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Sucrose-Specific Induction of the Anthocyanin Biosynthetic Pathway in *Arabidopsis thaliana*

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Abstract
Sugars act as signalling molecules, whose signal transduction pathways may lead to the activation or inactivation of gene expression. Whole-genome transcript profiling reveals that the flavonoid and anthocyanin biosynthetic pathways are strongly up-regulated following sucrose treatment. Besides mRNA accumulation, sucrose affects both flavonoid and anthocyanin contents. We investigated the effects of sugars (sucrose, glucose and fructose) on genes coding for flavonoid and anthocyanin biosynthetic enzymes in Arabidopsis thaliana. The results indicate that the sugar-dependent up-regulation of the anthocyanin synthesis pathway is sucrose-specific. An altered induction of several anthocyanin biosynthetic genes, consistent with in vivo sugar modulation of mRNA accumulation, is observed in the pgm (PHOSPHOGLUCOMUTASE) Arabidopsis mutant accumulating high levels of soluble sugars.
INTRODUCTION

Anthocyanins are plant secondary metabolites playing a key role as flower pigments, in signalling between plants and microbes, in responses related to nutrient availability, in male fertility of some species, in defence as antimicrobial agents and feeding deterrents, in the modulation of auxin transport, and in UV protection (Winkel-Shirley, 2001).

The anthocyanin biosynthetic pathway was described in different plants (Holton and Cornish, 1995), including *Arabidopsis Thaliana* (Shirley et al., 1995; Bharti and Khurana, 1997), and several transcription factors regulating the anthocyanin biosynthetic pathway have been identified (Nesi et al., 2000; Vom Endt et al., 2002; Davies and Schwinn, 2003; Mathews et al., 2003; Matsui et al., 2004; Broun, 2004; Park et al., 2004).

The interrelationships between developmental, environmental, and metabolic signal transduction pathways control the production of flavonoids. Anthocyanin biosynthesis was often observed in plants germinated or grown on a sugar-containing medium (Mita et al., 1997; Németh et al., 1998; Baier et al., 2004). The chalcone synthase (*CHS*) gene derived from *Petunia Hybrida* petals in transgenic Arabidopsis leaves was induced by sugars (Tsukaya et al., 1991), and Petunia corollas cultured in vitro without sucrose do not show any pigmentation (Weiss, 2000). Petunia and Arabidopsis *CHS* genes are, indeed, characterized by the presence of ‘sucrose boxes’ in the 5’ flanking regions that may be also found in the sucrose-inducible sporamin and amylase genes (Tsukaya et al., 1991). Arabidopsis grown on a sucrose-containing medium shows high levels of anthocyanins (Tsukaya et al., 1991; Ohto et al., 2001).

Genes coding for dihydroflavonol reductase (*DFR*) and anthocyanidin synthase (*ANS*), also known as leucoanthocyanidin dioxygenase (*LDOX*), were up-regulated and the accumulation of anthocyanins was strongly increased by sucrose in *Vitis Vinifera* cells (Gollop et al., 2001; Gollop et al., 2002), and signal transducers, such as Ca\(^{2+}\) and protein kinases/phosphatases were shown to be involved in this process (Vitrac et al., 2000).

The Arabidopsis *pho3* mutant, which has a defective copy of the sucrose transporter 2 (*SUC2*) gene (encoding a phloem-loading sucrose-proton symporter) leading to accumulation of soluble sugars and starch, showed growth retardation and anthocyanin accumulation (Lloyd and Zakhleniuk, 2004). The microarray analysis of *pho3* adult leaves evidenced an enhanced expression of PRODUCTION OF ANTHOCYANIN PIGMENT 1 (*PAP1*), PRODUCTION OF ANTHOCYANIN PIGMENT 2 (*PAP2*), and TRANSPARENT TESTA 8 (*TT8*) transcription factors, as well as of genes coding for anthocyanin biosynthesis enzymes, suggesting that sugars are in vivo triggers of the anthocyanin biosynthesis (Lloyd and Zakhleniuk, 2004).
It is presently unknown whether sugars co-ordinately induce most of the genes involved in the anthocyanin biosynthesis or if only a few genes play a pivotal role, and little is known about the sugar specificity for the anthocyanin biosynthesis induction in Arabidopsis. In this paper, we show evidence of the co-ordinated, sucrose-specific modulation of most of the genes involved in the anthocyanin biosynthesis. Furthermore, induction of several anthocyanin biosynthetic genes in the *pgm* (PHOSPHOGLUCOMUTASE) Arabidopsis mutant accumulating high levels of soluble sugars is described.
RESULTS

