Transcriptional-Metabolic Networks in \(\beta\)-Carotene-Enriched Potato Tubers: The Long and Winding Road to the Golden Phenotype\(^{1[C][W][OA]}\)

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Vitamin A deficiency is a public health problem in a large number of countries. Biofortification of major staple crops (wheat \([Triticum aestivum]\), rice \([Oryza sativa]\), maize \([Zea mays]\), and potato \([Solanum tuberosum]\)) with \(\beta\)-carotene has the potential to alleviate this nutritional problem. Previously, we engineered transgenic “Golden” potato tubers overexpressing three bacterial genes for \(\beta\)-carotene synthesis (CrtB, CrtI, and CrtY, encoding phytoene synthase, phytoene desaturase, and lycopene \(\beta\)-cyclase, respectively) and accumulating the highest amount of \(\beta\)-carotene in the four aforementioned crops. Here, we report the systematic quantitation of carotenoid metabolites and transcripts in 24 lines carrying six different transgene combinations under the control of the 355 and \(Patatin\) (\(Pat\)) promoters. Low levels of \(B-I\) expression are sufficient for interfering with leaf carotenogenesis, but not for \(\beta\)-carotene accumulation in tubers and calli, which requires high expression levels of all three genes under the control of the \(Pat\) promoter. Tubers expressing the \(B-I\) transgenes show large perturbations in the transcription of endogenous carotenoid genes, with only minor changes in carotenoid content, while the opposite phenotype (low levels of transcriptional perturbation and high carotenoid levels) is observed in Golden (\(Y-B-I\)) tubers. We used hierarchical clustering and pairwise correlation analysis, together with a new method for network correlation analysis, developed for this purpose, to assess the perturbations in transcript and metabolite levels in transgenic leaves and tubers. Through a “guilt-by-profiling” approach, we identified several endogenous genes for carotenoid biosynthesis likely to play a key regulatory role in Golden tubers, which are candidates for manipulations aimed at the further optimization of tuber carotenoid content.

Carotenoids are a group of widely distributed isoprenoid pigments exerting vital roles in photoprotection and as free radical scavengers. In addition, they represent essential components of light-harvesting and reaction center complexes of photosynthetic organisms (for review, see Hirschberg, 2001). When accumulated in chromoplasts, carotenoids confer their colors to many flowers and fruits, contributing substantially to plant-neighbor interactions (Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004). In addition, carotenoids are the precursors of several physiologically important apocarotenoids (Bouvier et al., 2005; Auldridge et al., 2006), like the phytohormone abscisic acid, originating from 9-cis-violaxanthin and -neoxanthin (Schwartz et al., 1997). A further example of plant apocarotenoids is represented by the strigolactones, which serve both as interspecific signaling molecules (Bouwmeester et al., 2007) and as regulators of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). In animals, the best-known apocarotenoids are vitamin A (retinol) and its derivatives, which are involved in different processes, including vision, immune response, development, and reproduction (Ross et al., 2000). This essential compound is either taken up directly from animal foodstuff or synthesized from dietary carotenoids with provitamin A activity, such as \(\beta\)-carotene, \(\alpha\)-carotene, and cryptoxanthin. Provitamin A carotenoids are converted into retinal via symmetrical cleavage reactions catalyzed by the \(\beta\)-carotene cleavage oxygenase I, which has been characterized from several species, including humans (for review, see Moise et al., 2005). The coverage of dietary vitamin A demand through plant carotenoids is particularly important in developing countries, where the consumption of animal products is low.

Vitamin A deficiency is the major cause of child blindness and increases the probability of disease and...
death from severe infections (http://www.who.int/nutrition/topics/vad/en/). Vitamin A deficiency is a widespread public health problem in developing countries, but it is also encountered among the elderly, heavy drinkers, and smokers in poor urban populations of developed countries (http://www.who.int/nutrition/topics/vad/en/). β-Carotene exhibits the highest provitamin A activity and accumulates in several plants such as carrot (Daucus carota), apricot (Prunus armeniaca), and peach (Prunus persica) but not in major crops such as wheat (Triticum aestivum), rice (Oryza sativa), and potato (Solanum tuberosum). In the Copenhagen Consensus 2008 list of priorities, vitamin A supplements for children and food biofortification rank first and third, respectively (http://www.copenhagenconsensus.com/Default.aspx). Therefore, the improvement of provitamin A content has been an important goal of plant biotechnology, and several successful efforts have been undertaken to improve the provitamin A content in major crops: “Golden” rice (Ye et al., 2000; Paine et al., 2005), tomato (Solanum lycopersicum); Romer et al., 2000; Rosati et al., 2000; Fraser et al., 2002; Dharmapuri et al., 2002), canola (Brassica napus; Shewmaker et al., 1999; Ravanello et al., 2003), maize (Ze a mays; Aluru et al., 2008), and flaxseed (Linum usitatissimum; Fujisawa et al., 2008).

In plants, the synthesis of carotenoids (Hirschberg, 2001; Fraser and Bramley, 2004; Dellapenna and Pogson, 2006) is initiated by the enzyme phytoene synthase (PSY), which mediates the condensation of two molecules of geranylgeranyl diphosphate, leading to the colorless carotene phytoene. The conjugated double bond system of phytoene is then extended by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) to yield lycopene. The pathway leading from phytoene to all-trans-lycopene involves at least one further enzyme, the carotene isomerase CrtISO. Lycopene is the substrate for the lycopene-β- and -ε-cyclases (LCY-Yb and LCY-e), which catalyze the formation of β- and α-carotene. β-Carotene and α-carotene are hydroxylated into zeaxanthin and lutein, respectively, by the action of heme (LUT1, LUT5) and nonheme (CHY1, CHY2) carotene hydroxylases. Zeaxanthin is a constituent of the xanthophyll cycle and can be reversibly converted into violaxanthin via antheraxanthin. These reactions are mediated by the enzymes zeaxanthin epoxidase (ZEP) and violaxanthin deepoxidase. In contrast to plants, some nonphotosynthetic bacteria, such as Erwinia, utilize only three enzymes (i.e. phytoene synthase CrtB, phytoene desaturase/isomerase CrtI, and lycopene cyclase CrtY) to perform the synthesis of β-carotene from geranylgeranyl diphosphate (Fig. 1).

