Light- and Carbon-Signaling Pathways. Modeling Circuits of Interactions[1]

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Here, we report the systematic exploration and modeling of interactions between light and sugar signaling. The data set analyzed explores the interactions of sugar (sucrose) with distinct light qualities (white, blue, red, and far-red) used at different fluence rates (low or high) in etiolated seedlings and mature green plants. Boolean logic was used to model the effect of these carbon/light interactions on three target genes involved in nitrogen assimilation: asparagine synthetase (*ASN1* and *ASN2*) and glutamine synthetase (*GLN2*). This analysis enabled us to assess the effects of carbon on light-induced genes (*GLN2*/*ASN2*) versus light-repressed genes (*ASN1*) in this pathway. New interactions between carbon and blue-light signaling were discovered, and further connections between red/far-red light and carbon were modeled. Overall, light was able to override carbon as a major regulator of *ASN1* and *GLN2* in etiolated seedlings. By contrast, carbon overrides light as the major regulator of *GLN2* and *ASN2* in light-grown plants. Specific examples include the following: Carbon attenuated the blue-light induction of *GLN2* in etiolated seedlings and also attenuated the white-, blue-, and red-light induction of *GLN2* and *ASN2* in light-grown plants. By contrast, carbon potentiated far-red-light induction of *GLN2* and *ASN2* in light-grown plants. Depending on the fluence rate of far-red light, carbon either attenuated or potentiated light repression of *ASN1* in light-grown plants. These studies indicate the interaction of carbon with blue, red, and far-red-light signaling and set the stage for further investigation into modeling this complex web of interacting pathways using systems biology approaches.

Light is an important environmental signal that is directly perceived by the plant through photoreceptors and is essential for driving photosynthesis. As such, light provides the reducing power for carbon fixation, nitrogen assimilation, amino acid biosynthesis, and other necessary metabolic pathways. Information about light quality, intensity, and duration is measured through numerous photoreceptors (Mancinelli, 1994; Smith, 1994). Phytochromes are the primary red-light photoreceptors. The blue-light, UV-A/B photoreceptors include the cryptochromes, phototropin, and other yet unidentified photoreceptors (for review, see Briggs and Huala, 1999). The various qualities of light perceived through these photoreceptors control diverse developmental programs in plants such as seed germination, hypocotyl elongation, shade avoidance, circadian rhythms, flowering, chloroplast differentiation, and cotyledon expansion (for review, see Fankhauser and Chory, 1997; Briggs and Huala, 1999; Neff et al., 2000). The most well-characterized photoreceptors are the phytochromes. In Arabidopsis, five different phytochromes exist (phyA–E), each containing both overlapping and unique biological functions. PhyA is predominately involved in physiological responses to continuous far-red light, whereas phyB is involved in responses to red light. Additionally, phyA mediates responses to very low fluences of red, blue, and far-red light. At different stages of plant development the influence of each photoreceptor may change (for review, see Moller et al., 2002).

Light perception and signaling through various photoreceptors has been intensely investigated. The identification of downstream components of photoreceptor-signaling pathways has revealed cross-talk between pathways of different light qualities as well as with other seemingly unrelated pathways (for review, see Moller et al., 2002; Nagy and Schafer, 2002). For example, SUB1 is both a component of a cryptochrome-signaling pathway and a modulator of a phytochrome-signaling pathway (Guo et al., 2001). Auxin, brassinosteroid, gibberellic acid, cytokinin, and ethylene signal transduction pathways are all influenced by light-signaling pathways either directly or indirectly (for review, see Moller et al., 2002). Additionally, sugars that serve as growth and signaling molecules have been shown to modulate phytochrome sensing and signaling pathways (Barnes et al., 1996; Dijkwel et al., 1997; Short, 1999).