Flavonols and Anthocyanins are Inversely Modulated by Sucrose

We analysed the anthocyanin and flavonol content in Arabidopsis seedlings grown in the absence or presence of exogenous sucrose. The histochemical analysis of flavonols (Fig. 1) shows the fluorescence that is characteristic of chlorophyll (red fluorescence), along with a limited orange fluorescence, which is typical of quercetin in cotyledons (Fig. 1A). The upper hypocotyl area exhibits a strong bright yellow fluorescence (chalcone-naringenin, Fig. 1C). Whereas sucrose does not affect the presence and distribution of flavonols in the roots (cfr. Fig. 1G, I with Fig. 1H, L), this disaccharide leads to a decreased presence of flavonols (mainly represented by chalcone-naringenin) in the hypocotyl and cotyledons (Fig. 1D and B). Interestingly, these tissues coincide with the anthocyanin accumulation site following sucrose feeding of Arabidopsis seedlings (Fig. 1F).

Sucrose Affects the mRNA Level in Anthocyanin Biosynthetic Pathway Genes

To identify the genes involved in the flavonol and anthocyanin biosynthesis that are regulated by sucrose, we performed a microarray experiment with seedlings treated with sucrose, compared to control seedlings. The rationale behind this experiment is that, besides the known genes involved in this pathway, there appears to be some gene redundancy for this cluster of genes (e.g. four 4-coumarate:CoA ligase (4CL) genes and six 4CL-like genes are represented in the Arabidopsis genome, http://www.arabidopsis.org/). The results of the microarray experiment are summarized in Figure 2. Interestingly, at least one gene up-regulated by sucrose was detected in each step of the biosynthetic pathway, with the exception of the flavonoid 3’5’-hydroxylase (F3’5’H), which is expressed at a very low level. This genome-wide overview of the effects of sucrose on the genes involved, or putatively involved, in the flavonoid and anthocyanin biosynthesis allowed us to select the genes to be further characterized in their response to sugar. One sucrose-induced gene was selected (AGI codes marked with a red triangle in Fig. 2) for each biosynthetic step, giving preference to well-characterized genes when more than one gene was up-regulated by sucrose (e.g. the TRANSPARENT TESTA 5 gene (TT5) corresponding to chalcone isomerase (CHI) was chosen among three sucrose-induced CHI genes). As far as the transcription factors are involved in the regulation of the anthocyanin synthesis pathway, the PAPI (At1g56650) transcript was 29-fold up-regulated by sucrose, whereas PAP2 (At1g66390), TT8 (At4g09820), TRANSPARENT TESTA 2
(TT2, At5g35550), ANTHOCYANINLESS 2 (ANL2, At4g00730) and MYB family transcription factor 4 (AtMYB4, At4g38620) mRNA level were unaffected by the treatment with sucrose, or the induction of sucrose was not confirmed by the biological replicate (TT8, ANL2) (see supplemental Table I).

We analysed the pattern of mRNA accumulation of the selected transcripts coding for proteins involved in the flavonoid biosynthesis pathway, selected on the basis of the microarray experiment results (Fig. 2). The results indicate that the mRNA level of several genes increases after the treatment with sucrose (Fig. 3A). The induction is particularly evident for those genes coding for enzymes that act at the level and downstream of CHS, namely CHS, CHI, flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), flavonol synthase (FLS), DFR, LDOX and UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT). The cinnamate 4-hydroxylase (C4H) mRNA level is barely affected by sucrose, whereas a transient induction of phenylalanine ammonia-lyase (PAL) by sucrose is observed (Fig. 3A). The 4CL mRNA level was below the detection threshold.

To evaluate whether the observed effects of sucrose could be ascribed to an osmotic effect, we verified the effects of mannitol on some of the genes studied. Arabidopsis seedlings were treated with sucrose (Fig. 3B, “Suc”), mannitol (Fig. 3B, “Man”), pre-treated mannitol (24h) followed by sucrose (Fig. 3B, “Man • Suc”), and pre-treated mannitol (24h) followed by mannitol (Fig. 3B, “Man • Man”). The expression of several genes involved in the flavonoid/anthocyanin biosynthesis was analysed, and the mRNA level of protein kinase 1 (KIN1), a stress-induced gene, was monitored (Kurkela and Franck, 1990). Genes involved in the flavonoid/anthocyanin synthesis were induced by sucrose. The expression of KIN1 is transient and strongly influenced by mannitol, whereas sucrose is unable to strongly induce this stress-related gene. A pre-treatment with mannitol (Fig. 3B, “Man • Suc”) mitigates the perception of stress, leading to an increase in the level of KIN1 mRNA (cfr. Fig. 3B “Man” with Fig. 3B “Man • Man”). This effect is not observed in the seedlings, which were pre-treated with mannitol and subsequently exposed to sucrose (Fig. 3B, “Man • Suc”): the sucrose-induction of flavonoid/anthocyanin genes is retained and is indistinguishable from the sucrose-alone treatment (cfr. Fig. 3B “Suc”), ruling out a stress response, such as the triggering of flavonoid gene induction by sucrose. These results suggest that the induction of flavonoid/anthocyanin synthesis genes is sugar-specific and unlikely to be stress-mediated.

