Metabolic responses to red/far-red ratio and ontogeny show poor correlation with the growth rate of sunflower stems

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Abstract

In sparse canopies, low red to far-red (R/FR) ratios reach only vertically-oriented stems, which respond with faster rates of extension. It is shown here that this signal also promotes stem dry matter accumulation in sunflower (Helianthus annuus) but not in mustard (Sinapis alba L.). Physically blocking internode extension growth also blocked internode recovery of labelled carbon fed to the leaves, indicating that increased carbon accumulation is partially a consequence of increased extension growth in sunflower. However, low R/FR also promoted carbon accumulation in the lower section of the internode, where extension growth was unaffected. Although the levels of many soluble metabolites and of cell-wall carbohydrates increased in the stem in response to low R/FR, allowing conservation of their concentration, sucrose was present at a lower concentration under low R/FR. This change is anticipated to favour carbon unloading from the stem phloem. Low R/FR also reduced the levels of selected fatty acids, fatty acid alcohols, and sterols. Compared with the lower section, the upper section of the internode showed higher levels of organic acids, amino acids, fatty acids, and sterols. It is concluded that the promotion of stem extension growth by low R/FR ratios causes increased dry matter gain in sunflower internodes by a mechanism that is largely independent of changes in metabolism, since, whilst both low R/FR and ontogeny alter the metabolic profile, the changes do not correlate with the observed growth responses.

Key words: Carbohydrates, metabolic profile, phytochrome, shade avoidance, sunflower.

Introduction

The growth of the stem is critical for the establishment of the position of plant foliage within the canopy, where small differences may determine large consequences in terms of light capture. One of the key cues that plants use to adjust the growth of the stem is the ratio of photon fluence rates between red light (R) and far-red light (FR) perceived by phytochromes (Smith, 2000). The R/FR ratio decreases with canopy density due to the selective optical properties of green leaves, which reflect and transmit significantly more FR than R (Ballaré et al., 1987). Tuning of the growth rate by the R/FR ratio is critical for shade-intolerant species because a constitutively shorter plant would easily become shaded by neighbours and a constitutively tall plant has high risks of stem fracture by wind (Casal et al., 1994).

Increased stem dry matter accumulation in the internode often accompanies the promotion of stem extension growth by low R/FR ratios (Kasperbauer, 1971; Holmes and Smith, 1977; Casal and Sánchez, 1992). However, when the shoot of mustard plants is exposed to low R/FR ratios, the changes in carbohydrate levels in the internode are not merely the consequence of changes in extension growth (Casal et al., 1995). Rather, it appears that phytochrome has multiple points of action in the mechanisms that modulate partitioning and the indirect effect via enhanced extension growth is only one of these points. In support of this view, low R/FR ratios reaching the leaves of mustard plants increase the activity of sucrose-phosphate synthase, a key enzyme in the regulation of sucrose export and, in experiments where only one of the two leaves of the first pair receive low R/FR, the activity increases in the low R/FR-treated leaf despite the lack of an extension-growth response by the stem (Yanovsky et al., 1995).
In sparse canopies, the stem receives low R/FR ratios before the leaves become shaded (Ballaré et al., 1987). In Amaranthus quitensis (Ballaré et al., 1991) and Helianthus annuus (sunflower) (Libenson et al., 2002), low R/FR ratios can increase stem extension growth and dry matter accumulation even if only applied to the growing stem while the rest of the shoot remains exposed to high R/FR. A first working hypothesis tested here is that when only the stem and not the leaves receive the low R/FR stimulus, the enhanced dry matter gain can be accounted for as a consequence of enhanced extension growth.

Low R/FR ratios can increase the length and dry weight of the internode but we remain relatively ignorant of the changes in metabolism that occur in these larger internodes compared with high R/FR controls. Hundreds of genes have been linked to shade-avoidance responses in recent transcriptome studies (Salter et al., 2003; Devlin et al., 2003). However, no studies have been conducted at the metabolome level, which has provided useful information about biological responses to environmental perturbations such as cold acclimation (Kaplan et al., 2007), salt stress (Kim et al., 2007), and water stress (Harrigan et al., 2007; Semel et al., 2007). Given that a major future challenge for plant breeding is the sustainable production of biomass to be converted into biofuels, it would be particularly important to explore the relationships between growth and metabolic composition. The analysis of multiple Arabidopsis accessions has revealed that a combination of metabolites rather than their individual levels correlates with biomass and growth (Meyer et al., 2007). Based on these findings, a second working hypothesis tested here is that the stem concentration of selected metabolites correlates with stem growth in sunflower internodes exposed to different R/FR ratios.