In recent years, three strategies have been followed to enhance the carotenoid content of potato tubers. The first one was a “blocking” strategy and relied on the posttranscriptional gene silencing of key biosynthetic steps: the CHY hydroxylase, acting on β-carotene (Diretto et al., 2007b; van Eck et al., 2007), the ZEP epoxidase, acting on zeaxanthin (Romer et al., 2002), and the LCY-e cyclase, acting on lycopene and starting the α-branch (Diretto et al., 2006; Fig. 1). The second one was a “push” strategy and relied on the overexpression of biosynthetic genes in a constitutive or tuber-specific manner. This included the overexpression of single genes such as CrtB (Ducreux et al., 2005), of 1-deoxy-D-xylulose 5-phosphate synthase (involved in the synthesis of the isoprenoid precursor IPP; Morris et al., 2006b) or of β-carotene ketolase (Morris et al., 2006a; resulting in the production of ketocarotenoids), or of combinations of two or three genes (minipathways; Gerjets and Sandmann, 2006; Diretto et al., 2007a). The third strategy, termed “sink engineering,” relied on the overexpression of regulatory genes causing differentiation of chromoplasts, which in turn act as storage structures for carotenoids (Lopez et al., 2008).

Quantitatively, the most important results have been obtained using the second strategy: through the tissue-specific overexpression of a minipathway containing the CrtB, CrtI, and CrtY bacterial genes, we have produced Golden potato tubers containing up to 47 μg g−1 dry weight of β-carotene (Diretto et al., 2007a). This value represents the highest β-carotene level ever reported in the four major staple crops: maize, rice, wheat, and potato. Golden tubers not only display a massive accumulation of carotenoids but also an increase of endogenous carotenoid biosynthesis transcripts.

In order to define the specific role of the various components of the minipathway (transgenes as well as promoters controlling them) in modulating carotenoid biosynthesis in the tuber, and in order to clarify the conditions leading to the Golden phenotype, we report here on the systematic molecular and biochemical characterization of transgenic plants harboring different combinations of the CrtB, CrtI, and CrtY genes under the control of constitutive (35S) and tuber-specific (Patatin [Pat]) promoters.

RESULTS AND DISCUSSION

Golden tubers were obtained using the plasmid pP-YBI, encoding the bacterial minipathway enzymes CrtB, CrtI, and CrtY, leading to β-carotene synthesis under the control of the tuber-specific Pat promoter (Diretto et al., 2007a; Table I). Additional transgenic plants were generated using five different plasmids, harboring CrtI alone (I) or in combination with CrtB (BI) and CrtY (YBI; Diretto et al., 2007a). In all cases, CrtB was put under the control of the tuber-specific Pat1 promoter, a strategy previously shown to result in an increase of β-carotene content (Ducreux et al., 2005). Genes mediating the next steps in the pathway (CrtI and/or CrtY) were put under the control of the constitutive 35S promoter (pK series) or of the tuber-specific Pat2 promoter (pP series; Diretto et al., 2007a; Table I). For each construct, 10 transgenic lines were selected and grown in the greenhouse under controlled
conditions. Based on their tuber carotenoid content (Diretto et al., 2007a), the lines obtained were designated as “expressor” or “nonexpressor,” exhibiting modified or unchanged carotenoid contents, respectively. The pK-YBI and pP-YBI constructs produced only two expressor lines, each with significantly increased carotenoid content. Therefore, two nonexpressor and two expressor lines for each construct were selected for further characterization in this work.

CrtY-B-I Expression Causes Unscheduled Carotenoid Accumulation in Vitro

During in vitro regeneration, the YBI expressor lines exhibited a phenotype that was not observed with any of the other transformants. Explants from pP-YBI expressor lines formed orange calli (Fig. 2A), while in vitro plantlets from pK-YBI expressor lines had a yellow-orange appearance (Fig. 2B). The carotenoid content of calli and plantlets was determined via diode array HPLC (Table II). Wild-type plantlets exhibited low carotenoid levels (approximately 75 μg g⁻¹ dry weight), of which about 13% was β-carotene. The carotenoid levels of pP-YBI plantlets were similar to those of the wild type, while the pK-YBI plantlets contained around 1.5-fold higher amounts of total carotenoids, of which approximately 16% was β-carotene, and 1.4-fold lower amounts of total chlorophylls, which explained their yellow-orange appearance. In contrast to the plantlets, the carotenoid content of pK-YBI calli was almost identical to that of wild-type calli, while the orange calli derived from pP-YBI expressor lines contained massive (up to 1 mg g⁻¹ dry weight) amounts of carotenoids, with β-carotene being the major carotenoid (about 75% of total carotenoids). The phenotype of the pK-YBI plantlets disappeared in greenhouse-grown plants.

Expression of CrtB-I Genes Induces Striking Alterations in Leaf Pigment Composition and Gene Expression

We reported previously that the pK-YBI expressor lines exhibit a pale leaf color (Diretto et al., 2007a). As shown in Figure 2C, this phenotype was already
observed in pK-I expressor leaves and became more pronounced in pK-BI and pK-YBI expressor leaves. Thus, the phenotype can be attributed to the expression of the CrtI transgene, which is expressed in all three types of leaves, and is intensified by the additional expression of CrtB and CrtY. In agreement with the visual phenotype, HPLC analysis showed that carotenoid content decreased significantly in leaves of pK expressor lines (Fig. 3A), while no significant variations were observed in leaves of expressor pP lines and of nonexpressor lines (Supplemental Table S1). Moreover, the reduction of pigment content was progressive, from pK-I to pK-YBI (Fig. 3A). It has been reported previously that overexpression of CrtI in tobacco (Nicotiana tabacum) and tomato leaves leads to alterations in pigment content and composition, including a decrease in total carotenoids and α-xanthophylls, an increase of β-carotene and β-xanthophylls, and a reduction of the chlorophyll-carotenoid ratio (Misawa et al., 1994; Romer et al., 2000). The changes in pigment composition reported here were quantitatively and qualitatively more severe: as shown in Supplemental Table S1, the amounts of chlorophylls and carotenoids were reduced approximately 25% and 34%, respectively. Moreover, the reduction affected all three carotenoid classes, α- and β-xanthophylls and β-carotene (Fig. 3A). The more pronounced phenotype reported here might be attributed to species-specific differences between tomato/tobacco and potato, or it may be the result of the codon-optimized CrtI gene used in this study (Al-Babili et al., 2006), probably leading to higher CrtI protein amounts. However, western-blot experiments were unable to reveal any CrtI protein in leaves of the expressor lines (data not shown).