A Specific Sucrose-Signalling Mechanism Requiring Low Sugar Concentrations Induces Anthocyanin Synthesis Genes
We investigated the sucrose specificity of the anthocyanin biosynthesis by testing the effects of a set of metabolic sugars (sucrose, glucose, fructose, as well as a 1:1 mixture of glucose + fructose) and non-metabolic sugars (sucrose analogues: turanose and palatinose). The anthocyanin synthesis induction is sucrose-specific, with a strong accumulation of these pigments in sucrose-treated seedlings only (Fig. 4A). The mRNA accumulation of transcripts related to flavonoid/anthocyanin synthesis is high in sucrose-enriched media, whereas neither turanose nor palatinose were able to induce flavonoid/anthocyanin genes (data not shown). The threshold for the induction of the genes involved in the flavonoid/anthocyanin synthesis was investigated by testing different sucrose concentrations. Three-day old seedlings were treated with sucrose concentrations ranging from 7.5 to 90 mM for 24h and the results show that 15 mM of sucrose are enough to enhance anthocyanin levels (Fig. 4B), although higher sucrose concentrations lead to a more marked anthocyanin accumulation, reaching a plateau between 60 and 90 mM. The mRNA levels of CHS, CHI and F3H readily increase when seedlings are treated with 7.5 mM of sucrose (Fig. 4C). Increasing the sucrose concentration up to 15 mM results in a increased mRNA level of F3’H, FLS, and LDOX, whereas 30-60 mM are required to significantly increase the mRNA level of C4H (Fig. 4C).

Northern analysis provides a semi-quantitative profile of expression, and a more accurate quantitation can be obtained by means of Real-Time RT PCR. Three-day old, light-grown, seedlings were fed for 12 h with Suc, Glc or Fru in concentrations ranging from 7.5 mM to 90 mM. The Real-Time RT PCR results indicate that sucrose is the most efficient trigger of mRNA accumulation for genes, whose products act downstream along the anthocyanin biosynthetic pathway (DFR, LDOX, UF3GT), as well as for PAP1 (Fig. 5). These genes are induced by sucrose several hundred folds, whereas genes upstream of DFR show a lower induction by sucrose, and can also be induced by glucose and, to a minor extent, by fructose (Fig. 5).

**In Vivo Sugar Modulation of Flavonoid and Anthocyanin Synthesis Genes**

The *pgm* Arabidopsis mutant has a defect in the plastidial PHOSPHOGLUCOMUTASE gene hampering starch synthesis in the chloroplasts. Therefore, the mutant is starch-less, but accumulates high levels of soluble sugars as a consequence of its photosynthetic activity during the day (Caspar *et al.*, 1985). Assuming that the experiments performed by treating seedlings with exogenous sugar reflect the ability of the plant to sense the endogenous sugars level, one would expect that the *pgm* mutant would show a sugar-controlled induction of genes, as soon as the endogenous sugar level increases beyond a certain threshold. Leaves were collected from plants at the rosette stage before
beginning the light treatment (0h), after five hours under light (5h) and after ten hours under light (10h). The pgm mutant exhibits a clear increase in its sucrose and glucose levels (up to 20 folds), whereas the wild-type shows a lower diurnal fluctuation in sucrose and glucose levels (Fig. 6A). The level of fructose increases almost equally in both the wild-type and pgm mutant (Fig. 6A). The highest sugar level was measured in the leaves of the pgm mutant, 10h after the beginning of the light treatment, with a measured concentration of about 10 mM soluble sugars (sucrose+glucose+fructose). The expression of several of the flavonoid and anthocyanin synthesis genes increased about ten to twenty folds after the 10h light treatment in the pgm leaves but not in the wild-type leaves (Fig. 6B), suggesting that the in vivo expression of PAP1, 4CL, CHS, CHI, F3H, DFR and LDOX is modulated by the rapid increase in sugar concentration observed in the pgm mutant (Fig. 6A). To confirm that the increases observed in the mRNA level of several of the genes studied is indeed the consequence of in vivo sugar sensing, we used the expression of the ApL3 gene, known to be sugar-modulated (Sokolov et al. 1998), as a marker of sugar-signalling occurring in leaves. The ApL3 mRNA level increases in response to sucrose, with a limited induction by glucose and fructose (Fig. 6C, heatmap), and is induced in vivo only in the pgm mutant (Fig. 6C, pgm, time: 10h).