**Materials and methods**

**Plant material**

Sunflower (Helianthus annuus) or mustard (Sinapis alba) seeds were sown on moistened cotton in clear plastic boxes and incubated at 25 °C. Three days later, the seedlings were transplanted to individual pots (10 cm diameter, 12 cm height) filled with soil and transferred to a greenhouse under natural radiation. The treatments started when the plants were 13 d old.

**R/FR and growth-restriction treatments**

To modify the R/FR ratios reaching the internode without affecting the light environment reaching the leaves, selective plastic filter tubes were placed around the first internode. Filters were of 2 cm in diameter. The height of the filter tube was adjusted according to internode height in order to ensure full coverage of this organ. The plastic filters used to obtain low R/FR ratios (<0.08) or sunlight R/FR ratios (i.e., control R/FR ratio=1.1) were described earlier by Libenson et al. (2002).

To restrict internode extension growth, a water-soluble vinylic adhesive (Plasticola Pico Blanco, Chemical Supply Co. Buenos Aires, Argentina) was applied as two parallel lines along the first internode (Casal et al., 1995). Approximately 15 mg of adhesive were applied per plant. No toxic effects of this treatment were observed (Casal et al., 1995).

**Growth measurements**

The length and diameter of the first internode were respectively measured with a ruler or a calliper at a precision of 0.5 mm. The increment in internode length was calculated as the difference between the values recorded at the beginning of the treatments and at the indicated time. In some experiments, the upper and lower internode sections were measured separately. For this purpose, a reference line in the middle of the internode was labelled at the beginning of the treatments.

To obtain internode dry weight, first internodes were harvested and dried at 72 °C for 3 d. Increments in dry weight were determined as the difference between values recorded at the beginning of the treatments and at the indicated times. At the beginning of the treatments, a group of plants was harvested to investigate the relationship between internode volume and dry weight in each experiment, and this relationship was used to estimate the initial dry weight of the internode in the remaining plants of that experiment.

**Radiolabelled carbon determination**

The first pair of leaves was painted with 14C urea (Sigma) diluted in 1 ml distilled water and two drops of Tween 20. Internodes were harvested 5 h after radioactive carbon application. Then internodes were homogenized in 3 ml of scintillation liquid (Optiphase Hisafe 3, LKB, Pharmacia, Sweden) and counts per minute (cpm) were measured with a scintillation counter.

**Extraction, derivatization, and analysis of metabolites by GC/MS**

Sunflower internodes were exposed to high or low R/FR ratios for 3 d and the upper and lower sections were harvested separately in liquid nitrogen. Samples (100 mg) were extracted in 1400 μl of methanol as described by Roessner et al. (2000) and 60 μl of ribitol (0.2 mg ml⁻¹ stock in water) were added as an internal quantitative standard. The mixture was extracted for 15 min at 70 °C and centrifuged 10 min at 14,000 rpm. The supernatants were transferred to a new assay tube and added 750 μl chloroform and 1500 μl of water before centrifugation for 15 min at 4000 rpm. The upper phase (polar phase) and the chloroform phase (apolar phase) were obtained separately, dried in a speed-vac for 3 h without heating, and stored at −20 °C. Both derivatization and subsequent chromatography and data elaboration were carried out exactly as described previously (Roessner et al., 2001; Fiehn et al., 2000). For the polar phase, the sunflower extracts were compared against previously determined tomato extracts (Schauer et al., 2006) in a so-called recombination experiment. In this experiment, tomato and sunflower extracts were injected on the GC-MS independently, followed by a 50:50 mixture of the extracts of both species. This controls that there are no major shifts in chromatographic behaviour between the different biological matrices and, in addition, allows robust quantitative recovery within the peaks identified to be checked. In the case of the sunflower extracts both parameters were deemed of high quality. For the lipid phase, assignments were made on the basis of co-elution and mass spectral properties alone and therefore the certainty of their assignment should be regarded as less absolute.