Sugars initiate changes in the expression of genes involved in diverse functions such as embryogenesis, flowering, seedling development, and senescence. Some genes encoding proteins involved in or relating to photosynthesis are strongly induced by light yet repressed by carbon (e.g. chlorophyll a/b binding

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protein, plastocyanin, and small subunit of Rubisco; Koch, 1996) or induced by both light and sugar (e.g. Gln synthetase, nitrate reductase, and Asn synthetase 2; Koch, 1996; Lam et al., 1998; Oliveira and Coruzzi, 1999). More specific interactions between carbon and light have been observed by the ability of Suc to suppress a phyA-specific, far-red-light-induced block of greening. Hence, Suc may antagonize or suppress the phyA-signaling pathway(s) in this case (Barnes et al., 1996). A class of Suc-uncoupled (*sun*) mutants have been identified that exhibit reduced Suc repression of light-induced genes and are defective in the ability of Suc to inhibit the far-red block of greening (Dijkwel et al., 1997). One of the *SUN* genes is identical to *ABI4*, a gene involved in abscisic acid signal transduction, suggesting the involvement of abscisic acid in sugar responses (Huijser et al., 2000). Additionally, use of a phyA or phyB pathway may be influenced by Suc where it may modify or change the preference of common downstream components of a phyA or phyB pathway (Short, 1999).

In contrast to the perception and transduction of light, our understanding of sugar perception and signaling is less well studied (for recent review, see Rolland et al., 2002). Although there is evidence that hexokinases HXK1 and -2 may act as sensors involved in sugar sensing and transduction in plants (Jang et al., 1997; Sheen et al., 1999), other mechanisms have also been proposed (Halford et al., 1999; for review, see Rolland et al., 2002). Many sugar-regulated genes have been categorized as being regulated through an HXK-dependent or -independent pathway, where other sugar-sensing pathways most likely also exist (Sheen et al., 1999). Although not much is known about downstream components of carbon-signaling pathways, a number of components are proposed to play a role including protein phosphatases, transcription factors, and numerous kinases (calcium-dependent, mitogen-activated, and SNF1-related; Sheen et al., 1999; Smeekens, 2000; Rolland et al., 2002). Mutants defective in sugar sensing and signaling have been isolated and demonstrate interactions with hormone signal transduction pathways (see Rolland et al., 2002). For example, Glc-insensitive mutants *gin5* and *gin6* show the involvement of abscisic acid in sugar responses (Arenas-Huertero et al., 2000), where they are allelic to genes (*aba3* and *abi4*) involved in abscisic acid biosynthesis or signal transduction, respectively (for review, see Rolland et al., 2002). As more information is gleaned about any signal transduction pathway, signaling pathways can no longer be studied in a "linear" manner, but the influence and integration of numerous pathways must be considered.

Light- and sugar-signaling pathways have been shown to regulate the transcription of genes involved in metabolism. For example, the assimilation of nitrogen into amino acids is a process partially controlled at the transcriptional level by light and sugar signaling. Gln synthetase (*GLN2*) and ferredoxin-Glu synthase, Fd-GOGAT (*GLU1*) are two enzymes involved in the assimilation of ammonia into Gln and Glu, whose genes are induced by light (Coschigano et al., 1998; Oliveira et al., 1999). In contrast, light represses transcription of the genes *ASN1* and *GDH1*, which encode the enzymes Asn synthetase and Glu dehydrogenase (Melo-Oliveira et al., 1996; Lam et al., 1998). Interestingly, exogenously supplied Suc has been shown to mimic the effect of light. Suc supplied to dark-adapted plants increases expression of *GLN2* and represses *ASN1* expression (Lam et al., 1998; Oliveira et al., 1999). Reciprocal regulation of the *GLN2*/*ASN1* genes by light reflects changes in the levels of their cognate amino acids, Gln and Asn. Gln levels are high in light-grown plants, and Asn levels are high in dark-grown/adapted plants (Lam et al., 1995; Ngai and Coruzzi, 1998). *ASN1* is the major gene controlling Asn synthesis in Arabidopsis, where expression of *ASN1* in the dark serves to enable the conversion of Gln to Asn, which is used for N-transport when C-skeletons are limiting (Lam et al., 1995). However, light activates the expression of *ASN2*, another member of the *ASN* gene family. *ASN2* is induced by both light and Suc (Lam et al., 1998). It is postulated that *ASN2* serves to synthesize low levels of Asn in the light, used for protein synthesis, whereas *ASN1* is responsible for making high levels of Asn for N-transport/storage in the dark (Lam et al., 1998). It has been shown that light regulation of the genes *GLN2*, *ASN1*, and *ASN2* are mediated in part via phytochrome in etiolated seedlings (Lam et al., 1994; for review, see Lam et al., 1996; Hsieh, 1999) and also through the indirect effects of light (e.g. light-induced increases in carbon).