The comparison of the response of flavonoid and anthocyanin synthesis genes to low sugar concentration (7.5 mM; see Fig. 5) with their induction in the pgm mutant (Fig. 6B) indicates that the genes, which do not show any induction in the pgm mutant (PAL, C4H, FLS, F3’H and UF3GT), are those showing a lower induction by the lowest sugar concentration tested (7.5 mM, Fig. 5). When the induction observed in pgm is plotted against the induction by 7.5 mM sugars (data from Fig. 5), it is possible to observe that all the genes lacking induction in the pgm mutant are grouped together, and are those showing a lower induction by 7.5 mM exogenous sugars (Fig. 6D, red dots).
DISCUSSION

The expression of anthocyanin biosynthetic genes in grape berry skin appears to be highly coordinated during berry development (Boss et al., 1996), and the expression of grape DFR is responsive to sucrose (Gollop et al., 2002). Since sugars accumulate during grape berry development (Boss et al., 1996), it is tempting to speculate that sugars are endogenous triggers modulating the expression of anthocyanin biosynthetic genes, possibly through the involvement of sugar-modulated regulatory genes. The ectopic expression of the transcription factor PAP1 (also AtMYB75) and of the related gene PAP2 results in an enhanced expression of the flavonoid biosynthetic genes PAL, CHS and DFR (Borevits et al., 2000). The DFR expression is also under the control of TT2, interacting with TT8 (Nesi, et al. 2001). Furthermore, the functional TRANSPARENT TESTA GLABROUS 1 (TTG1), encoding a WD40 repeat protein, is required for the normal expression of the DFR anthocyanin gene (Walker et al., 1999; ). AtMYB4 is another player in the biosynthesis of anthocyanins down-regulating C4H and 4CL genes (Jin et al., 2000), and ANTHOCYANINLESS 2 (ANL2) is involved in the accumulation of anthocyanins in sub-epidermal tissues (Kubo et al., 1999).

Our results suggest that sugars act as signalling molecules, activating the PAP1 gene by means of a sucrose-specific signalling pathway. This is supported by the following experimental evidences: (i) only PAP1 mRNA level was strongly sucrose-inducible, as previously suggested by Kranz et al. (1998), whereas other players involved in the anthocyanin biosynthesis regulation were unaffected by the treatment with sucrose (see Supplemental Table I); (ii) sucrose, but neither glucose nor fructose, increases the mRNA level of PAP1 and of anthocyanin biosynthetic genes (Fig. 5, DFR, LDOX, UF3GT), and triggers an increased anthocyanin synthesis (Fig. 4A); (iii) the effects of sucrose on PAP1 mRNA level are unrelated to a generic osmotic response (Fig. 3B). We cannot, however, rule out the possibility of an involvement of other regulatory genes, since the sugar-response expression pattern (Fig. 5) reveals that the response of genes upstream of DFR is clearly distinct from the response of PAP1.

A metabolite could control a pathway through selected steps. Regarding sugar regulation of the anthocyanin pathway, the effect is likely achieved through the last few steps, which are the most sensitive to sugar and show selective response to sucrose (Fig. 5). Only sucrose can elicit a clear increase in the anthocyanin content of Arabidopsis seedlings (Fig. 4A), and this is likely due to the induction, that is highly specific for sucrose, of DFR, LDOX, and UF3GT (Fig. 5). This correlates nicely with the disappearance of naringenin chalcone from the seedling hypocotyl, and its replacement with anthocyanins (Fig. 1). Gollop et al. (2001; 2002) reported that the induction of
anthocyanin biosynthesis genes by sugars is rather unspecific, and glucose, fructose and sucrose treatments result in the induction of DFR and ANS (LDOX) in grape. The sugar-sensing mechanisms operating in grape are poorly studied, and it is reasonable to assume that they might be distinct from the sugar-sensing mechanisms active in Arabidopsis. Seedlings younger than 2-d show some degree of sensing unspecificity, and also glucose is able to induce DFR, LDOX, and UF3GT (data not shown), thought not with the hundred-fold induction observed in the 3-d-old seedlings used in our experiments. Furthermore, it is worth outlining that the effects of glucose treatments longer then 12h partly mirror the effects obtained with sucrose, and feeding Arabidopsis seedlings with radiolabeled glucose reveals that this monosaccharide is readily converted into sucrose within few hours (data not shown).

