Arabidopsis Seedling Growth, Storage Lipid Mobilization, and Photosynthetic Gene Expression Are Regulated by Carbon:Nitrogen Availability[1]

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The objective of the current work was to establish the degree to which the effects of carbon and nitrogen availability on Arabidopsis seedling growth and development are due to these nutrients acting independently or together. Growth of seedlings on low (0.1 mM) nitrogen results in a significant reduction of seedling and cotyledon size, fresh weight, chlorophyll, and anthocyanin content but a slight increase in endogenous sugars. The addition of 100 mM sucrose (Suc) to the nitrogen-depleted growth media results in a further reduction in cotyledon size and chlorophyll content and an overall increase in anthocyanins and endogenous sugars. Storage lipid breakdown is almost completely blocked in seedlings grown on low nitrogen and 100 mM Suc and is significantly inhibited when seedlings are grown on either low nitrogen or high Suc. Carbohydrate repression of photosynthetic gene expression can only be observed under low nitrogen conditions. Low (0.1 mM) nitrogen in the absence of exogenous carbohydrate results in a significant decrease in *chlorophyll a/b-binding protein* and *ribulose bisphosphate carboxylase small subunit* gene transcript levels. Thus, carbon to nitrogen ratio rather than carbohydrate status alone appears to play the predominant role in regulating various aspects of seedling growth including storage reserve mobilization and photosynthetic gene expression.

Successful seedling establishment after germination requires efficient utilization of both endogenous storage reserves and resources from the environment. To achieve this, seedlings must adapt both developmental and metabolic programs to the prevailing environmental conditions (Holdsworth et al., 1999; Eastmond and Graham, 2001). Light, through a complex system of photoreceptors and signal transduction pathways, is one of the most important environmental parameters affecting seedling developmental programs (Chory, 1993; Neff and Van Volkenburgh, 1994; Mustilli and Bowler, 1997; Howell, 1998). Following the recent demonstration that light can compensate for the glyoxylate cycle in Arabidopsis seedlings (Eastmond et al., 2000a), it is now apparent that through photosynthesis and carbohydrate production light can also make an important contribution to metabolic programs during the early post-germinative growth period.

The availability of macronutrients such as nitrogen is another important environmental parameter influencing seedling growth and development. For example, growth of tobacco (*Nicotiana tabacum*) seedlings under nitrogen-limiting conditions results in a dramatic redirection of biomass allocation to roots versus shoot and an accumulation of soluble carbohydrate (Paul and Stitt, 1993). Feeding exogenous Suc to nitrogen-starved seedlings led to a further increase in endogenous carbohydrate and a decrease of the Rubisco protein and chlorophyll content in shoots. Altering nitrogen metabolism in castor bean (*Ricinus communis*) cotyledons resulted in marked changes in the allocation of carbon between carbohydrate synthesis and respiratory pathways (Geigenberger and Stitt, 1991). Nutrient depletion experiments in barley (*Hordeum vulgare*), pea (*Pisum sativum*), *Lemna gibba*, and tobacco suggest that nitrogen deficiency limits growth, respiration, and utilization of carbohydrates more than it limits photosynthesis (Thorsteinsson et al., 1987; Thorsteinsson and Tillberg, 1990; Paul and Stitt, 1993).

In addition to their metabolic function, soluble sugars play an important role in the regulation of many genes involved in physiological and developmental processes including photosynthesis, nitrate assimilation, assimilate storage, and the mobilization of starch and lipids (for reviews, see Graham, 1996;

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Koch, 1996; Jang et al., 1997; Smeekens and Rook, 1997). Among various genes induced by sugars are those associated with nitrate assimilation such as nitrate reductase and genes encoding the high (*NRT*2) and low (*NRT*1) affinity nitrate uptake systems (Cheng et al., 1992; Lejay et al., 1999). On the other hand, exogenous sugars repress other nitrate metabolism-associated genes such as the Gln-dependent Asn synthetase gene (*ASN*1) of Arabidopsis (Lam et al., 1994). Sugars are also known to repress many of the genes involved in photosynthesis related processes (Sheen, 1990; Von Schaewen et al., 1990; Krapp et al., 1993; Krapp and Stitt, 1995).

Sugars can also interfere with developmentally regulated gene expression during germination and seedling establishment. Examples include Suc and Glc repression of the light-independent, transient expression of the plastocyanin gene (*PC*) during early seedling development; the maintenance of higher chlorophyll *a/b* binding protein (*CAB*1) mRNA levels in older seedlings by exogenous supplied Suc; and the inhibition of light induction of the ribulose bisphosphate carboxylase small subunit gene (*RBCS*) in dark-adapted Arabidopsis seedlings by Suc or Glc (Brusslan and Tobin, 1992; Dijkwel et al., 1996). Sugars have also been shown to repress expression of the genes encoding the key glyoxylate cycle enzymes malate synthase and isocitrate lyase in a cucumber cell culture and in a mesophyll protoplast transient expression system (Graham et al., 1994a, 1994b). The glyoxylate cycle plays a central role in the conversion of carbon, derived from lipid breakdown, into Suc during post-germinative growth of oilseeds such as Arabidopsis (Eastmond and Graham, 2001). However, feeding exogenous Suc to young Arabidopsis seedlings has little impact on the levels of expression of either fatty acid β-oxidation or glyoxylate cycle genes (Hooks et al., 1999; Rylott et al.; 2001). Exogenous Suc does however decrease the rate at which storage lipid is broken down in young seedlings (Eastmond et al., 2000a).