This study represents the first systematic approach to investigate the interactions between light- and carbon-signaling pathways. *ASN1*, *ASN2*, and *GLN2* serve as sentinel genes for the examination of light and carbon interactions. Expression profiles of these genes were analyzed in plants treated with different wavelengths of light at low- or high-fluence rates in the presence or absence of a carbon source. Etiolated and light-grown plants were analyzed to investigate possible differences in light- and carbon-signaling cross-talk in these very different stages of development. Because these pathways are expected to be complex, their interactions were analyzed and modeled using Boolean circuits. Depending on the developmental stage of the plant and the gene analyzed, it is shown carbon can attenuate, potentiate or enhance light responses at specific wavelengths and fluence rates.

RESULTS

"Experimental Space" for Investigating Light and/or Carbon Signaling

The experiments represented in Table I were designed in a systematic manner (a) to further investi-

Table I. *Experimental space for investigating light and/or carbon signaling*

Variables or inputs for each experiment are indicated as present (Y) or absent (N). The carbon source was 1% (w/v) Suc, and fluence rates for white light were 70 μE m^{-2} s^{-1} and 2 or 100 μE m^{-2} s^{-1} for blue, red, and far-red light.

Experiment No.	Carbon	White Light	Blue Light		Red Light		Far-Red Light	
			Low-Fluence	High-Fluence	Low-Fluence	High-Fluence	Low-Fluence	High-Fluence
1	N	Y	N	N	N	N	N	N
2	N	N	Y	N	N	N	N	N
3	N	N	N	Y	N	N	N	N
4	N	N	N	N	Y	N	N	N
5	N	N	N	N	N	Y	N	N
6	N	N	N	N	N	N	Y	N
7	N	N	N	N	N	N	N	Y
8	N	N	N	N	N	N	N	N
9	Y	N	N	N	N	N	N	N
10	Y	Y	N	N	N	N	N	N
11	Y	N	Y	N	N	N	N	N
12	Y	N	N	Y	N	N	N	N
13	Y	N	N	N	Y	N	N	N
14	Y	N	N	N	N	Y	N	N
15	Y	N	N	N	N	N	Y	N
16	Y	N	N	N	N	N	N	Y

gate the individual light qualities and quantities regulating genes involved in nitrogen assimilation and (b) to investigate the influence of carbon on these specific light-signaling pathways in an attempt to further our understanding of light- and carbon-signaling interactions. The experimental setup consisted of using carbon (supplied exogenously as Suc) as a binary input (±) combined with various light qualities. Experiments 1 through 8 were designed to investigate the influence of individual light qualities in the absence of an exogenously supplied carbon source. Experiments 9 through 16 were designed to investigate the influence of carbon on the individual light qualities.

Light Overrides Carbon Regulation of *ASN1* and *GLN2* But Not *ASN2* in Etiolated Seedlings

Quantitative real-time PCR was used to monitor the transcript abundance of *ASN1*, *ASN2*, and *GLN2* in etiolated plants treated with light and/or Suc (Fig. 1). Control transcripts from a putative clathrin coat assembly protein (At4g24550; *CLH*) were also detected and used as a normalization control. *CLH* was chosen as a control gene because these transcripts remained unchanged in plants at different developmental stages (etiolated versus light grown) and in response to light, carbon, or nitrogen (G.M. Coruzzi laboratory, unpublished data). Plants were grown in the absence or presence of 1% (w/v) Suc for 7 d in continuous darkness. After this growth period, plants were maintained in continuous darkness or illuminated with white light (WL) at 70 μE m^{-2} s^{-1} or with blue, red, or far-red light separately at 2 or 100 μE m^{-2} s^{-1} for an additional 3 h (Fig. 1; see Table I for experimental design). The results shown in Figures 1 and 2 are from five replicates.