As low as 7.5 mM sucrose is enough to induce the mRNA accumulation of most of the genes studied, indicating that the sensing mechanism is compatible with physiological sucrose concentrations, and is likely to be operating in vivo. The expression of PAP1 shows a characteristic fluctuation during dark/light periods, the higher expression being reached around midday (Harmer et al., 2000), which is confirmed by our analysis of wild-type Arabidopsis leaves (Fig. 6B, PAP1; also observed in the Benshime ecotype, data not shown). The increase in sugar content during the light treatment peaks at 5h in the wild-type, and it is tempting to speculate that the PAP1 expression is sugar-modulated in vivo. The expression of most of the genes involved in the flavonoid and anthocyanin biosynthesis is, however, unaffected in wild-type Arabidopsis leaves during the light treatment, a likely consequence of the limited fluctuation in the leaf content of sucrose and glucose (Fig. 6A). The level of soluble sugars in wild-type Arabidopsis leaves increases from about 1 mM at time=0h to a maximum of 6 mM (reached at time=5h), whereas the pgm mutant shows a rise in soluble sugars from the very low level of 0.4 mM (time=0) to a maximum of 10 mM (time=10h). These values are compatible with the increase observed in the mRNA level of several genes in the pgm mutant after 10h of growth under light (Fig. 6B), as well as with their threshold for induction by sugars (Fig. 5). Indeed, the induction of genes in the pgm mutant correlates with gene sensitivity to exogenous sugars (Fig. 6D), and the pattern of expression of the ApL3 gene, known to be sugar-modulated, mirrors that of anthocyanin-related genes, by showing an up-regulation in the pgm mutant (Fig. 6B-C). The suggestion that sugars play a role in the modulation of the anthocyanin synthesis pathway in leaves is consistent with the microarray analysis of pho3 adult leaves accumulating high levels of soluble sugars and showing higher levels of expression of genes coding for anthocyanin biosynthesis enzymes (Lloyd and Zakhleniuk, 2004); these genes exhibit a very low expression level in sugar-depleted pgm leaves at the end of the night (Thimm et al. 2004).
The description of the anthocyanin biosynthesis - as specifically responsive to sucrose acting as a signalling molecule - allows to include this physiological process among the other sucrose-specific processes that have been described up to now (Dijkwel et al. 1996; Smeekens and Rook, 1997; Chiou and Bush, 1998; Lalonde et al., 1999; Rolland et al., 2002; Vaughn et al., 2002; Koch, 2004). Furthermore, these results provide evidence for the occurrence of an in vivo sucrose-sensing mechanism, which modulates the anthocyanin biosynthesis in Arabidopsis Thaliana. Further work is needed to elucidate the molecular basis of this process, and the possible interactions with the hormonal signalling network.
MATERIALS AND METHODS

Plant and Growth Condition

Arabidopsis thaliana seeds were sterilized for 7 min in 1.7% (v/v) bleach solution, incubated overnight in 4% PPM (Plant Preservative Mixture, Plant Cell Technology, Washington DC, USA). PPM contains two isothiazolone class biocides, namely methylchloroisothiazolinone and methylisothiazolinone (Paul et al., 2001) in full strength sterilised Murashige-Skoog (MS) salt solution with gentle shaking, abundantly rinsed in sterile water and transferred into 2.5 ml liquid growing media (MS half strength solution +/- sugars) with 0.05% PPM in 6-well plates. Plates were incubated in the darkness at 4°C for 2 days and finally transferred to continuous light (90µm photons/ m²) with gentle swirling for experiment-time in a plant growth chamber at 22°C. Treatments were performed by adding sugar solution to selected wells and water to the control wells.

Probe Design and Preparation

PCR primers were designed to amplify the most specific region inside the Affymetrix target region (sequence alignment was checked by the free web interfaced software ClustalW http://www.ebi.ac.uk/clustalw/). For the design of the primers we used the free web interfaced software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Oligonucleotides were purchased from MWG-Biotech (High Point, NC). Primer sequences for each gene are listed in the Supplemental Table II. PolyA(+)RNA was purified by Oligotex (Qiagen, Valencia, CA, USA) from total RNA extracted from 4 days old Arabidopsis seedling incubated for 6 hours in the presence of 90 mM sucrose. About 150 ng of purified polyA (+) RNA was reverse transcribed with random primers by Improm-II (Promega, Madison WI, USA) for 1 hour at 42°C. PCR amplification on 15 ng cDNA (or 150 ng genomic DNA for intronless probed region) was performed with 400 nM specific primers and 2X PCR MIX (Promega, Madison WI, USA). PCR condition were as follow: 94°C for 2 min, 30 PCR cycles - 94°C 45 sec, primers annealing temperature (see Supplemental Table II) 45 sec, 72°C for 45 sec with a final extension of 8 min at 72°C.

RNA Isolation and Gel Blots
RNA extraction was performed by using the aurantricarboxylic acid method as previously described (Perata et al., 1997). The amount of total RNA loaded per lane for electrophoresis was 20 µg. RNA was electrophoresed on 1% (w/v) agarose glyoxal gels, and blotted on nylon membrane (BrightStar-Plus®, Ambion, Austin, Texas USA) by using the procedure suggested by the manufacturer. Membranes were prehybridized and hybridized using the NorthernMax-Gly® kit (Ambion, Austin, Texas USA). Radiolabeled probes were prepared from gel-purified cDNAs by random primer labeling (Takara Chemicals, Shiga, Japan) with \([\alpha^{32}P]-dCTP\). Equal loading was checked by reprobing with an rRNA cDNA probe (not shown). RNA blots were scanned using a Cyclone Phosphoimager (Packard Bioscience, Perkin Elmer, Foster City, CA, USA). mRNA level was quantified using the Optiquant software (Packard Bioscience, Perkin Elmer, Foster City, CA, USA).