Arabidopsis is the plant of choice for a genetic approach to dissect the mechanisms controlling plant growth and development. Seedlings in particular are ideal for genetic screens since they can be grown under a multitude of different conditions on agar plates. Various genetic screens have been developed in an effort to isolate mutants in the signal transduction mechanisms involved in sugar-mediated repression and induction of gene expression (for review, see Graham and Martin, 2000; Smeekens, 2000). In contrast to the significant amount of work that has focused on dissecting the mechanism(s) that mediate sugar responses in Arabidopsis seedlings, there has been much less done to understand the effects of altering carbon and nitrogen together. The current study shows that seedling growth, development, metabolism, and gene expression respond to the combined effects of carbon and nitrogen availability.

RESULTS

The Effect of Nitrogen and Carbon Availability on Early Post-Germinative Growth of Arabidopsis Seedlings

To investigate the influence of nitrogen supply on the early stages of post-germinative growth in Arabidopsis, wild-type Columbia (Col0) seeds were germinated and grown for 6 d on modified Murashige and Skoog medium containing 60, 6, 0.6, or 0.1 mM nitrogen. Seedlings germinated on 60 mM nitrogen had expanded green cotyledons, a green hypocotyl, and emerging primary leaves at d 6 (Fig. 1). A reduction of the nitrogen concentration to 0.1 mM led to a reduction of seedling and cotyledon size, no visible primary leaves, and a lighter green appearance (Fig. 1). The addition of 100 mM Suc to the 60 mM nitrogen containing media did not enhance the growth over 6 d compared with seedlings on 60 mM nitrogen and

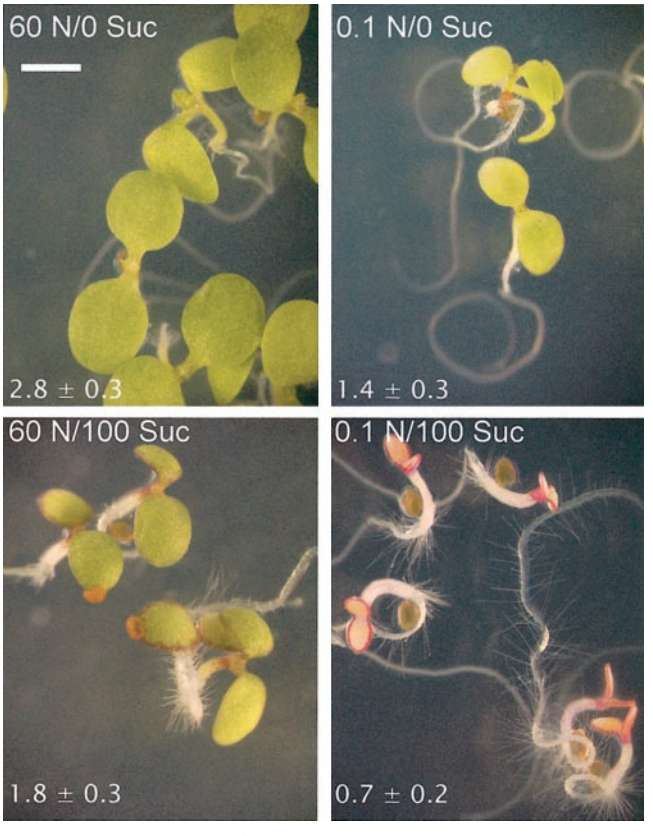

Figure 1. Phenotypes of Arabidopsis seedlings germinated and grown for 6 d on media containing different concentrations of nitrogen and Suc. 60 N/0 Suc, 60 mM nitrogen and 0 Suc; 0.1 N/0 Suc, 0.1 mM nitrogen and 0 Suc; 60 N/100 Suc, 60 mM nitrogen and 100 mM Suc; 0.1 N/100 Suc, 0.1 mM nitrogen and 100 mM Suc. Scale bar = 2 mm. The mean width of approximately 20 cotyledons ± the SD is shown in each case. 0.1 mM nitrogen/100 mM sorbitol treatment resulted in seedlings similar to those grown on 0.1N/0 Suc. Seedlings grown on 100 mM sorbitol did not show the decrease in cotyledon size and purple pigmentation present in seedlings grown on 100 mM Suc and 0.1 mM nitrogen but were similar to seedlings grown on 0.1 mM nitrogen alone (not shown).

no Suc. Instead, cotyledons of seedlings grown on 100 mM Suc and 60 mM nitrogen were less expanded and darker green with a proportion having a purple halo (Fig. 1). Seedlings grown in the presence of 100 mM Suc and 0.1 mM nitrogen exhibited strong purple pigmentation and smaller cotyledons with purple halos after 6 d (Fig. 1).