Figure 1A, 1 shows the high level accumulation of *ASN1* transcripts in dark-grown plants in the absence of Suc. Illumination of these plants with WL, blue, red, or far-red light at either a low- or high-fluence rate decreased *ASN1* transcript levels (Fig. 1, A–C, 1 versus 2–4). The presence of Suc in the media caused a dramatic decrease in the amount of *ASN1* transcripts in the absence of light (Fig. 1A, 5). Illumination of these plants in all light conditions in the presence of Suc further reduced *ASN1* transcripts to almost undetectable levels (Fig. 1, A–C, 5 versus 6–8).

ASN2 transcript levels in dark Suc-free-grown plants were low, and illumination with any of the light qualities or fluency rates used in this study had no significant effect on *ASN2* transcript abundance (Fig. 1, D–F, 1 versus 2–4). The presence of Suc on etiolated seedlings increased *ASN2* transcripts in the absence of light (Fig. 1D, 5). Illumination of these plants with most light qualities and quantities increased the level of *ASN2* transcripts (Fig. 1, D–F, 5 versus 6–8). Interestingly, illumination with blue light at 100 μE m^{-2} s^{-1} resulted in a decrease of *ASN2* transcripts below that observed for dark-grown plants (Fig. 1D).

Consistent with the reported reciprocal regulation of *ASN1* and *GLN2* (Lam et al., 1994), *GLN2* mRNA levels were low in etiolated seedlings grown in the absence of Suc (Fig. 1G, 1). Illumination of these plants with any of the light qualities and quantities in this study increased *GLN2* transcripts, albeit to varying degrees (Fig. 1, G–I, 1 versus 2–4). *GLN2* mRNA levels increased in etiolated plants grown in the presence of Suc (Fig. 1G, 1 versus 5). Illumination of these plants with all light qualities and quantities except for blue light at 100 μE m^{-2} s^{-1}, increased *GLN2* transcripts (Fig. 1, G–I, 5 versus 6–8). An effect of