Compared with pK-I leaves, the pK-BI leaves showed lower pigment content and paler color. This suggested that CrtB was contributing to the phenotype and thus that it was expressed in leaves, in spite of its control by the Pat1 tuber-specific promoter. Indeed, real-time reverse transcription (RT)-PCR analyses revealed low, but detectable, CrtB transcript levels in leaves of the pK-BI and pK-YBI lines (Supplemental Table S2). Comparably low, but detectable, levels of expression in leaves were measured for CrtI and CrtY under the control of the Pat2 promoter (Supplemental Table S2). These observations suggest that both Pat promoters used in this work were “leaky” and allowed low levels of expression in leaves. In the case of the CrtB gene, these low levels of expression resulted in an enhancement of the leaf phenotype caused by CrtI expression. It is also notable that coexpression of CrtI and CrtB led to lower amounts of CrtI transcript, compared with the expression of CrtI as a single transgene (Supplemental Table S2).

To evaluate possible transcriptional perturbations of endogenous carotenoid genes, and to determine their contribution to leaf pigment composition, their transcript levels were monitored by real-time RT-PCR. All gene expression data were normalized to the housekeeping gene, ubiquitin, resulted in similar expression trends (data not shown). The results are analytically shown in Supplemental Table S2 and summarized in Figure 3B. Overexpression of CrtI led to a repression of “early” transcripts mediating lycopene synthesis and to an induction of transcripts in the α- and β-branched. These transcriptional alterations are consistent with the general reduction of carotenoid content (Fig. 3A). Combined expression of CrtI and CrtB further repressed early carotenoid transcripts and LCY-e (Fig. 3B) and, at the pigment level, further reduced β-carotene and β-xanthophyll contents, while α-xanthophyll content was increased (Fig. 3A). Finally, the additional expression of the CrtY β-cyclase partially restored the levels of β-xanthophylls (Fig. 3A) but further repressed the transcripts of xanthophyll biosynthesis enzymes (Fig. 3B).

These data indicate that leaf carotenoid levels are directly related to the expression levels of early genes, particularly PSY1, PSY2, and PDS (Fig. 3), suggesting that the early biosynthetic flux leading to lycopene (Fig. 1) in transgenic leaves is mainly controlled by the endogenous enzymes. In contrast, the levels of α- or

| Table 1. Gene constructs analyzed |
|----------------------------------|
| Plasmid | Promoter-Gene Combinations |
| pk-I    | 35S:CrtI                   |
| pk-BI   | Pat1:CrtB, 35S:CrtI       |
| pk-YBI  | Pat1:CrtB, 35S:CrtY, 35S:CrtY |
| pP-I    | Pat2:Crt, Pat2:Crt        |
| pP-BI   | Pat1:CrtB, Pat2:Crt       |
| pP-YBI  | Pat1:CrtB, Pat2:Crt, Pat2:CrtY |

Figure 2. Phenotypes of transgenic plants. A, In vitro phenotypes of explants. B, In vitro phenotypes of regenerated plantlets. C, Leaf phenotypes of expressor plants grown in the greenhouse. Wt, Wild type.
Table II. HPLC analysis of chlorophyll and carotenoid contents in wild-type and Golden calli and plantlets

Values shown are µg g⁻¹ dry weight. Carotenoid composition was measured via diode array HPLC on a minimum of four different calli or stems. Fold change with respect to the wild type is reported for each pigment and for each line. Fold change with respect to the wild type was not calculated in cases in which a metabolite was not detected (indicated by dash).

| Sample | Chlorophyll a | Chlorophyll b | Total Chlorophyll | Violaxanthin + Neoaxanthin + Other Xanthophylls | Lutein | β-Carotene | α/β-Xanthophyll | Total Carotene |
|--------|---------------|---------------|-------------------|-----------------------------------------------|-------|------------|----------------|---------------|
| Calli  |               |               |                   |                                               |       |            |                |               |
| Wild type | 8.8 ± 1.7     | 1.7 ± 0.5     | 10.9 ± 2.0        | 10.2 ± 1.9                                    | 10.5 ± 1.7 | 6.1 ± 1.3 | 1.0            | 48.1 ± 2.5    |
| pK-YBI 41 (E) | 9.1 ± 1.6     | 1.2 ± 0.4     | 10.7 ± 1.7        | 10.6 ± 1.7                                    | 10.3 ± 3.0 | 8.9 ± 1.1 | 1.0            | 50.8 ± 4.8    |
| Fold change | 1.0           | 0.7           | 0.9              | 1.0                                          | 1.0    | 1.5        | 1.0            | 1.0           |
| pK-YBI 42 (E) | 8.0 ± 1.4     | 1.1 ± 0.4     | 9.4 ± 1.7         | 10.9 ± 1.9                                    | 11.0 ± 2.7 | 8.8 ± 1.8 | 1.0            | 49.2 ± 3.3    |
| Fold change | 0.9           | 0.7           | 0.9              | 1.1                                          | 1.1    | 1.4        | 1.0            | 1.0           |
| pP-YBI 17 (E) | 0.00          | 0.0           | 0.00             | 0.00                                         | 206.9 ± 38.8 | 605.0 ± 90.22 | –              | 811.8 ± 84.8  |
| Fold change | –             | –             | –                | –                                           | 19.8   | 99.0       | –              | 16.9          |
| pP-YBI 30 (E) | 0.00          | 0.0           | 0.0              | 0.00                                         | 302.8 ± 49.8 | 787.3 ± 76.3 | –              | 1,090.1 ± 128.4 |
| Fold change | –             | –             | –                | –                                           | 29.0   | 128.9      | –              | 22.6          |
| Plantlets |               |               |                   |                                               |       |            |                |               |
| Wild type | 250.1 ± 29.6  | 102.7 ± 18.1  | 361.3 ± 40.8      | 213.0 ± 4.9                                   | 427.2 ± 6.5 | 103.0 ± 1.5 | 0.5            | 74.3 ± 8.5    |
| pK-YBI 41 (E) | 173.1 ± 18.2  | 66.7 ± 10.3   | 241.3 ± 37.4      | 24.7 ± 4.0                                    | 71.2 ± 7.8 | 18.1 ± 1.8 | 0.4            | 113.9 ± 25.8  |
| Fold change | 0.7           | 0.7           | 0.7              | 1.2                                          | 1.7    | 1.8        | 0.7            | 1.5           |
| pK-YBI 42 (E) | 162.4 ± 28.0  | 53.5 ± 6.4    | 137.5 ± 3.0       | 22.9 ± 5.2                                    | 74.5 ± 10.3 | 18.4 ± 4.3 | 0.3            | 115.8 ± 30.0  |
| Fold change | 0.7           | 0.5           | 0.7              | 1.1                                          | 1.7    | 1.8        | 0.6            | 1.6           |
| pP-YBI 17 (E) | 237.3 ± 41.4  | 95.3 ± 16.8   | 334.1 ± 45.5      | 20.0 ± 2.0                                    | 42.1 ± 6.4 | 11.4 ± 2.1 | 0.5            | 73.5 ± 5.4    |
| Fold change | 1.0           | 0.9           | 0.9              | 0.9                                          | 1.0    | 1.1        | 1.0            | 1.0           |
| pP-YBI 30 (E) | 255.3 ± 32.8  | 114.1 ± 27.4  | 370.2 ± 52.7      | 22.2 ± 4.0                                    | 44.7 ± 6.6 | 12.1 ± 2.1 | 0.5            | 79.0 ± 6.8    |
| Fold change | 1.0           | 1.1           | 1.0              | 1.0                                          | 1.1    | 1.2        | 1.0            | 1.1           |