Nitrogen availability is known to affect root growth (Stitt, 1999). The mean primary root length of 6-d-old seedlings increased from 30.6 to 37.8 mm when the concentration of nitrogen in the media was decreased from 60 to 6 mM (Fig. 2). However, a further decrease of the nitrogen concentration in the growth media to 0.6 or 0.1 mM led to a significant decrease in the mean primary root length to 26.0 and 11.9 mm, respectively (Fig. 2). This suggests that the 0.6 and 0.1 mM nitrogen conditions are limiting growth.

Decreasing nitrogen in the growth medium also influenced gain in fresh weight of 6-d-old seedlings (Fig. 3). In the absence of exogenous Suc, the average fresh weight per seedling decreased from 0.53 mg in the 60 mM nitrogen medium to 0.16 mg in the 0.1 mM nitrogen medium. The fresh weight gain in the zero exogenous Suc plus 60 mM nitrogen treatment was greater than the 100 mM Suc plus 60 mM nitrogen treatment, which is in agreement with the phenotype of the seedlings shown in Figure 1. The fresh weight of seedlings grown on 100 mM sorbitol in the presence of 0.1 mM nitrogen was similar to that of seedling grown on 0.1 mM nitrogen treatment and no exogenous carbohydrate source (Fig. 3). Seedlings grown on 100 mM sorbitol and 0.1 mM nitrogen did not show the decrease in cotyledon size and purple pigmentation present in seedlings grown on 100 mM Suc and 0.1 mM nitrogen but were similar to seedlings grown on 0.1 mM nitrogen alone (not shown). This suggests that the purple pigmentation phenotype of the 100 mM Suc, 0.1 mM nitrogen-grown seedlings is not due to osmotic effects.

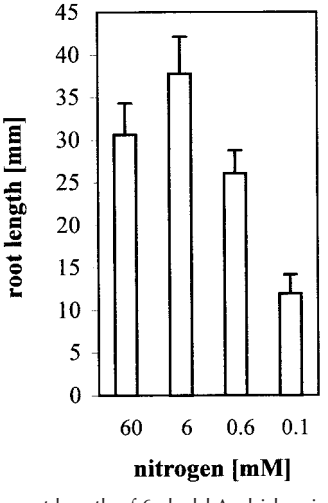

Figure 2. Primary root length of 6-d-old Arabidopsis seedlings grown in the presence of various nitrogen concentrations (60–0.1 mM) and 100 mM Suc. SD bars are shown; $n = 20$.

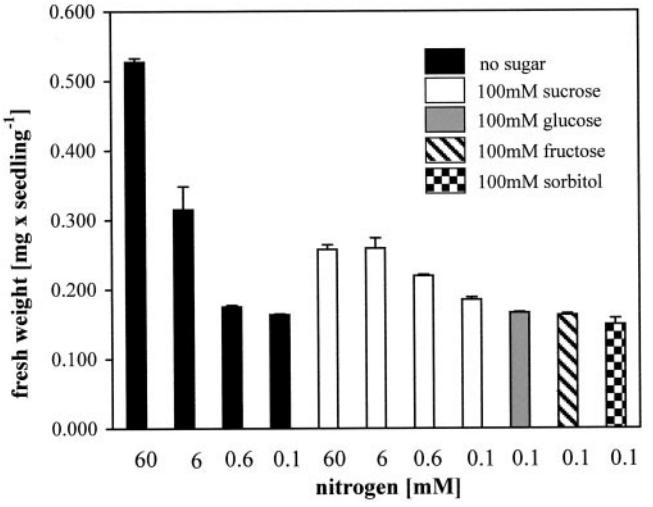

Figure 3. Fresh weight of Arabidopsis seedlings germinated for 6 d in the presence of various nitrogen concentrations (60–0.1 mM) and in the absence or presence of different carbohydrate sources. Each bar presents the average fresh weight of Arabidopsis seedlings. The average fresh weight was estimated in three independent experiments using three to five batches of 20 seedlings in each experiment. Error bars show the SD of the average fresh weight over the three independent experiments.

The Effect of Nitrogen and Carbon Availability on the Mobilization of Triacylglycerol (TAG) Storage Reserves

Breakdown of storage lipids in Arabidopsis seedlings grown on one-half-strength Murashige and Skoog medium plus 29 mM Suc is significantly delayed compared with seedlings on the same medium without Suc (Eastmond et al., 2000a). To establish if carbohydrate to nitrogen ratio rather than carbon supply alone influences the rate of storage lipid breakdown in growing seedlings, eicosenoic acid content was monitored in seedlings grown for 6 d in the presence of various carbohydrate to nitrogen treatments (Fig. 4). Eicosenoic acid is a marker for storage TAG in Arabidopsis seeds (Lemieux et al., 1990). Levels of eicosenoic acid fell to near zero after 6 d when seedling were grown in 60 mM nitrogen and zero exogenous Suc (Fig. 4). The supply of 100 mM exogenous Suc delayed the breakdown of eicosenoic acid and resulted in significant levels being maintained after 6 d growth, which is in agreement with previous reports (Eastmond et al., 2000a). Growth on low levels of exogenous nitrogen (0.1 mM) and zero exogenous Suc also resulted in a significant delay of eicosenoic acid breakdown. The greatest effect on eicosenoic acid breakdown was observed in seedlings grown on low nitrogen (0.1 mM) and high Suc (100 mM). After 6 d, levels of this marker fatty acid were still approximately 80% of that found at d 0 (Fig. 4). Total fatty acid levels followed a similar