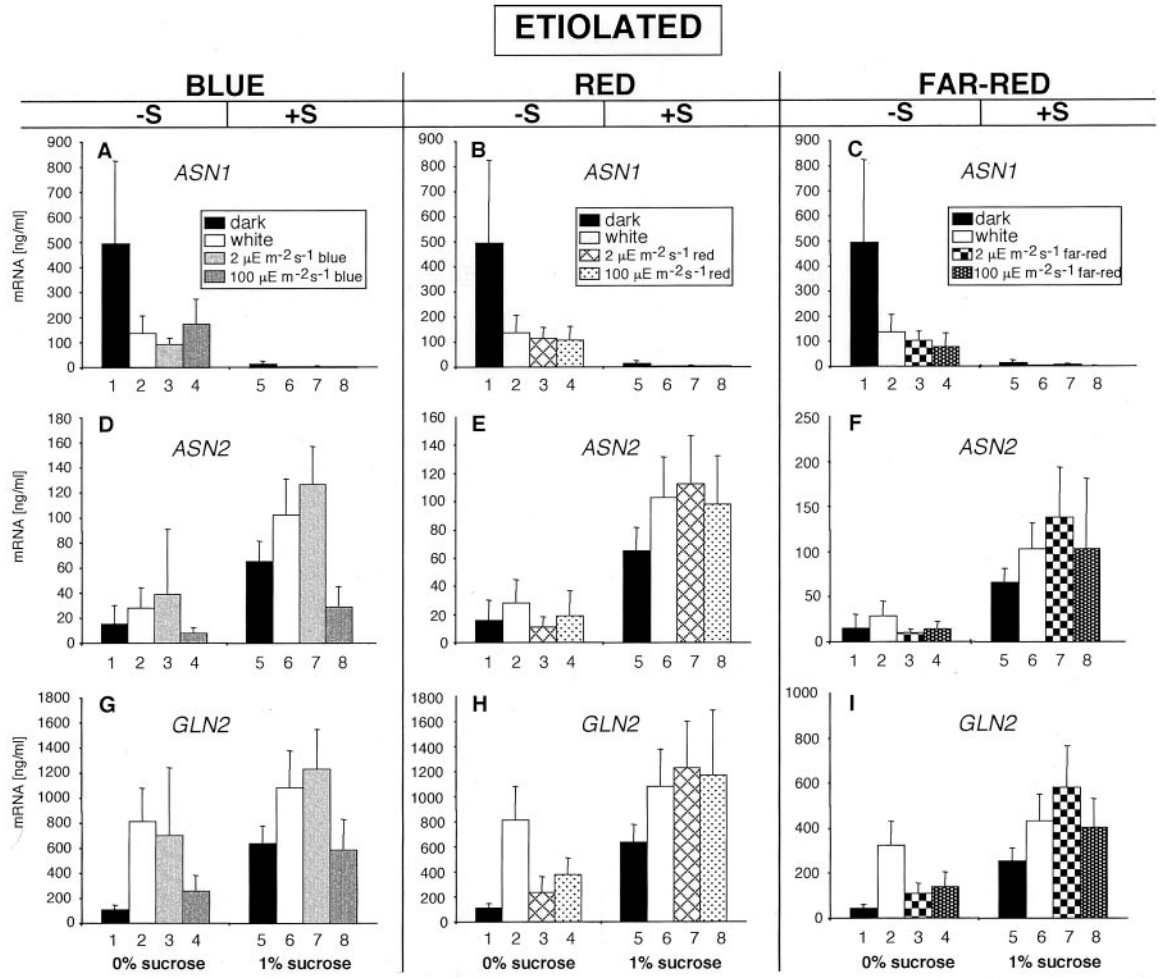

Figure 1. Analysis of *ASN1*, *ASN2*, and *GLN2* transcript accumulation in etiolated seedlings. A through C, *ASN1* transcript levels in 7-d-dark-grown plants in the presence or absence of carbon and illuminated with WL, blue (A), red (B), or far-red (C) light. D through F, *ASN2* transcript levels in 7-d-dark-grown plants in the presence or absence of carbon and illuminated with WL, blue (D), red (E), or far-red (F) light. G through I, *GLN2* transcript levels in 7-d-dark-grown plants in the presence or absence of carbon and illuminated with WL, blue (G), red (H), or far-red (I) light. The carbon source used was 1% (w/v) Suc. All transcripts were measured using real-time quantitative PCR and normalized to a putative clathrin coat-assembly protein (At4g24550). The data represent the mean and SD of at least five separate experiments.

high-fluence blue light in the presence of Suc but not in the absence can also be observed for *ASN2*.

Carbon Overrides Light Regulation of *GLN2* and *ASN2* But Not *ASN1* in Light-Grown Plants

As with the analysis for etiolated seedlings, quantitative real-time PCR was used to characterize Suc and/or light-modulated changes in *ASN1*, *ASN2*, and *GLN2* transcript abundance in 14-d-light-/dark-grown plants (Fig. 2). After dark adaptation, plants were maintained in continuous darkness or illuminated with WL at 70 μE m^{-2} s^{-1}, or with blue, red, or far-red light separately at 2 or 100 μE m^{-2} s^{-1} for an additional 3 h.

ASN1 mRNA levels were high in dark-adapted plants in the absence of Suc (Fig. 2A, 1), and illumination of these plants with most light qualities and fluence rates used in this study decreased *ASN1* transcripts to varying degrees (Fig. 2, A–C, 1 versus 2–4). One exception is the illumination with 2 μE m^{-2} s^{-1} of far-red light, which appears to be unable to repress *ASN1*. The presence of Suc on plants in the dark, resulted in a decrease in *ASN1* transcript levels compared with those observed for *ASN1* in plants in the absence of Suc (Fig. 2A, 5). Illumination of dark-adapted plants in the presence of Suc with all of the light qualities and quantities further reduced *ASN1* mRNA levels (Fig. 2, A–C, 5 versus 6–8).