β-branch xanthophylls tend to be inversely related to the levels of transcripts in the corresponding branches (Fig. 3). This might be the result of branch-specific negative feedback mechanisms, in which the compounds of one branch repress the transcripts in the other branch. The existence of such a mechanism would explain how leaves manage to maintain a constant α-xanthophyll/β-xanthophyll ratio.

In summary, overexpression of CrtI represses leaf carotenoid levels, and this phenotype is potentiated by coexpression of CrtB. Our interpretation is that the all-trans desaturation/isomerization pathway catalyzed by the CrtI enzyme competes with the endogenous poly-cis pathway catalyzed by the plant enzymes (Fig. 1; Bartley et al., 1999; Giuliano et al., 2002). Also, through an unknown mechanism, CrtI overexpression results in the repression of endogenous early genes (Fig. 3B). One possible hypothesis is that this repression is mediated by the accumulation of trace amounts of all-trans-lycopene, or of one of its degradation products, in leaves, which are undetected by the chromatographic system used here. Both the reduced synthetic flux, resulting from repression of early endogenous genes, and the competition at the enzyme level for the precursor pool result in inefficient synthesis of the final products β-carotene, β-xanthophylls, and α-xanthophylls (Fig. 3A).

Why does CrtB potentiate this effect? Our hypothesis is that, even at the low expression levels observed in pK-BI leaves, the enzyme forms a complex with CrtI and diverts, through metabolic channeling, a larger part of the geranylgeranyl diphasate pool into the all-trans pathway, resulting in greater competition with the endogenous, poly-cis desaturases (Bartley et al., 1999). Since CrtI is an enzyme of bacterial origin, it is unlikely that it forms an efficient complex with the endogenous cyclases; thus, the lycopene it forms would be less readily available to the latter enzymes, resulting in lower levels of final products in the pathway (Fig. 3A). Finally, it is possible that the carotenoids produced by the bacterial enzymes are incorrectly compartmentalized within the plastid membranes and are thus less readily assembled into pigment-protein complexes, resulting in lower overall carotenoid accumulation.

It is noteworthy that these metabolic effects are observed in spite of the fact that the levels of expression of the transgenes are low, compared with those of endogenous genes (Fig. 3B), and that the levels of the CrtI protein are undetectable by western blotting (data not shown). Possibly, higher expression levels would result in lethal bleaching of leaves and are counterselected immediately after transformation. Since CrtB potentiates the leaf-bleaching phenotype induced by CrtI, only events with low expression of both transgenes would survive, explaining why coexpression of CrtB reduces CrtI expression, as noted above.

The Golden Phenotype Requires Coordinated Expression of the Y-B-I Genes in Tubers

Carotenoid composition and gene expression in tubers were analyzed, respectively, by HPLC and
real-time RT-PCR. The detailed data are shown in Supplemental Tables S3 and S4 and summarized in Figure 4. An increase in total carotenoid content was observed in all expressor lines, with a maximum level (approximately 20-fold) in Golden (pP-YBI) tubers. In most cases, the differences in carotenoid composition of tubers overexpressing the same transgene combination under the control of the 35S or the Pat promoter (pK versus pP series) were quantitative rather than qualitative: the pK series showed more modest modifications of carotenoid content (Fig. 4A), corresponding to lower transgene expression levels in tubers (Fig. 4B). As stated above, we believe that this lower expression results from a counterselection of high-expressing lines during “in vitro” culture, expressing CrtI (Romer et al., 2000) but, in contrast to tomato fruits, results in the accumulation of β-xanthophylls rather than of β-carotene (Fig. 4A). The absence of β-carotene accumulation in CrtI tubers is probably due to the high expression of endogenous transcripts encoding β-xanthophyll synthases in this tissue (Fig. 4B), which is sufficient to convert β-carotene into downstream β-xanthophylls.

Simultaneous overexpression of CrtB and CrtI resulted in the accumulation of β-xanthophylls (Fig. 4A), but to lower levels than overexpression of CrtI alone. These lower levels are likely the result of the lower transgene expression in B-I versus I tubers (Fig. 4B; Supplemental Table S4). As stated above, we believe that this lower expression results from a counterselection of high-expressing lines during “in vitro” culture.

Figure 3. Expression of endogenous carotenoid metabolites and transcripts in the different segments of the leaf carotenoid pathway. A, Metabolite data (from Supplemental Table S1). B, Transcript data (from Supplemental Table S2). Transcript data are normalized to the housekeeping tubulin transcript. Early transcripts include PSY1, PSY2, PDS, ZDS, and CrtISO. α-Xanthophyll and β-xanthophyll synthases include LCY-e, LCY-b, LUT1, and LUT5 and LCY-b, CHY1, CHY2, LUT5, ZEP, and NXS, respectively. See also Figure 1. DW, Dry weight. [See online article for color version of this figure.]