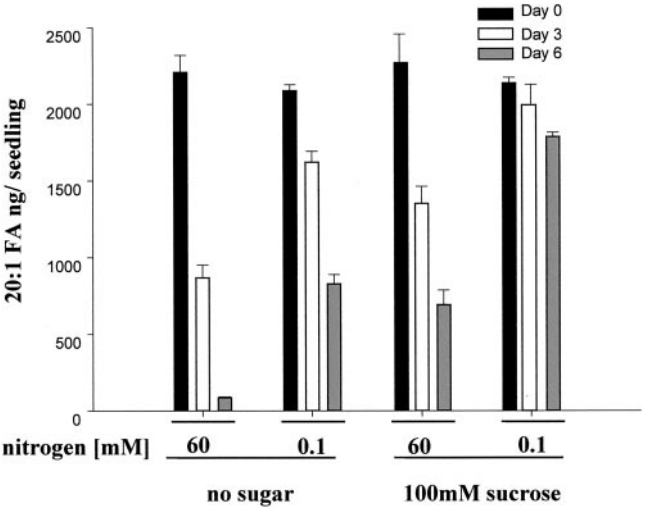

Figure 4. Effect of carbohydrate to nitrogen balance on storage lipid breakdown. Seeds were germinated on media with either 60 mM or 0.1 mM nitrogen in the presence or absence of 100 mM Suc. Samples of 20 seeds or seedlings were taken at the start of the experiment (d 0) and after 3 and 6 d of germination. Total lipids were extracted and measured. All experiments were done in triplicate. The levels of eicosenoic acid (C20:1, $n = 11$) are shown as an indicator of TAG content of the seedlings. SEs are shown. In all cases total fatty acid levels followed a similar pattern to that of eicosenoic acid (not shown).

pattern to that of eicosenoic acid in all cases (data not shown).

The Effect of Nitrogen and Carbon Availability on Soluble Sugar, Chlorophyll, and Anthocyanin Levels

A major product of TAG mobilization during postgerminative growth is Suc. Feedback inhibition by endogenous Suc or a related metabolite is therefore one form of metabolic control that could operate to regulate lipid breakdown under conditions where it is not needed. We analyzed total soluble sugars in seedlings grown under the various carbohydrate to nitrogen growth conditions to establish the impact of nitrogen-limiting growth conditions on endogenous sugar levels. Growth on exogenous sugars resulted in overall increased levels of endogenous sugars measured. However the amount of endogenous sugars per seedling also increased with decreasing nitrogen concentration in either the presence or absence of exogenous sugars (Fig. 5A). There is a positive correlation, significant at the 0.05 probability level ($r = 0.928$), between the amounts of soluble sugars and the storage lipid marker eicosenoic acid (Fig. 4), thus supporting the hypothesis that endogenous sugars can directly or indirectly impose a feedback inhibition on storage lipid mobilization during postgerminative growth.

In contrast to the increase in endogenous sugars, the amount of chlorophyll in 6-d-old seedlings fell with decreasing nitrogen concentration and this effect was even more pronounced when 100 mM Suc was present in the growth media (Fig. 5B). The almost complete failure of seedlings grown on low nitrogen and high Suc to accumulate chlorophyll is probably a result of reduced synthesis of proteins associated with the photosynthetic apparatus. This effect does not appear to be due to osmotic stress because the 100 mM sorbitol, 0.1 mM nitrogen osmotic control did not show the same repression of chlorophyll synthesis but rather had levels similar to the zero exogenous Suc, 0.1 mM nitrogen treatment (Fig. 5B).

Anthocyanins are secondary metabolites that are predominantly synthesized in the upper epidermis in response to various stresses and are responsible for