**Determination of reducing sugars, starch, and wall carbohydrates**

First internode samples were harvested and stored immediately in liquid nitrogen. Each internode was homogenized separately using...
a mortar and pestle with 4 or 5 ml of distilled water. The homogenates were autoclaved for 5 h at 120 °C and 1 atm and centrifuged (20 min, 15,000 rpm). The supernatant aliquots were used to quantify reducing sugars following the Somogy-Nelson method (Nelson, 1944). Sucrose content was estimated as the difference in reducing sugars between the samples and aliquots of the supernatant incubated with 100 μL of invertase enzyme (Sigma, Chemical) in citrate buffer (100 mM pH 4.5) for 30 min at 55 °C. Starch was determined as described by Casal et al. (1995). For wall carbohydrates determinations, pellets were washed three times with distilled water and incubated with 3 ml sulphuric acid (70% v/v) for 24 h at 25 °C. Cell wall carbohydrates were measured using the anthrone method (Yemm and Willis, 1954).

Statistics
Data were analysed by ANOVA after verification of normality and homogeneity of variances. Data corresponding to reducing sugars, sucrose, wall carbohydrates, and radioactivity were log transformed to obtain homogeneity of variances.

Results
The relationship between low R/FR-stimulated stem extension growth and dry matter gain depends on the species

Sunflower and mustard plants were grown in a greenhouse in pots isolated from nearby neighbours. Plastic filters were placed around the first internode to obtain either a high R/FR ratio (1.1; i.e. sunlight R/FR ratio) or low R/FR ratio (0.08) reaching that organ. These treatments started when the internode was less than 2.5±0.1 (sunflower) or 1.2±0.1 (mustard) cm tall. Low R/FR ratios increased extension growth of sunflower and mustard internodes (Fig. 1A). The increase in internode extension growth was accompanied by an increase in dry matter accumulation in sunflower internodes, but not in mustard (Fig. 1B).

The first internode of sunflower plants was exposed to high or low R/FR ratios and, 3 d later, 14C urea was applied to the first pair of leaves as a source of radioactive carbon. The internode was harvested 5 h later. The radioactive carbon recovered in the first internode indicates that the enhanced dry matter accumulation in low R/FR-treated sunflower internodes was at least partially due to increased translocation of carbon from the leaves to the stem (Fig. 1B, inset).

The above results show that, in sunflower, the stem that extends faster also accumulates more carbon. To test this correlation by independent means, simultaneously with the radioactive carbon, synthetic glue was applied at both sides of the first internode of sunflower plants in order to restrict internode extension growth (Casal et al., 1995). In plants with internodes exposed to high R/FR, the physical restriction of extension growth (mean ± SE, cm, control: 0.7±0.2, +glue: 0.2±0.1, P <0.05) also reduced internode radioactive carbon accumulation (log cpm, mean ± SE, control: 3.4±0.1, +glue: 3.1±0.1, P <0.05). A similar result was observed in plants with low R/FR-treated internodes (data not shown). In summary, at this level of resolution there is a correlation between stem extension growth and dry matter (carbon) accumulation in sunflower, but not in mustard.

Stem dry matter accumulation depends on current and previous light conditions

To investigate if dry matter accumulation depends on the current and/or previous light conditions, the first internode of sunflower plants was exposed to high or low R/FR ratios and, after 2 d, the R/FR treatments of half of the plants were altered, so that those plants that received high R/FR ratios (pretreatment) received low R/FR ratio (treatment) and vice versa (Fig. 2A). First internode extension growth responded only to the current light condition (Fig. 2B). However, first internode dry matter gain responded to both current and pretreatment light conditions (Fig. 2C). Therefore, dry matter gain during the treatment period has two components, one related to the current rate of extension growth (promoted by low R/FR during the treatment period), and another related to the initial size of the internode (promoted by low R/FR during the pretreatment).