Transcript levels of *ASN2* were low in dark-adapted plants in the absence of Suc and could be increased, albeit at varying levels, by illumination with most light qualities except far-red at 2 μE m^{-2} s^{-1} (Fig. 2, D–F, 1 versus 2–4). Dark-adapted plants in the presence of Suc had higher levels of *ASN2* mRNA compared with those in the absence of Suc (Fig. 2D,

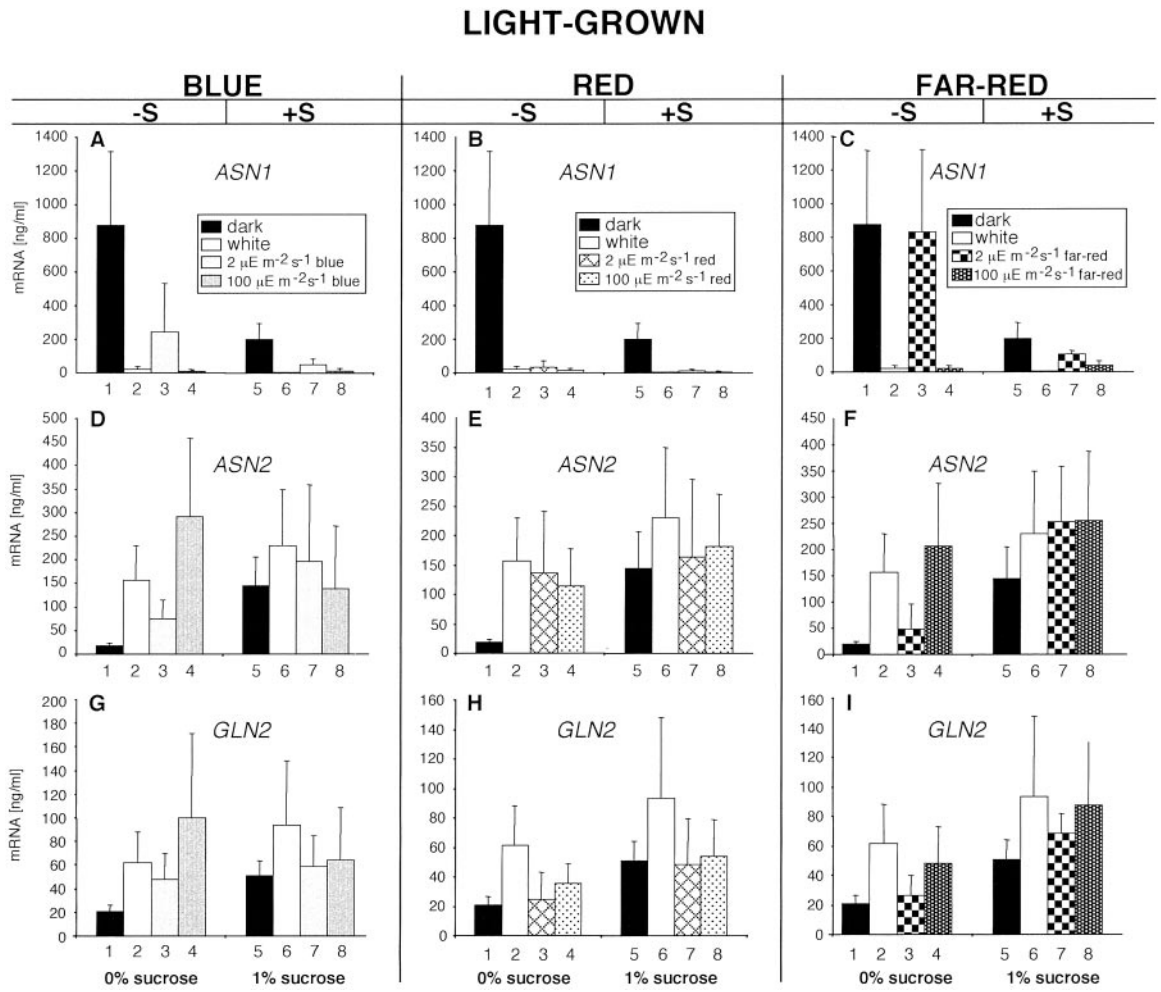

Figure 2. Analysis of *ASN1*, *ASN2*, and *GLN2* transcript accumulation in light-grown plants. A through C, *ASN1* transcript levels in 14-d-light-/dark-grown plants in the presence or absence of carbon and illuminated with WL, blue (A), red (B), or far-red (C) light. D through F, *ASN2* transcript levels in 14-d-light-/dark-grown plants in the presence or absence of carbon and illuminated with WL, blue (D), red (E), or far-red (F) light. G through I, *GLN2* transcript levels in 14-d-light-/dark-grown plants in the presence or absence of carbon and illuminated with WL, blue (G), red (H), or far-red (I) light. The carbon source used was 1% (w/v) Suc. All transcripts were measured using real-time quantitative PCR and normalized to a putative clathrin coat-assembly protein (At4g24550). The data represent the mean and SD of at least five separate experiments.