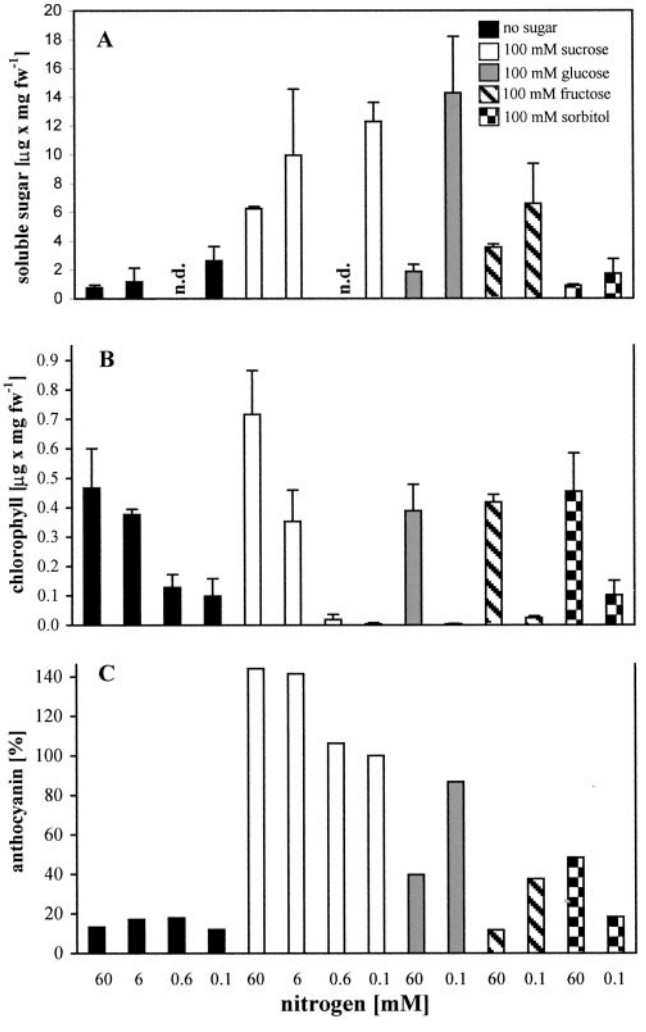

Figure 5. Soluble sugar content (A), chlorophyll amount (B), and anthocyanin levels (C) in 6-d-old Arabidopsis seedlings. Seedlings were germinated either in the absence of a carbohydrate source or in the presence of 100 mM Suc, Glc, or Fru. Germination in the presence of 100 mM sorbitol was used as an osmotic control. The nitrogen concentrations in the media were as follows: 60 mM, 6 mM, 0.6 mM, or 0.1 mM. Sugars, chlorophyll, and anthocyanins were extracted and measured as described in "Material and Methods." Error bars indicate the SD as described ("Materials and Methods").

the purple coloration in plant leaves. Because the seedling phenotype showed a dramatic change from green to purple under high Suc low nitrogen conditions, we were interested in establishing what effect the various metabolic conditions had on anthocyanin levels in seedlings. Overall, the levels of anthocyanins in 6-d-old seedlings grown in the presence of 100 mM Suc are significantly higher than in seedlings grown in media without Suc. Anthocyanin levels in seedlings grown on 100 mM Suc fell slightly with decreasing nitrogen concentration (Fig. 5C), but overall there is a positive correlation with the amount of endogenous soluble sugar that is significant at the 0.05 probability level ($r = 0.80$). The purple phenotype under high Suc low nitrogen conditions (Fig. 1) appears to be due to the elevated levels of anthocyanins becoming more visible in the almost complete absence of chlorophyll (Fig. 5, B and C).

The Effect of Nitrogen and Carbon Availability on the Expression of Genes Associated with Photosynthesis and Anthocyanin Production

Sugars can operate to repress certain classes of genes in higher plants, including those associated with photosynthesis, and induce others such as chalcone synthase, which is associated with anthocyanin biosynthesis (for review, see Koch, 1996; Graham and Martin, 2000). Northern analysis was used to establish if the effects observed on chlorophyll and anthocyanin amounts under the different carbon and nitrogen growth conditions were reflected at the level of gene expression. Transcript levels of the small subunit of Rubisco (*RBCS*) and the chlorophyll a/b binding protein (*CAB*) were taken as markers for photosynthetic gene expression and chalcone synthase (*CHS*) was used as a marker for anthocyanin biosynthesis (Fig. 6).

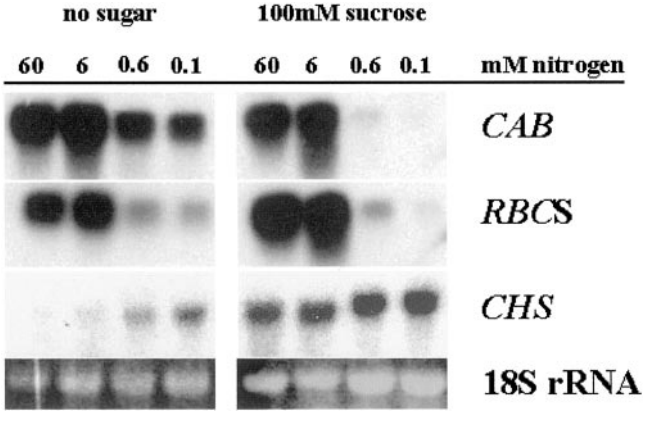

Figure 6. Northern analysis of *CAB*, *RBCS*, and *CHS* gene expression in seedlings germinated for 6 d on media containing 60 mM, 6 mM, 0.6 mM, or 0.1 mM nitrogen in either the absence or presence of 100 mM Suc. Photographs of RNA gels show the amount of 18S rRNA in each lane as a loading control.

CAB and *RBCS* transcript levels were similar in 6-d-old seedlings grown in the presence of 60 or 6 mM nitrogen irrespective of whether Suc was present or absent from the growth media. A significant drop in photosynthetic gene transcript abundance could only be observed when the levels of nitrogen supply were as low as 0.6 or 0.1 mM (Fig. 6). The decrease in transcript abundance at these lower nitrogen concentrations was more extreme in the presence of 100 mM exogenous Suc. These changes in photosynthetic gene expression approximated the changes observed for seedling chlorophyll content under the same growth conditions (Fig. 5B). They also broadly reflect the effects on development of photosynthetic tissue. For example, seedlings grown on high nitrogen plus or minus exogenous Suc have well-developed cotyledons and photosynthetic gene expression is high, whereas seedlings grown in low nitrogen plus 100 mM exogenous Suc have poorly developed purple cotyledons and photosynthetic gene expression is very low (Fig. 1, and Fig. 6). This highlights the importance of interpreting any effects of nutrients on gene expression in the context of related developmental changes.