Low R/FR promotes carbon accumulation in the upper and lower sections of the internode but it affects extension growth only in the upper section

To investigate carbon partitioning in further detail, the first internode of sunflower plants was exposed to high or
low R/FR ratios and, 3 d later, $^{14}$C urea was applied to the first pair of leaves as a source of radioactive carbon. The upper and lower sections of the internode (marked immediately prior to the beginning of the R/FR treatments) were harvested separately 5 h later. Low R/FR ratios increased extension growth (Fig. 3A) and radioactive carbon recovery (Fig. 3B) of the upper section of the internode. Compared to the upper half, the lower half showed reduced extension growth and carbon accumulation. Low R/FR ratios increased carbon accumulation, but not extension growth, in the lower internode section (Fig. 3).

Several metabolites conserve constant concentration despite differential stem growth rate

The first internode (less than 2.5 cm tall) was treated with high or low R/FR ratios and harvested 3 d or 6 d later. The diameter of the internode was unaffected by R/FR ratio (mean ±SE, mm, 3 d of treatment: high R/FR = 3.8 ± 0.1; low R/FR = 3.9 ± 0.1; 6 d of treatment: high R/FR = 4.3 ± 0.1; low R/FR = 4.4 ± 0.1). Therefore, the volume of the internode increased in response to low R/FR (mean ±SE, cm$^3$, 3 d of treatment: high R/FR = 0.48 ± 0.04; low R/FR = 0.60 ± 0.04; $P < 0.05$; 6 d of treatment: high R/FR = 0.72 ± 0.07; low R/FR = 1.1 ± 0.1; $P < 0.01$). When the amount of reducing sugars or cell-wall carbohydrates is plotted against the volume of the internode, the points corresponding to the different harvest dates and R/FR conditions fall on the same line ($r^2$ for reducing sugars = 0.94; $r^2$ for wall carbohydrates = 0.99; Fig. 4A). This indicates that low R/FR increased the absolute levels of these carbohydrates maintaining (within the resolution achieved here) the same proportion observed in high R/FR-treated plants.

The diameter of upper and lower internode sections were similar (mean ±SE, mm, high R/FR ratio: lower section = 3.5 ± 0.1; upper section = 3.5 ± 0.1; low R/FR ratio: lower section = 3.6 ± 0.1; upper section = 3.6 ± 0.1). Therefore, low R/FR increased the volume of the upper section of the internode but not in the lower section (mean ±SE, cm$^3$, upper section: high R/FR = 0.47 ± 0.04; low R/FR = 0.62 ± 0.03; $P < 0.05$; lower section: high R/FR = 0.26 ± 0.05; low R/FR = 0.30 ± 0.02). Reducing sugars per unit volume were similar in high and low R/FR ratios (Fig. 4B). The upper section of the internode showed reduced levels of cell wall carbohydrates per unit volume when compared to the lower section of the internode ($P < 0.05$, Fig. 4B). Starch levels were very low in the first internode (less than 0.1 mg per unit volume) and no differences could be detected between sections or R/FR ratio conditions.

To investigate metabolic changes in further detail, the first internode of sunflower plants was exposed to high or
low R/FR ratios for 3 d before the upper and lower internode sections were harvested for metabolic profiling of polar and non-polar fractions by gas chromatography/mass spectrometry (GC/MS). The analysis allowed us to identify the chemical nature of 14 non-polar metabolites and 47 polar metabolites (see Supplementary Table S1 at JXB online). Approximately half of these metabolites (six non-polar, 24 polar) showed no significant changes in levels per unit fresh weight, despite the effects of R/FR ratio and ontogeny on extension growth and carbon accumulation.

Ontogeny and R/FR ratio affect the stem metabolic profile

Figures 5 and 6, respectively, include the eight non-polar and 23 polar metabolites that show significant main effects and/or interaction in a two-way ANOVA involving the factorial combination between the upper and lower sections of the internode and high and low R/FR ratios. The correlations between metabolite levels (Figs 5, 6) and either extension growth or log carbon accumulation (Fig. 3) were analysed. Of the 31 metabolites that showed significant changes in levels, only saccharate, correlated with extension growth and only galacturonate MX1 correlated with log carbon accumulation ($r^2 >0.9$, $P <0.05$).

