshold of 5%. The Bonferroni method (with a type I error equal to 5%) keeps a strong control of the false positives in a multiple-comparison context (Ge et al., 2003). Microarray studies and statistical analyses are described in more detail in the Supplementary Data.

**Data Deposition**

Microarray data from this article were deposited at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, no. GSE7998) and at CATdb (http://urgv.evrn.inra.fr/CATdb/, Project R504-02_NTR) according to the ‘Minimum Information about a Microarray Experiment’ standards.

**Gene Expression Analysis by RT–PCR and GUS Analysis**

Total RNA was extracted from frozen plant organs using the TRIzol Reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer’s protocol. For reverse transcriptase (RT)–PCR, 5 µg of DNase I-treated total RNA was used for first-strand synthesis of cDNA by using oligo(dT) primer reverse transcription with the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV–RT) as described by the manufacturer’s protocol (First Strand RT–PCR kit, ProSTAR™, Stratagene). PCRs (25–28 cycles) were performed as described in Reichheld et al. (2005). Primers used for RT–PCR experiments are described in the Supplemental Table 2.

For the GUS assay, plants were cultivated in vitro under the same conditions as described previously. GUS histochemical staining was performed according to Reichheld et al. (2002).

**Spectrophotometric Analysis and Anthocyanin Determination**

Pigment extracts were prepared by incubating 0.05 g of 17-day-old plants in 0.5 mL of 80% (v/v) ethanol at 65°C for 15 min. The extracts were centrifuged for 15 min at 12,000 g, and the supernatants used to obtain the absorption spectra with a spectrophotometer (Beckman DU 800).

Anthocyanin content was evaluated as reported before (Feinbaum and Ausubel, 1988). Tissues were blended in liquid nitrogen and mixed in the presence of one volume ethanol containing 1% HCl. After mixing, one volume of methanol/water (ratio 1/3) was added, followed by one volume of chloroform. After centrifugation at full speed to pellet membranes and precipitated proteins, the clear supernatant was mixed to one volume of methanol/water (1/3). Absorbance at 530 nm was measured to evaluate the amount of anthocyanins in all samples.

**Accession Numbers**

Sequence data for NTRA, NTRB, ACT2, ACC1, TT3, TT4, TT5, TT6, TT7, FLS1, and MYB75 can be found in the EMBL/GenBank data libraries under accession numbers At2g17420, At4g35460, At3g18780, At1g36160, At5g07990, At5g08640, At3g55120, At3g51240, At5g07990, At5g08640, and At1g56650, respectively.

**SUPPLEMENTARY DATA**

Supplementary Data are available at Molecular Plant Online.

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