**RNA Isolation, cRNA Synthesis and Hybridization to Affymetrix Genechips**

Total RNA was extracted from the seedlings samples, using the Ambion RNAqueous kit (Ambion, Austin, Texas USA). RNA quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on Affymetrix Arabidopsis ATH1 GeneChip arrays as previously described (Loreti et al., 2005). Hybridization, washing, staining, and scanning procedures were performed by Biopolio (University of Milano Bicocca, Italy) as described in the Affymetrix technical manual. Expression analysis via the Affymetrix Microarray Suite software (version 5.0) (Affymetrix Inc., Santa Clara, CA ) was performed with standard parameters. Two independent, replicated experiments were performed for each experimental condition, and the output of the Affymetrix Microarray Suite software for each independent experiment was subjected to further analysis by using Microsoft Excel (Microsoft, Redmond, WA, USA). Signal values (indicating the relative abundance of a particular transcript) and detection call values (indicating the probability that a particular transcript is present) were generated by Microarray Analysis Suite 5.0 software (Affymetrix Inc., Santa Clara, CA). Probe pair sets (genes) called "Absent" were removed from subsequent analyses. Furthermore, genes with “Absent” for the detection value in the baseline data and “Decrease” for the change call were excluded from the list. Similarly, genes with “Absent” for the detection call in the experimental data and “Increase” for the Change value were also excluded from the list. Differences in transcript abundance, expressed as signal log ratio, were calculated using the Microarray Analysis Suite 5.0 software change algorithm. Signal log ratio was assumed to be correct only if the corresponding “change call” indicated a significant change (“I”: Increase; “D”: Decrease, generated by Microarray Analysis Suite 5.0 software). Expression data
were filtered to select only genes showing a coinciding change-call in the two biological replicates samples for each experimental condition.

**Real Time RT- PCR**

RNA was extracted from seedlings grown on MS 0.5X solution (control) or in the same medium supplemented with 90 mM sugars as indicated in Figure legends. Total RNA, extracted with the RNAqueous kit (Ambion, Austin, Texas, USA) according to the manufacturer’s instruction, was subjected to a DNase treatment using the TURBO DNA free Kit (Ambion). Two micrograms of each sample were reverse transcribed into cDNA with the “High capacity cDNA archive kit” (Applied Biosystems, Foster City, CA, USA). RealTime PCR amplification was carried out with the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, USA), using the primers described in Supplemental Table III. ubiquitin10 (UBQ10) was used as endogenous control. Taqman probes specific for each gene were used. Probe sequences are reported in Supplemental Table III. PCR reactions were carried out using 50 ng of cDNA and “TaqMan Universal PCR Master Mix” (Applied Biosystems) following the manufacturer’s protocol. Relative quantitation of each single gene expression was performed using the comparative CT method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems).

**Anthocyanins Quantitation**

Arabidopsis seedling extraction was performed as described by Ronchi et al. (1997) with minor modifications. In brief, seedlings were grinded in 1 volume HCl 1% (v/v) in methanol with the addition of 2/3 volume of distilled water. Extracts were recovered and 1 volume of chloroform was added to remove chlorophylls through mixing and centrifugation (1 min at 14000 g). Anthocyanins containing aqueous phase was recovered and absorption was determined spectrophotometrically (A535 nm). Mean values were obtained from three independent replicates.

**Flavonoids Staining**

Flavonoids staining was performed as described by Peer et al. (2001). Three-days old Arabidopsis seedlings, treated for 72 hrs with 90mM sucrose or minimal MS medium (control), were stained for 5 to 15 min using saturated (0.25%, w/v) diphenylboric acid-2-aminoethyl ester (DPBA) with 0.005% Triton X-100 and were visualized with an epifluorescence microscope equipped with an
fluorescein isothiocyanate FITC filter (excitation 450–490 nm, suppression LP 515 nm). Photographs of seedlings were taken using colour slide film (Kodak Elite, ASA 400, Kodak, Rochester, NY) after 5 min of staining.

Sugar Analysis

Samples were rapidly frozen in liquid nitrogen and ground to a powder. Samples were then extracted and assayed by coupled enzymatic assay methods measuring the increase in $A_{340}$ as described by Guglielminetti et al. (1995).