Figure 4. Expression of endogenous carotenoid metabolites and transcripts in the different segments of the tuber carotenoid pathway. A, Metabolite data (from Supplemental Table S3). B, Transcript data (from Supplemental Table S4). Transcript data are normalized to the housekeeping tubulin transcript. Early transcripts, α-xanthophyll, and β-xanthophyll synthases are as in Figure 3. DW, Dry weight. [See online article for color version of this figure.]
due to the leaky expression of the Pat promoter in leaves. At the transcriptional level, CrtB-I overexpression resulted in a large up-regulation of α- and β-xanthophyll synthases and of NXS (Fig. 4B; Supplemental Table S4), which, however, does not result in a corresponding increase of α- and β-xanthophylls (Fig. 4A), NXS has been previously shown to catalyze the synthesis of neoxanthin in vitro (Al-Babili et al., 2000), but it is also almost identical, in its primary structure, to tomato CYC-b and to pepper (Capsicum annuum) capsanthin-capsorubin synthase, which both exhibit lycopene β-cyclase activity (Hugueney et al., 1995; Ronen et al., 2000). Therefore, it is likely that NXS is a lycopene β-cyclase and that its increased expression in tubers overexpressing CrtB-I is responsible for the lack of accumulation of lycopene (Fig. 4A; Supplemental Table S3), the product of the CrtB-I minipathway. The accumulation of β-carotene and β-xanthophylls instead of lycopene was also observed in Golden rice overexpressing PSY and CrtI in the endosperm (Ye et al., 2000) and was suggested to be the result of the activities of the downstream genes LCY-b and CHY (Schaub et al., 2005). Additionally, it is possible that potato tubers and/or rice endosperm are unable to accumulate lycopene because of the lack of specialized “sink” structures. In fact, lycopene-accumulating plastids are characterized by a different ultrastructure compared with other carotenoid-accumulating chromoplasts (Deruere et al., 1994).

The simultaneous overexpression of CrtB, CrtI, and CrtY led to the accumulation of β-carotene. However, this accumulation was relatively modest (on the order of 2 μg g⁻¹ dry weight) when CrtI and CrtY expression was driven by the 35S promoter, leading to low expression levels in tubers (Fig. 4). In contrast, expression of all three transgenes under the control of the Pat promoter control resulted in a much higher (10- to 100-fold) expression of the transgenes (Fig. 4B), in a massive (20-fold) increase of total carotenoids (Fig. 4A), and in a dramatic shift in the xanthophyll-carotene ratio, with phytoene, α-carotene, and β-carotene accounting for 50% of total carotenoids (Fig. 4A).

Antibodies raised to the CrtI proteins were used for western-blot analysis: extracts of Golden tubers show a signal with an apparent molecular mass of about 55 kD, corresponding to that of the mature CrtI protein (Supplemental Fig. S1). This implies that in Golden tubers, the CrtI protein is correctly imported into plastids. In contrast, pK-YBI tubers showed very low, or undetectable, signal. A similar lack of signal was observed in tubers and leaves of all the other lines (data not shown).

The levels of transcripts encoding carotenoid biosynthesis enzymes were modified in all transgenic tubers. (1) The most highly expressed phytoene synthase, PSY1, was down-regulated in all transgenic tubers, with the exception of pK-YBI (Supplemental Table S4). The second dedicated gene in the pathway, PDS, was up-regulated in most transgenic tubers, with the exception of Golden (pP-YBI) tubers. This regulation of endogenous early genes can be attributed to the CrtI transgene, which is expressed in all transgenic tubers. A similar repression of PSY1 and induction of PDS have been observed in tomato fruits overexpressing CrtI (Romer et al., 2000). (2) LCY-b was up-regulated in the B-I and Y-B-I series, especially in the Golden pP-YBI tubers, while the β-cyclase paralog NXS was down-regulated in the latter but induced in all other BI and YBI expressor lines (Fig. 4B; Supplemental Table S4). LCY-e was repressed in the B-I series but up-regulated in the Y-B-I series, particularly in Golden tubers (Fig. 4B). The high levels of expression of LCY-b and LCY-e in Golden tubers are consistent with the accumulation of α- and β-carotene (Fig. 4A).

Apart from the nonuniform response of CHY2, other transcripts encoding α- and β-xanthophyll synthases were strongly up-regulated in B-I tubers (Fig. 4B). Particularly CHY1, encoding a β-branch nonheme hydroxylase, was induced in all B-I and Y-B-I lines (Supplemental Table S4); P450 carotenoid hydroxylases (LUT5, LUT1) were strongly up-regulated in B-I and to a lower extent in pK-YBI tubers, while LUT1 was strongly repressed in Golden tubers (Supplemental Table S4). The induction of hydroxylases in B-I tubers and the repression of LUT1 in Golden tubers provide a rationale for the lack of β-carotene accumulation in the former and for α-carotene accumulation in the latter.

In summary, most transgene combinations induced the levels of endogenous carotenoid transcripts, with the exception of PSY1 in most tubers and LUT1 and NXS in Golden tubers. The transcriptional alterations observed (Fig. 4B) were generally consistent with the HPLC data (Fig. 4A). However, they do not explain some biochemical phenotypes, such as phytoene accumulation in Golden tubers. The simultaneous overexpression of CrtB and CrtI genes resulted in the highest deregulation/induction of endogenous transcripts, which however did not result in a comparable increase in carotenoid content. In contrast, Golden tubers show a relatively modest induction of endogenous transcripts, affecting mainly the β-branch, and a high expression level of all three transgenes (Fig. 4B). This high expression of all three transgenes, combined with a minor perturbation of endogenous gene expression, can be considered as a “golden ratio,” allowing the accumulation of high levels of β-carotene in tubers.