CHS gene expression showed an induction with decreasing nitrogen availability in both the absence and presence of exogenous Suc (Fig. 6). The overall levels of *CHS* transcript were higher in the presence of exogenous Suc over the range of nitrogen concentration, which agrees with the elevated anthocyanin levels detected in seedlings grown under the same conditions (Fig. 5C).

Glc But Not Fru Has an Effect Similar to Suc on Seedlings Grown under Different Nitrogen Conditions

The addition of 100 mM Glc to low nitrogen (0.1 mM)-containing media resulted in high endogenous sugar levels (Fig. 5A), an almost total absence of chlorophyll (Fig. 5B), a strong increase of anthocyanins (Fig. 5C), and seedlings with red/purple cotyledons (not shown). Thus the presence of 100 mM Glc caused effects similar to 100 mM Suc if nitrogen was low in the media. However, in seedlings grown in the presence of 100 mM Fru and low nitrogen (0.1 mM), the endogenous soluble sugar content was 50% lower than that of seedlings on low nitrogen and high Glc or Suc (Fig. 5A), there was a less pronounced reduction in chlorophyll content (Fig. 5B), and anthocyanin content did not increase to the same extent (Fig. 5C). In agreement with this seedlings grown on Fru did not exhibit the red/purple cotyledon phenotype of seedlings grown in the presence of Glc or Suc but instead the cotyledons remained pale green (not shown). However, the low sugar content alone cannot explain the lower concentration of anthocyanins because a similar sugar content was found in seedlings germinated on 60 mM nitrogen and 100 mM Suc, and these seedlings contain higher levels of anthocyanins.

DISCUSSION

In this study we demonstrate that the effects of sugars on a number of parameters including morphology, growth, storage reserve mobilization, chlorophyll levels, and photosynthetic gene expression are largely dependent on nitrogen availability in young Arabidopsis seedlings. Decreasing the availability of nitrogen alone affects all of these parameters, and the provision of exogenous sugars on these effects is additive. Therefore, carbohydrate to nitrogen ratios play a central and interactive role in regulating the processes underpinning seedling establishment.

Arabidopsis seedling development was normal in the presence of 60 or 6 mM total nitrogen and no exogenous carbon. Cotyledons were green and expanded, and primary leaves developed. Chlorophyll accumulated, photosynthetic genes such as *CAB* and *RBCS* were induced, and storage lipids (eicosenoic acid) were rapidly broken down indicating a normal transition from heterotrophic to photoautotrophic growth. Reduction of the nitrogen content in the growth media to 0.6 or 0.1 mM led to several phenotypic, physiological and metabolic changes in seedlings. Seedling morphology was significantly altered with overall size, root length, and fresh weight all being reduced. Decreased levels of chlorophyll and photosynthetic gene expression in 6-d-old seedlings mirrored these changes. Levels of soluble sugar increased slightly as the availability of nitrogen in the growth media decreased and breakdown of storage lipids at d 3 after imbibition was significantly delayed compared with seedlings germinated on 60 mM nitrogen. Both the increase in endogenous sugars and the delay in lipid mobilization suggest a restricted use of carbon resources under nitrogen limited growth conditions. These results are in agreement with previous studies showing accumulation of carbohydrates in leaves and roots of mature plants after nitrogen withdrawal (Thorsteinnson and Tillberg, 1990; Henry and Raper, 1991; Paul and Driscoll, 1997). In tobacco seedlings, nitrogen-deficient growth conditions resulted in elevated levels of hexose, hexose phosphates, and 3-phosphoglyceric acid in both shoots and roots, but a decrease of Rubisco and chlorophyll was only observed when exogenous Suc was provided in the growth media (Paul and Stitt, 1993). This is in contrast to the results presented here, where nitrogen limited growth conditions alone are sufficient to inhibit the accumulation of chlorophyll and repress the expression of photosynthetic genes (Figs. 5B and 6). Addition of 100 mM Suc or Glc to nitrogen-limiting growth media caused an even greater reduction of photosynthetic gene expression and chlorophyll content than that caused by nitrogen limitation alone (Figs. 5B and 6). Tobacco seedlings grown on exogenous Suc and low nitrogen showed similar effects with decreased Rubisco and chlorophyll content in shoots (Paul and Stitt, 1993).

The observed decrease in chlorophyll and photosynthetic gene expression in nitrogen starved seedlings occurs under conditions where the sugar levels are only one-half that of seedlings grown on high nitrogen (60 mM) and high carbon (100 mM Suc or Glc), yet these latter seedlings show no decrease in either chlorophyll or photosynthetic gene expression (Figs. 5, A and B, and 6). Therefore, either a non-sugar-mediated mechanism operates to reduce the amount of photosynthetic machinery in nitrogen starved seedlings or the sensitivity to sugars changes with nitrogen status. This latter option is possible given that carbon flux through glycolysis or other pathways linked to sugar signal generation could well be responsive to nitrogen status. It is also important to note that decreasing the nitrogen availability also has significant effects on seedling growth and morphology (Figs. 1 and 2) and the observed decreases in chlorophyll and photosynthetic gene expression could be secondary to these developmental changes. This highlights the importance of considering the overall effects on plant growth and development of experimental treatments that are designed to investigate direct involvement of nutrients such as sugars in regulating specific aspects of metabolism and related gene expression. This is particularly true of Arabidopsis mutant screening conditions that employ high concentrations of exogenous sugars to identify mutants that are disrupted in sugar sensing and signaling (for review, see Smeekens, 2000). Mutants that show an altered response to such treatments could do so for a variety of reasons.