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LITERATURE CITED


FIGURE LEGENDS

Figure 1. Effect of sucrose on flavonoid and anthocyanin content and distribution in Arabidopsis seedlings. A-D, G-L effects of exogenous sucrose on flavonoid content and distribution by DPBA staining of 3-days old Arabidopsis seedlings, viewed through an FITC filter. When reacting with flavonoids, DPBA emits orange fluorescence (quercetin), bright yellow fluorescence (naringenin-chalcone) and bright green fluorescence (kaempferol); chlorophylls exhibit a red autofluorescence. A,C,G and I: histochemical results of 3d-old seedlings grown for additional 72h on control medium; the cotyledons (shown in panel A) display the fluorescence characteristic of quercetin; the cotyledonary node (shown in panel C) show the bright yellow fluorescence of chalcone-naringenin; the root (shown in panel G) contains mostly chalcone-naringenin and the root tip (shown in panel I) contains mostly kaempferol. B,D,H and L: 3d-old seedlings grown for additional 72 h on 90 mM sucrose. Seedlings show a reduced bright yellow fluorescence and the chlorophyll red fluorescence in cotyledons (shown in panel B). The cotyledonary node (shown in panel D) shows only the chlorophyll red fluorescence. Sucrose does not affect the flavonols presence and distribution in the roots (cfr. Panels G, I and H,L). E-F. Anthocyanin accumulation in Arabidopsis seedlings grown on control medium (shown in panel E) or grown for additional 72 h on 90 mM sucrose (shown in panel F). Bar in panel A, B, C, D, G, H, I and L = 500 µm; bar in panel E and F = 200 µm.

Figure 2. Effects of sucrose on the mRNA accumulation for genes coding for flavonoid and anthocyanin biosynthetic enzymes in Arabidopsis seedlings. Arabidopsis seeds were germinated for 3 days and subsequently treated without (C) or with (S) 90 mM sucrose for 6h. Genes coding for enzymes involved in the flavonoid and anthocyanin pathways were identified by searching the Arabidopsis annotation in TAIR (http://www.arabidopsis.org/). Microarray data (averaged transcript level from two biological replicates) were visualised using the Heatmapper Plus software (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi ).

The output of the software is shown, with the genes involved in each metabolic step represented by their respective AGI codes. A black square (and black shades) indicates a gene whose transcript level is low. A yellow square (and yellow shades) indicates a gene whose transcript level is relatively high within the group of genes putatively coding for the same function. AGI code marked with a red triangle indicate the genes chosen for further studies.

Arabidopsis mutants have been isolated on the basis of modified seed pigmentation and are therefore known as tt (for transparent testa) mutants (Koornneef, 1981, 1990) and TT loci identified have been characterized. When available, the TT gene codes are reported in figure.
Figure 3. Pattern of mRNA accumulation for genes coding for flavonoid and anthocyanin biosynthesis enzymes in Arabidopsis seedlings (A). Three days-old Arabidopsis seedlings were grown for additional 0 to 96h on a Murashige and Skoog medium (Control) or on a sucrose-enriched medium (Sucrose). RNA was extracted, electrophoresed, and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (data not shown). A representative experiment is shown. Evaluation of the osmotic effect on the mRNA level of several anthocyanins biosynthetic genes and on the stress-induced KIN1 gene (B). Three-days old Arabidopsis seedlings were treated for 0 to 120h with sucrose (Suc), mannitol (Man), pre-treated (24h) with mannitol followed by sucrose for 0 to 120h (Man → Suc), pre-treated (24h) with mannitol followed by mannitol for 0 to 120h (Man → Man). RNA was extracted, electrophoresed, and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (data not shown). A representative experiment is shown.

Figure 4. A. Effects of a set of metabolic sugars (Sucrose 90 mM, Glucose 90 mM, Fructose 90 mM, 1:1 mixture of Glucose 45 mM + Fructose 45 mM) and of non metabolic sugars (Turanose and Palatinose, 90 mM) on anthocyanin accumulation. B. Effect sucrose concentrations ranging from 7.5 to 90 mM on anthocyanin accumulation. C. mRNA accumulation for genes coding for flavonoids and anthocyanin biosynthesis enzymes in Arabidopsis seedlings. Seedlings were sugar-treated for 48h (A) or 24h (B-C). RNA was extracted, electrophoresed, and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (not shown). A representative experiment is shown.