Low R/FR ratios decreased the levels of sucrose, tetradecanoic acid, pentadecanoic acid, and octodecanoic acid (three saturated fatty acids) in the upper and lower sections of the internode, and the levels of asparagine 3 and octodecanol only in the upper section. Low R/FR ratios increased the levels of galacturonate, glutarate, saccharate, fructose, and inositol in the upper and lower sections of the internode, and the levels of glutamate, pyroglutamate, hexadecanol, and campesterol only in the lower section (Figs 5, 6).

The levels of three amino acids (alanine, asparagine 3, and glycine), 10 organic acids (aspartate, glutamate, citrate, galacturonate, glycerate, malate, quinate, saccharate, salicynate, threonate), phosphate, inositol, sucrose, glucose-6-P, hexadecanol, campesterol, and beta-sitosterol were higher in the upper than in the lower section of the internode. The levels of raffinose and three saturated fatty acids (tetradecanoic acic, octadecanoic acid, and tetraicosanoic acid) decreased in the upper, compared to the lower section of the internode.

Daily kinetics of extension growth and sugar levels

The kinetics of extension growth and soluble carbohydrate levels (reducing sugars, sucrose) during the photoperiod were also compared. In the high R/FR ratio controls, extension growth peaked around midday (Fig. 7A). The promotion by low R/FR was maximal during the earliest hours of the photoperiod. The levels of reducing sugars per unit volume remained relatively stable and unaffected by R/FR ratio (Fig. 7B). The levels of sucrose were higher in high than low R/FR-treated internodes throughout the photoperiod (Fig. 7B).

Discussion

When low R/FR ratios promote stem growth, the effects on stem carbon accumulation depend on the organs that
receive the R/FR-ratio signal and on the species. When the whole shoot of mustard plants is exposed to low R/FR ratios, the stem shows increased rates of both extension and carbon accumulation (Casal et al., 1995). These effects on internode carbon gain are not simply the consequence of enhanced extension growth because they are observed even if extension growth is physically restricted (Casal et al., 1995). Low R/FR ratios reaching mustard leaves increase their ability to export sucrose (Yanovsky et al., 1995). In accordance with this scenario, when only the stem of mustard plants received low R/FR ratios, the rate of internode extension increased but the rate of dry matter gain showed no significant response (Fig. 1). Conversely, in sunflower, low R/FR ratios applied only to the first internode were enough to increase not only stem extension growth but also stem dry matter accumulation (Libenson et al., 2002) and stem recovery of radioactive carbon applied to the leaves (Fig. 1B). Low R/FR ratios increased internode levels of many metabolites (including reducing sugars and of cell wall carbohydrates) to the same extent as internode volume or fresh weight, maintaining constant the concentration of these metabolites (see Fig. 4A; Supplementary Table S1 at JXB online).

In contrast to the observations in mustard (Casal et al., 1995), in sunflower, physical restriction of stem extension growth simultaneously impaired stem carbon accumulation. Furthermore, when sunflower internodes were shifted from high to low R/FR ratios and vice versa, the rate of stem extension growth responded to the prevailing light conditions, but the rate of internode dry matter accumulation responded to both current and previous conditions (Fig. 2). These observations are consistent with a scenario where the enhanced download of carbon in the internodes exposed to low R/FR could partially be the consequence of the enhanced rate of stem extension growth and of the subsequent consolidation of previous growth by deposition of additional cell wall material (note increased cell wall carbohydrates per unit volume in the lower, compared with the upper section Fig. 4B). The relationship between internode carbon accumulation and the prevailing growth rate may be accounted for by the fact that, in actively growing cells, wall loosening reduces turgor pressure favouring the download of sugars from the phloem by mass flow (Cosgrove, 1987; Schmalstig and Cosgrove, 1990). In turn, the relationship between internode carbon accumulation and internode length (i.e. previous growth rate) may be accounted for by the differential use of soluble carbohydrates in the synthesis of structural polysaccharides for deposition following extension. The additional metabolism of sugars in longer internodes would dilute solutes inside the cell and this is also predicted to favour the entrance of carbohydrates from the phloem.