The data suggest that both negative and positive feedback mechanisms may be acting to influence the endogenous transcript levels in Golden tubers. The high levels of carotenoids and β-carotene contained in these tubers seem to trigger a repression of PSY1, which is likely a key gene in tuber carotenogenesis, and an induction of β-xanthophyll synthases and lycopene α-cyclase. The latter regulatory events are predicted to counteract the accumulation of high levels of carotenoids and β-carotene (Fig. 1). Thus, it is possible that, by silencing of the CHY1, CHY2, LUT5, and LCY-e genes, the levels of β-carotene in golden
tubers may increase even further. This has been experimentally confirmed for the LCY-e and CHY genes (Diretto et al., 2006, 2007b; van Eck et al., 2007).

Correlation and Network Analysis of Transcripts and Metabolites in Leaves and Tubers

To further explore relationships between gene expression and the accumulation of selected metabolites in the different transgenic lines, we used three different approaches: hierarchical clustering and correlation matrices and networks of the transcript and metabolite levels in leaves and tubers.

Hierarchical clustering (Claverie, 1999) generates a heat map, in which the genes or metabolites (rows) and transgenic lines (columns) are clustered together according to their fold changes with respect to the wild type. The clustering algorithm treats each row or column as numerical vectors and rearranges their position in the data table according to a predefined measure of similarity (Pearson distance). Different shades of red and green indicate different levels of increase or decrease, respectively (Fig. 5). In the leaf and tuber heat maps, all expressor lines containing the same transgene combination clustered together on the left side of the heat map, forming a distinct group from the cluster of nonexpressor lines and of the wild type. Golden (pP-YBI) tubers showed the largest overall perturbations and were found on the extreme left, followed by BI, pK-YBI, and pK-I tubers. Leaves of the pK lines, expressing one or more transgenes under the control of the constitutive 35S promoter, show the largest transcriptional/metabolic perturbations and cluster preferentially on the left side, away from the wild type (Fig. 5A). Leaves of the two Golden lines cluster instead very close to wild-type leaves, indicating very low levels of metabolic-transcriptional perturbation.

The Crt transgenes are coregulated in both leaf and tuber heat maps, clustering at the top in the dendrograms. In leaves (Fig. 5A), CrtI and CrtY are most tightly coregulated, in agreement with the fact that their expression is controlled by identical promoters; however, the transgene showing the closer coregulation with endogenous genes is CrtB. In tubers (Fig. 5B), all three transgenes are coregulated with each other and with three carotenoids (phytoene, α-carotene, and β-carotene), which appear de novo in Golden tubers. While the clustering of CrtB with phytoene and of CrtY with β-carotene are expected on the basis of the biochemical reactions they catalyze (Fig. 1), their clustering with α-carotene is less expected, since synthesis of the latter compound requires the combined action of β- and e-cyclase.

All leaf carotenoids and tuber xanthophylls cluster separately from the transgenes as well as from the endogenous genes, albeit some closer correlations are observed with the latter: in pK leaves, a cluster comprising early transcripts (PSY1, PSY2, CrtISO) as well as β-branch transcripts (LCY-b, NXS, CHY2) is corepressed with carotenoid metabolites (Fig. 5A). In Golden tubers, transcripts from all three biosynthetic branches (ZDS, CrtISO, LCY-b, CHY1, ZEP, LCY-e) are coinduced with xanthophylls (Fig. 5B). Some transcripts also show coregulation with each other: leaf clusters include PDS/LUT5/CHY1/LUT1, ZDS/LCY-e/ZEP, and PSY1/PSY2/NXS (Fig. 5A), while tuber clusters include PDS/CrtISO, LCY-b/CHY1 ZEP/ZDS, and LUT1/LUT5 (Fig. 5B).

We then applied pairwise correlation analysis (Steuer, 2006), a method that calculates the Pearson correlation coefficient for the fold change of all transcript-metabolite pairs in the expressor lines with respect to their nonexpressor counterparts (Fig. 6). Positive correlations are shown in different shades of red, while negative correlations are shown in different shades of blue. Besides the correlations described above, novel and significant correlations (greater than +0.60 and less than −0.60) emerge from this analysis. Leaf carotenoids positively correlate with most endogenous carotenoid transcripts (Fig. 6A), while negative correlations are observed with transgene transcripts, particularly CrtI; among endogenous transcripts, PSY1, PSY2, CrtISO, LCY-b, and CHY2 show the highest number of positive correlations to metabolites and other transcripts, thus indicating a “master switch” role of these genes. In tubers (Fig. 6B), PSY1 shows significant negative correlations to the end products of the β-branch, violaxanthin and neoxanthin, and of the α-branch, lutein. This is an indication that this transcript may be under negative feedback control by carotenoid end products. NXS shows a significant negative correlation with violaxanthin, which is the neoxanthin precursor; this is consistent with the function proposed for this gene as a neoxanthin synthase (Al-Babili et al., 2000; Bouvier et al., 2000). Besides the above correlations, Crt transgenes display the largest number of negative and positive correlations to endogenous transcripts/metabolites in both tissues: CrtI shows a remarkably high number of negative correlations in leaves (Fig. 6A), while CrtB and CrtY display a comparable number of positive correlations in tubers. As noted previously, these two transgenes show very high correlations with phytoene, α-carotene, and β-carotene, the three compounds that appear de novo in Golden tubers.

Each matrix was transformed into a correlation network to highlight potential differences in the topology and strength of the overall transcript-metabolite correlations in different tissues and transgene combinations. A correlation network is a graph depicting all-versus-all correlations in a group of variables showing quantitative variation (for review, see Albert, 2007; Krishnan et al., 2008). In this case, these variables are the fold variations in transcript-metabolite levels in transgenic expressor lines with respect to the wild type. The correlation network (Fig. 7) is composed of a number of nodes: metabolites (diamonds), endogenous genes (circles), transgenes (triangles). The color of a line, or edge, joining two nodes corresponds to a positive (red) or negative (blue) correlation, while its...
thickness represents the strength of the correlation (i.e. the absolute value of the Pearson correlation coefficient $|\rho|$ of that particular pair). The present network is defined by the following parameters (G. Giuliano, F. Scossa, and G. Diretto, unpublished data): “node strength” (ns) is the average of the absolute values of all correlation coefficients of a given node with all other nodes:

$$ns = \text{Avg}(|\rho|)$$

and “network strength” (NS) is the average of all node strengths:

$$NS = \text{Avg}(ns)$$
The sizes of the symbols in Figure 7 are proportional to the node strengths, while the absolute values of such strengths are shown in Supplemental Table S5. Network strength is high (greater than 0.70) in leaves and tubers for most transgene combinations (with the exception of the pP-I tuber network, which has a network strength of 0.59). This is perhaps not surprising, since we are dealing with a group of compounds and transcripts involved in the same metabolic pathway and thus expected to be coregulated. Additionally, network strength increases from single to triple constructs, indicating that the latter increase the overall level of coregulation between the nodes of the network (transgenes, endogenous genes, and carotenoid metabolites). However, this increase occurs with different kinetics in pK leaves and pP tubers. The major increase in network strength in leaves (from 0.72 to 0.92) is observed between pK-BI and pK-YBI plants (Fig. 7). In other terms, the addition of the CrtY transgene to the CrtB and CrtI ones brings a sizable increase of the total coregulation in the network. This increase is observed in the absence of significant changes in leaf metabolite levels (Fig. 3A); that is, it is mainly due to transcriptional perturbation.
This increase is the result of a generalized increase in the strength of all nodes (Fig. 7; Supplemental Table S5) rather than of specific ones. The colors of the edges indicate that transgenes show negative correlations, and endogenous genes show positive correlations, with most other nodes.

In contrast, the major increase in tuber network strength is observed between pP-I and pP-BI plants, while the major increase in carotenoid content is observed between pP-BI and pP-YBI tubers, in the absence of an increase in network strength. Again, this means that the addition of the CrtB transgene to the CrtI one causes a major increase of the coregulation within the tuber network (Fig. 7), and again, this is mainly due to increases in coregulation of transcript rather than metabolite levels (Fig. 4). Unlike in leaves, transgenes show mostly positive correlations with carotenoid levels, while endogenous genes show more variable trends: the PSY1 and LUT1 nodes are weak in pP-BI tubers and become very strong, with mostly negative correlations to the rest of the network, in Golden tubers. We define such strong nodes, showing a large number of either positive or negative correlations, as “hubs” (G. Giuliano, F. Scossa, and G. Diretto, unpublished data). Thus, in the Golden tuber network, the CrtB, CrtI, and CrtY transgenes are positive hubs and the PSY1, LUT1, and NXS endogenous genes are negative ones (Fig. 7).

Figure 7. Correlation networks of carotenoid transcripts and metabolites in transgenic leaves and tubers. Networks are visualized as circles, with each node representing an endogenous transcript (turquoise circles), a transgene (green triangles), or a metabolite (yellow diamonds). Lines joining the nodes represent correlations; direct correlations are shown in red, while inverse correlations are in blue. Node sizes are proportional to the respective node strengths, which are shown in Supplemental Table S5. Number of nodes (N) and network strength (NS) are shown on top of each network.
In addition to the real-time analysis of carotenoid genes, a global transcriptomic analysis of Golden tubers is under way using the Potato Oligo Chip Initiative microarray (Kloosterman et al., 2008). The preliminary results indicate transcriptional perturbations in a range of metabolic pathways (such as glycolysis, starch synthesis and mobilization, lipid biosynthesis and degradation, biosynthesis of secondary metabolites other than carotenoids), albeit the frequency of transcriptional alterations in those pathways is substantially lower than in the carotenoid pathway. As an example, we show in Supplemental Table S6 the regulation of genes in those pathways that are metabolically adjacent to the dedicated carotenoid pathway: 1-deoxy-D-xylulose 5-phosphate and mevalonate pathways, chlorophyll and gibberellin biosynthesis, biosynthesis of quinones, tocopherol, and apocarotenoids. The metabolic relations between these pathways and the carotenoid pathway are shown in Supplemental Figure S2. As can be seen, several differentially regulated genes are found in these pathways, especially in the mevalonate, tocopherol, and gibberellin pathways.

CONCLUSION

We used a combined approach based on the quantitative biochemical and molecular characterization of carotenoid metabolites and transcripts in 24 transgenic potato lines containing the CrtB, CrtI, and CrtY genes in different combinations. Our data allow a general and extensive assessment of carotenoid regulation in potato leaves and tubers in six different genetic manipulation trials. CrtI expression, alone or in combination with CrtB and CrtY, strongly interferes with leaf carotenogenesis, both at the metabolite and transcript levels. Phytoene, α-carotene, and β-carotene accumulate only in Golden tubers: at the transcript level, this phenotype can be explained by several processes: CrtY transgenic expression, which, contrary to what was observed in rice and canola, is necessary for β-carotene accumulation; down-regulation of LUT1, which possibly represses the transformation of α-carotene into lutein; and a golden ratio value in the transcription of endogenous genes. The accumulation of phytoene is common in many transgenic lines and therefore appears to be a bottleneck for further metabolism. It could be due to compartmentment of this carotenoid within the plastid, making it metabolically inert.

This study also provides additional potential targets for the rational design of further metabolic engineering experiments. Network correlation analysis showed an unexpectedly high degree of overall correlation in transcript metabolite levels, which is increased by the sequential addition of transgenes. This increase in correlation does not often correspond to major changes in metabolite levels (i.e., it is predominantly observed at the transcript levels). Transcript levels seem to "react" to the perturbation introduced by the genetic manipulations. This is, to some extent, expected: since regulatory relations between transcripts and metabolites are aimed at maintaining metabolite homeostasis, a "reaction" of the endogenous transcript network is likely to counteract an induced perturbation in metabolite abundance. Through a "guilt-by-profiling" approach (Tian et al., 2008), we have identified a series of endogenous genes showing such a regulatory behavior in Golden tubers. Besides PSY1, LCY-e, CHYs, and ZEP, which have been previously shown by us and others to be effective in modulating β-carotene and total carotenoid contents, the cytochrome P450 genes (LUT1 and LUT5) were identified as potential modulators of potato carotenogenesis. Such genes are obvious candidates for manipulations aimed at the further optimization of the carotenoid content of Golden tubers. Differential regulation of genes for general isoprenoid metabolism is also found in Golden tubers. Although this is unlikely to play a major role in carotenoid accumulation, it probably reflects a regulatory cross talk between carotenoid biosynthesis and wider isoprenoid metabolism that deserves further investigation.