Figure 5. Effect of different metabolic sugars on the expression of genes involved in flavonoid/anthocyanin synthesis. Three days old Arabidopsis seedlings were grown for 12h on a MS standard medium (Control) or standard medium supplemented with sucrose, glucose, fructose, at concentrations ranging from 7.5 to 90 mM. mRNA accumulation has been analysed by Real-time RT-PCR. Data (averaged transcript level from two biological replicates) were visualised using the Heatmapper Plus software (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi). Each treatment is represented by a row of coloured boxes, and each sugar concentration is represented by a single column. Data are expressed as fold change (1=control). Effects of sugars on gene expression range from pale to saturated yellow. Black indicates no change in gene expression. The value for image contrast was set to “automatic” for inductions lower than 500 fold, while it was fixed at 500 for induction higher than 500 fold.
Figure 6. Expression of flavonoid and anthocyanin synthesis genes in leaves of Arabidopsis WT and pgm mutant. Leaves collected from plants at the rosette stage before the beginning of the light treatment (0h), after five hours under light (5h) and after ten hours under light (10h). The results are mean ± SD (n=3). A. Changes in sugar concentration in leaves collected from WT and pgm mutant plants. Sucrose, glucose and fructose were quantified and changes in their amount was calculated (fold change 1=Time 0h). B. mRNA accumulation was analysed by Real-time RT-PCR and changes (fold change) in their amount was calculated (fold change 1=Time 0h). C. Effect of sugars on ApL3 mRNA level. Arabidopsis seedlings were grown and mRNA analysed by Real-time RT-PCR as described in Figure 5. Data are expressed as fold change (fold change 1=control). Effects of sugars on gene expression range from pale to saturated yellow (heatmap; see Fig. 5 legend for details). Black indicates no change in gene expression. The value for image contrast was set to “automatic”. The histogram shows ApL3 mRNA accumulation in leaves of Arabidopsis WT and pgm mutant. mRNA was analysed by Real-time RT-PCR and changes (fold change) in their amount was calculated (fold change 1=Time 0h). D. Scatter plot showing the induction observed in pgm (data from Fig. 6 B-C) plotted against the induction by 7.5 mM sugars (data from Fig. 5). Red dots identify PAL, C4H, FLS, F3’H, and UF3GT. The blue dot identifies ApL3. Black dots identify PAP1, 4CL, CHS, CHI, F3H, DFR, and LDOX.
Figure 3
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<td>6.5</td>
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<tr>
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<td>5.1</td>
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</tr>
<tr>
<td>Fru</td>
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<td>3</td>
<td>5.4</td>
<td>6</td>
<td>7.7</td>
</tr>
</tbody>
</table>

**PAL**

| Suc | 1 | 1.6 | 1.4 | 1.7 | 2.1 |
| Gln | 1 | 1.4 | 1.6 | 2.5 | 2.2 |
| Fru | 1 | 1.6 | 1.9 | 2.7 | 2.1 |

**C4H**

| Suc | 1 | 6.6 | 7.0 | 5.9 | 15.0 |
| Gln | 1 | 9.8 | 12.5 | 14.9 | 9.7 |
| Fru | 1 | 0.9 | 1.2 | 1.8 | 1.8 |

**4CL**

| Suc | 1 | 14.9 | 21.9 | 22.8 | 27.2 |
| Gln | 1 | 15.6 | 28.3 | 36.8 | 37.8 |
| Fru | 1 | 4 | 5.5 | 8.5 | 8.3 |

**CHS**

| Suc | 1 | 6.2 | 7.1 | 6.4 | 6.8 |
| Gln | 1 | 5.8 | 6.7 | 9.5 | 7.9 |
| Fru | 1 | 3.7 | 4 | 3.6 | 3.8 |

**CHI**

| Suc | 1 | 7.2 | 24.1 | 25.8 | 31.1 |
| Gln | 1 | 5.6 | 18.9 | 19.7 | 12.7 |
| Fru | 1 | 3.2 | 4 | 6.2 | 8.3 |

**F3H**

| Suc | 1 | 4.6 | 2.4 | 4.5 | 8.8 |
| Gln | 1 | 3.5 | 4.8 | 8.6 | 4.2 |
| Fru | 1 | 2.8 | 3.1 | 9.5 | 6.3 |

**FLS**

| Suc | 1 | 2.0 | 2.3 | 3.0 | 4.2 |
| Gln | 1 | 1.7 | 2.1 | 2.6 | 3 |
| Fru | 1 | 1.3 | 2.9 | 6 | 4.8 |

**F3'H**

| Suc | 1 | 2.7 | 338 | 685 | 929 |
| Gln | 1 | 7 | 9 | 41 | 96 |
| Fru | 1 | 2 | 3 | 2 | 1 |

**DFR**

| Suc | 1 | 42 | 357 | 1209 | 1840 |
| Gln | 1 | 1 | 1 | 195 | 252 |
| Fru | 1 | 1 | 1 | 9 | 4 |

**LDOX**

| Suc | 1 | 1 | 7 | 109 | 198 |
| Gln | 1 | 3 | 6 | 20 | 41 |
| Fru | 1 | 1 | 5 | 4 | 5 |

**UF3GT**

| Suc | 1 | 20 | 45 | 89 | 290 |
| Gln | 1 | 1 | 2 | 1 | |
| Fru | 1 | | | | |

**PAP1**
Figure 6