In addition to the predicted indirect effects of R/FR on internode carbon gain via its effects on extension growth, the R/FR ratio appears to have more direct effects. In fact, low R/FR ratios increased radioactive carbon recovery in the upper internode section, which showed increased internode extension growth, and also in the lower sections, where extension growth (and final size) was unaffected (Fig. 3). The reduction in sucrose concentration observed in response to low R/FR (Fig. 7), which is particularly strong in the upper section of the internode (Fig. 6) could be part of a mechanism to enhance a concentration gradient favouring phloem download by diffusion. In addition, a lower concentration could affect osmotic potential and reduce apoplastic water uptake and cell turgor pressure, also favouring the download of sugars from the phloem by mass flow.

Low R/FR ratios decreased the levels of three saturated fatty acids per unit fresh weight in sunflower stems (Fig. 5). It is interesting to note that, in soybean, shaded leaves (i.e. leaves exposed to low R/FR) also have reduced contents of polar lipids (Burkey et al., 1997). Low R/FR could increase lipid breakdown and/or reduce lipid synthesis since a high proportion of transcripts encoding proteins involved in fatty acid metabolism are down-regulated by low R/FR ratios (Devlin et al., 2003). Lipids are converted to sugars to generate energy and this appears to be important to maintain plant growth. In fact, icl mutants of Arabidopsis, lacking isocitrate lyase, grow poorly because they are unable to convert lipids to sugars (Eastmond et al., 2000) and seedlings of the mls mutants, lacking malate synthase (another key enzyme of the
glyoxalate cycle), grow faster because they can use lipids more rapidly and accumulate more sugars to sustain growth (Eastmond et al., 2000; Cornah et al., 2004). However, the effects of R/FR on saturated fatty acids occurred both in the upper and lower sections of the internode, despite the fact that R/FR only affected the growth rate of the upper section.

For many metabolites, the upper section of sunflower internodes showed higher levels per unit fresh weight than the lower section. These metabolites include organic acids, amino acids, fatty acid alcohols, and sterols (Figs 5, 6). In sugarcane stems, several amino acids and organic acids associated with the tricarboxylic acid cycle show higher abundance in the top section of the stem and decrease in concentration with tissue maturity (Glassop et al., 2007). In pea seedlings, lipid synthesis and lipid soluble compounds are higher in the expanding than in fully expanded leaves (Hellgren and Sandelius, 2001). In Arabidopsis, a subset of 85 genes involved in lipid metabolism is up-regulated in the epidermis of the upper section of the stem (Suh et al., 2005).

In the current climate of evaluating the potential use of crop plants for the production of biofuels, a better understanding of the physiological relationships between extension growth, carbon accumulation, and metabolic profile is essential. The results reported here indicate that increased stem extension can drive increased dry matter accumulation, but this depends on the species. Many soluble metabolites and cell wall carbohydrates respond in quantitative concert with the changes in extension growth, i.e. maintaining relatively constant rates per unit tissue

![Fig. 6. Polar metabolic profile of the first internode of sunflower plants is affected by internode R/FR ratio and ontogeny. Data are mean ±SE of six replicate samples. Values are normalized to the lower section of high R/FR-treated internodes. * P < 0.05, ** P < 0.01, *** P < 0.001, NS: not significant.](https://academic.oup.com/jxb/article-abstract/59/9/2469/464491)

![Fig. 7. Time-course of extension growth (A) and reducing sugars and sucrose (B) in sunflower internodes exposed to high or low R/FR ratios. Data are means ±SE of 24 replicate plants. * P < 0.05, statistics of reducing sugars yielded no significant differences.](https://academic.oup.com/jxb/article-abstract/59/9/2469/464491)
volume or fresh weight. Other metabolites, in particular, lipophilic metabolites organic acids or amino acids, show changes in response to the environment (R/FR ratio) and/or the ontogeny of the organ bearing no consistent association with growth.

Supplementary data

Supplementary data can be found at JXB online. Supplementary Table S1 contains the data of total polar (A) and apolar (B) metabolites identified by GC/MS in the upper and lower internode sections exposed to high or low R/FR ratios. Data are the average of six biological replicates and are expressed relative to the signal in the lower internode section exposed to high R/FR ratios.

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