The significant levels of photosynthetic gene expression in 6-d-old seedlings grown on 100 mM Suc and 60 mM nitrogen is in stark contrast to the repression of photosynthetic genes by similar levels of sugars previously reported for numerous experimental systems (Sheen, 1990; Von Schaewen et al., 1990; Krapp et al., 1993; Krapp and Stitt, 1995). This stimulatory effect of Suc under high nitrogen conditions is also in contrast to the additive repression effect of Suc that occurs when it is added to the growth media of seedlings grown under nitrogen-limiting conditions (Figs. 5B and 6). The transport and metabolism of sugars is likely to be very different under nitrogen-limiting and nitrogen-sufficient conditions. For example, under nitrogen-limiting conditions, a significant amount of available carbon will be directed to the roots to support increased root growth, whereas under high nitrogen conditions, the growing shoot will represent a strong metabolic sink for available carbon (Paul and Stitt, 1993). These changes in carbon flux through different metabolic and/or transport pathways could result in changes in the type or amount of sugar related signals being generated or altered sensitivity or response to the signals.

The appearance of red/purple cotyledon pigmentation and the accumulation of anthocyanins in Arabidopsis cotyledons are only marginally influenced

by the nitrogen concentration in the growth media but they are dependent on the supply of exogenous sugars (Figs. 1 and 5C). Similarly, the induction of *CHS* expression depends primarily on the presence of Suc in the growth media. A marginal increase of *CHS* expression on Suc free media correlates with an increase in the endogenous sugar concentration with decreasing nitrogen. Anthocyanin levels remained low in seedlings germinated in the presence of 100 mM sorbitol excluding the possibility of osmotic effects influencing their accumulation. Induction of *CHS* gene expression by sugars has previously been reported (Tsukaya et al., 1991). Expression of the petunia *CHS-A* promoter-β-glucuronidase (*GUS*) gene fusion in transgenic Arabidopsis plants is induced by 300 mM Suc, Glc, or Fru (Tsukaya et al., 1991). The increased steady-state *CHS* mRNA levels observed in the current study are therefore likely to be regulated at the transcriptional level, as is the case for the *CHS-A* promoter-*GUS* gene fusion.

The decrease in storage lipid breakdown with decreasing nitrogen availability and the additive effect of exogenous sugar demonstrates that reserve mobilization is subject to nutrient availability. The inhibition of lipid mobilization could arise either as a direct consequence of carbon and nitrogen availability affecting some aspect of this process or indirectly through the effect on seedling growth and morphology. During normal post-germinative seedling growth of Arabidopsis the majority of storage lipids are mobilized in the 3 to 4 d after imbibition when the radicle and cotyledons are just emerging from the seed coat (Eastmond and Graham, 2001). In the high carbon, low nitrogen treatment in which lipid mobilization is almost completely blocked the seedling roots, cotyledons, and hypocotyl have emerged from the seed coat and undergone considerable development after 6 d (Fig. 1). It would therefore appear that seedling morphology is not playing a predominant role in regulating lipid reserve mobilization but rather nutrient availability is having a direct effect on some aspect of the mobilization process. Previous work has shown that the genes encoding the glyoxylate cycle enzymes malate synthase and isocitrate lyase, which play an integral role in lipid mobilization during post-germinative oilseed growth, are subject to sugar-mediated repression in cucumber cell cultures (Graham et al., 1994b). Growth of Arabidopsis seedlings on media containing one-half-strength Murashige and Skoog medium and 29 mM exogenous Suc has recently been shown to delay lipid breakdown (Eastmond et al., 2000a). However, this delay is slight in comparison with the effects observed when both nitrogen and carbon availability are altered as shown in the current work (Fig. 4). In the marine diatom, *Phaeodactylum tricornutum*, storage lipid mobilization was shown to be affected by both carbohydrate and nitrogen status (Larson and Harrison, 1997). In that study addition of nitrate to nitrate-depleted growth media resulted in a decrease in intracellular carbohydrate followed by degradation of fatty acids associated with storage lipids and an increase in isocitrate lyase enzyme activity. Therefore, control of storage lipid mobilization by both carbon and nitrogen status could be a universal mechanism common to both algae and higher plants. It is not clear at what level such a mechanism may operate. Control could be exerted specifically at the first committed step of lipid mobilization, which involves a TAG lipase. The gene encoding this enzyme has not yet been identified in higher plants. There could alternately be a down-regulation of genes encoding fatty acid β-oxidation and glyoxylate cycle enzymes in response to high carbon, low nitrogen conditions. Sugars alone do not appear to repress glyoxylate cycle or β-oxidation genes during post-germinative seedling growth (Hooks et al., 1999; Rylott et al., 2001). The use of tools such as the β-oxidation *ACX3* promoter:*GUS* reporter gene transgenic lines (Eastmond et al., 2000b) should establish whether altering carbohydrate to nitrogen ratios affects the transcriptional control of genes involved in lipid mobilization.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis genotype Col0 was obtained from the Nottingham Arabidopsis Stock Center, UK. Surface-sterilized seeds were sown on Murashige and Skoog medium (Murashige and Skoog, 1962) modified with different concentrations of sugars and total nitrogen. The ratio of potassium nitrate to ammonium nitrate was maintained in each experiment as previously described for Murashige and Skoog medium (Murashige and Skoog, 1962). Potassium chloride was added to the medium to compensate for the lower potassium ion concentration in reduced potassium nitrate containing media. To ensure a homogenous germination, seeds were kept at 4°C for 96 h before transfer to the growth room. Seeds were germinated and grown for 6 d under continuous, cool fluorescent white light (100 μE m^{-2} s^{-1}) at 20°C to 21°C.