MATERIALS AND METHODS

Unless indicated differently, molecular biology methods were as described (Sambrook et al., 1989). Construct design, plant transformation, and selection were described previously (Diretto et al., 2007a). Wild-type cv Desiree and CrtI transgenic rooted potato (Solanum tuberosum) plantlets were adapted in a transgenic greenhouse in pots (diameter, 25 cm) in a soil mixture composed of one-third sand and two-thirds sterile soil (Terraplant 2; BASF). Photoperiod was set at 14 h of light and 10 h of dark, with temperature set at 24°C during the light period and at 16°C during the dark period; irrigation was performed two times per week and always at the same time of the day. Three plants for each construct were grown, and a random block design strategy was used. Pooled leaf samples from three plants for each line were collected 45 d after transplantation and frozen at −80°C. During the late phase of growth, the day temperature was kept around 20°C in order to promote tuberization. During tuberization, irrigation was reduced in order to prevent tuber decay. After flowers fell and vines turned yellow (growth stage V), plants were cut and tubers were kept in the soil for 10 d in order to promote tuber hardening. Tubers from the lower two-thirds of the pot ("deep" tubers) were collected separately from superficial ones, washed in water, dried briefly at room temperature, cut in pieces, pooled, and frozen at −80°C. All carotenoid and RT-PCR measurements were conducted on at least four different deep tubers from three plants for each line (12 tubers in total) to avoid single tuber-specific variations.

HPLC assays were performed according to established protocols (Diretto et al., 2007a) using acetone-petroleum ether/diethyl ether (2:1, v/v) extraction; for leaf samples, Tris-HCl was added to the acetone phase in order to stabilize chlorophylls. All total carotenoid measurements in different lines were normalized using spectrophotometry.

RNA isolation, real-time RT-PCR conditions, and primers were described previously (Diretto et al., 2007) and were performed according to established practices (Giuliano et al., 1993; LdVardi et al., 2006). Briefly, total RNA isolation was performed using a Tris-borate/phenol-chloroform-isomyl alcohol (25:24:1)-based extraction followed by potassium acetate/lithium chloride/sodium chloride precipitation steps (Lopez-Gomez and Gomez-Lim, 1992) and DNase I purification at 37°C for 20 min. RNA purity was checked using a Nanodrop ND-1000 (Nanodrop Technologies), and only RNAs showing A260/A280 and A260/A230 ratios between 1.8/2.0 and 2.0/2.2, respectively, were selected. RNA integrity was assayed using an Agenent 2100 Bioanalyzer, and only RNAs with a 28S/18S ratio between 1.5 and 2.0 were selected. A total of 500 ng of total RNA was retrotranscribed to cDNA using a murine leukemia virus reverse transcriptase/RNase inhibitor/oligo(dT)-based
mix (Applied Biosystems). cDNA quality was assessed using two pairs of primers for a reference gene (tubulin) in order to obtain a 1.0-kb amplicon in both standard and real-time PCR conditions. Three independent biological replicates for each independent line were used, and the “threshold cycle” values were determined for each gene using a standard curve. Primer pairs for each gene were designed using the same parameters (e.g. primer temperature of 60.0°C ± 1.0°C, length of 18–25 bases, GC content between 40% and 60%) in order to generate a unique 120- to 150-bp PCR product. Normalization to a housekeeping gene (tubulin, ubiquitin, or actin) and to wild-type values was applied to raw data to obtain relative expression levels.

For western-blot analysis, 120 mg (fresh weight) of ground tuber tissue was incubated at 98°C in 150 µL of sample buffer (65 mM Tris-HCl, pH 6.75, 4% [w/v] SDS, 10% [v/v] β-mercaptoethanol, and 20% [v/v] glycerol) for 15 min. Samples were then centrifuged for 20 min at 18,000×g. Ten micrograms of supernatant was applied to SDS-PAGE (acrylamide concentration of 12%; Laemmli, 1970). Applied protein amounts were verified by Coomassie Brilliant Blue staining of a gel run in parallel. For immunodetection, anti-CrtI antibodies, raised in rabbit against CrtI expressed in Escherichia coli (kindly provided by Syngenta), were used. For detection, horseradish peroxidase-coupled secondary anti-rabbit antibodies and the ECL system (Amersham Biosciences Europe) were employed according to the manufacturer’s protocol.

Statistical analysis (one-way ANOVA plus Tukey’s pairwise comparison) was performed using the PAST software (Hammer et al., 2001). Hierarchical clustering was performed on data tables organized as follows: each row representing a gene or a metabolite, columns for the different transgenic lines, and data points showing the associated log2 fold changes with respect to the wild type. Clustering was performed using Genesis version 1.7.2 (http://genome.tugraz.at/genesicient/; Sturm et al., 2002) and calculated on both rows and columns using Pearson correlation and average linkage as agglomeration rule. Correlation matrices were built in Microsoft Excel based on the data tables as described above and were then visualized with Heatmapper plus (Bio-array resource for Arabidopsis Functional Genomics; http://www.bar.utoronto.ca/; Verhaak et al., 2006). Correlation networks were assembled manually from the corresponding matrices. Each data pair in the matrix was converted to a single line (edge) connecting two nodes. In all network diagrams, edge thickness is proportional to the absolute value of the Pearson correlation coefficient (1/ρ), while node sizes are proportional to their node strengths. Direct (ρ > 0) and inverse (ρ < 0) correlations are shown in red and blue, respectively. Different node shapes were used to distinguish endogenous transcripts from transgenes and metabolites. Networks were visualized as circle layouts with Cytoscape version 2.6.2 (www.cytoscape.org; Cline et al., 2007).

Supplemental Data

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

Supplemental Figure S1. Expression of the CrtI protein in tubers.

Supplemental Figure S2. Isoprenoid metabolism in Golden tubers.

Supplemental Table S1. HPLC analysis of leaf chlorophyll and carotenoid content.

Supplemental Table S2. Real-time RT-PCR analysis of endogenous carotenoid gene expression in leaves.

Supplemental Table S3. HPLC analysis of tuber carotenoid content.

Supplemental Table S4. Real-time RT-PCR analysis of endogenous carotenoid gene expression in tubers.

Supplemental Table S5. Node strengths of the network shown in Figure 7.

Supplemental Table S6. Expression in Golden tubers of isoprenoid metabolism genes.

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