Cotyledon and Root Growth Analysis

Cotyledon width was determined by measurement of digital images of 6-d-old seedlings using the software application Adobe Photoshop (Adobe Systems, Mountain View, CA). Primary root length was determined by arranging seeds in a row on appropriate media on a Petri dish, orienting this in a vertical position, and measuring root length with a ruler after 6 d of growth.

RNA Analysis

Seedlings were harvested and frozen in liquid nitrogen. Total RNA was extracted and analyzed as described previously (Kay et al., 1987; Sambrook et al., 1989). Hybrid-

ization using nylon membranes (Hybond N+, Amersham, Buckinghamshire, UK) was performed in the presence of 50% (v/v) formamide, 5× SSPE, 5× Denhardt's solution, 0.5% (w/v) SDS, and 100 mg μL^{-1} herring sperm DNA at 42°C using randomly primed [^{32}P]dCTP probes. Membranes were washed under stringent conditions (65°C, 0.2× SSPE, 0.5% [w/v]SDS) and subsequently autoradiographed. The membranes were rehybridized several times. Membranes were stripped for 2 h at 65°C in 0.1% (w/v) SDS, 1 mM EDTA, and 5 mM Tris, pH 7.5, and rehybridized. Probes used were the Arabidopsis chlorophyll *a/b* binding protein gene 2 (*CAB*2; Leutwiler et al., 1986), an expressed sequence tag for the small subunit of Rubisco (*RBC*S; GenBank ID: T04228) and a genomic clone of Arabidopsis chalcone synthase (Trezzini et al., 1993).

Fresh Weight Measurement

Six-day-old seedlings were harvested in batches of 20 seedlings, rinsed in distilled water, and blotted for a short period of time on 3MM filter paper (Whatman, Clifton, NJ) before determination of the weight. The weight of three to five seedling batches of 20 seedlings was determined for each measurement. The experiment was repeated three times on independently prepared plates and at different days. The average and SD of the three experiments was calculated.

Determination of Eicosenoic Acid

Arabidopsis genotype Col0 seeds were germinated on media containing either 60 or 0.1 mM nitrogen with or without 100 mM Suc at 100 μmol photons m^{-2} s^{-1}. Upon transfer into the growth room 20 seeds were taken for each treatment for lipid analysis (d 0). After 3 and 6 d of germination, a further 20 seedlings were taken for lipid analysis (d 3 and 6 samples, respectively). All samples were immediately frozen in liquid nitrogen and stored at −80°C until extraction. Experiments were carried out in triplicate.

Total lipids were extracted and measured with a gas chromatograph based on the method described in Browse et al. (1986). The levels of eicosenoic acid (C20:1; $n = 11$) were taken as an indicator of TAG content of the seedling.

Measurement of Soluble Sugars, Chlorophyll, and Anthocyanins

Six to 7-d-old seedlings were harvested, washed several times in sterile, de-ionized water, dry-blotted on paper towels, weighed, and frozen in liquid nitrogen. Between 25 and 100 mg of seedling material were homogenized and used for the given extraction procedure. All samples were measured in duplicates and data presented are average values of at least three independent experiments.

Soluble Sugars

Frozen homogenized seedling material was extracted three times in 500 μL of 80% (v/v) ethanol at 80°C. Extracts were pooled, the volume was reduced under vacuum, and the concentration for Glc, Fru, and Suc were measured as described by Stitt et al. (1989).

Chlorophyll

Frozen homogenized material was extracted in 80% (v/v) acetone at dim light and on ice. The process was repeated until chlorophyll was completely extracted from the seedlings. Extracts were pooled and chlorophyll was measured as described in Arnon (1949).

Anthocyanin Measurement

Frozen homogenized material was extracted overnight at 4°C under gentle shaking in 300 μL of 7% (v/v) hydrochloric acid in methanol. Sterile, de-ionized water (200 μL) was added to the extract and mixed. Chloroform (500 μL) was added to each sample, mixed, and centrifuged at 13,000 rpm for 2 min. The top layer (400 μL) was transferred to a fresh microtube, and 600 μL of 1% (v/v) hydrochloric acid in methanol was added and centrifuged as above to remove remaining particles. The supernatant was used for absorbency measurements at 530 and 657 nm. Relative anthocyanin concentrations were calculated as absorbency (530 nm) minus absorbency (657 nm).

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