5). Illumination of dark-adapted plants with WL or far-red light (2 or 100 μE m^{-2} s^{-1}) was able to significantly increase *ASN2* transcript levels, where blue and red light had minimal effects (Fig. 2, D–F, 5 versus 6–8).

GLN2 mRNA levels were low in dark-adapted plants in the absence of Suc (Fig. 2G, 1), and illumination with most light qualities and quantities except red and far-red light at 2 μE m^{-2} s^{-1} increased *GLN2* transcript abundance (Fig. 2, G–I, 1 versus 2–4). As observed with *ASN2*, albeit more modest, the presence of Suc in dark-adapted plants resulted in an increase of *GLN2* transcripts (Fig. 2G, 5). Only illumination of these plants with WL or far-red light at 2 or 100 μE m^{-2} s^{-1} was able to increase *GLN2* transcript levels above those observed for dark-adapted plants in the presence of Suc alone (Fig. 2I, 5 versus 7 and 8). Red- or blue-light illumination of plants in the presence of Suc was unable to alter *GLN2* transcript levels beyond those observed for plants in the presence of Suc (Fig. 2, G and H, 5 versus 6–8).

Boolean Circuits Determine Significant Regulators of *ASN1*, *ASN2*, and *GLN2*

To model the interactions of light and carbon signaling, we use Boolean logic to analyze the data generated from the experiments shown in Table I. In brief, two base conditions, no light/no carbon (Table I, experiment 8) and no light/carbon (Table I, experiment 9) were used for comparison against all non-base conditions (all other experiments). Specific thresholds were assigned where expression levels relative to the base condition were categorized as inductive, super-inductive, repressive, or super-repressive. On the basis of an unpaired *t* test (P =

0.05), a particular Boolean input is deemed statistically significant and affects the output, whereas the absence of a Boolean input is due to either statistical insignificance or due to no effect of the input.

Figure 3 shows Boolean circuits for *GLN2*, *ASN2*, and *ASN1* in etiolated plants in the absence or presence of carbon. Figure 3A shows that WL OR red light low fluence (RLF) OR red light high fluence (RHF) OR far-red light low fluence (FRLF) OR far-red light high fluence (FRHF) OR blue light low fluence (BLF) OR blue light high fluence (BHF), singly, in the absence of carbon each induce expression of *GLN2*. In the presence of carbon, all light qualities at different fluence rates induce *GLN2*, except for BHF. Interestingly, light has no significant effect on *ASN2* expression levels in the absence of carbon, whereas in the presence of carbon, light becomes inductive for all light qualities with the exception of BHF, where it is repressive (Fig. 3B). *ASN1* is repressed by all light qualities at any quantity in the absence of carbon, whereas only WL, RHF, and BHF are repressive in the presence of carbon, and FRHF becomes super-repressive (Fig. 3C).

Figure 4 shows Boolean circuits for *GLN2*, *ASN2*, and *ASN1* in 14-d-light-grown plants in the absence or presence of carbon. In the absence of carbon, WL, RHF, FRHF, BLF, and BHF each induce *GLN2* expression (Fig. 4A). In the presence of carbon, FRHF remains and FRLF becomes inductive. *ASN2* is super-induced by BHF and FRHF and induced by WL, RLF, RHF, or BLF in the absence of carbon (Fig. 4B). In the presence of carbon, *ASN2* is induced only by FRLF or FRHF, as shown also for *GLN2*. *ASN1* is repressed by BLF and super-repressed by WL, BHF, RHF, or FRHF in the absence of carbon (Fig. 4C). In the presence of carbon, the super-repression of *ASN1* by WL,

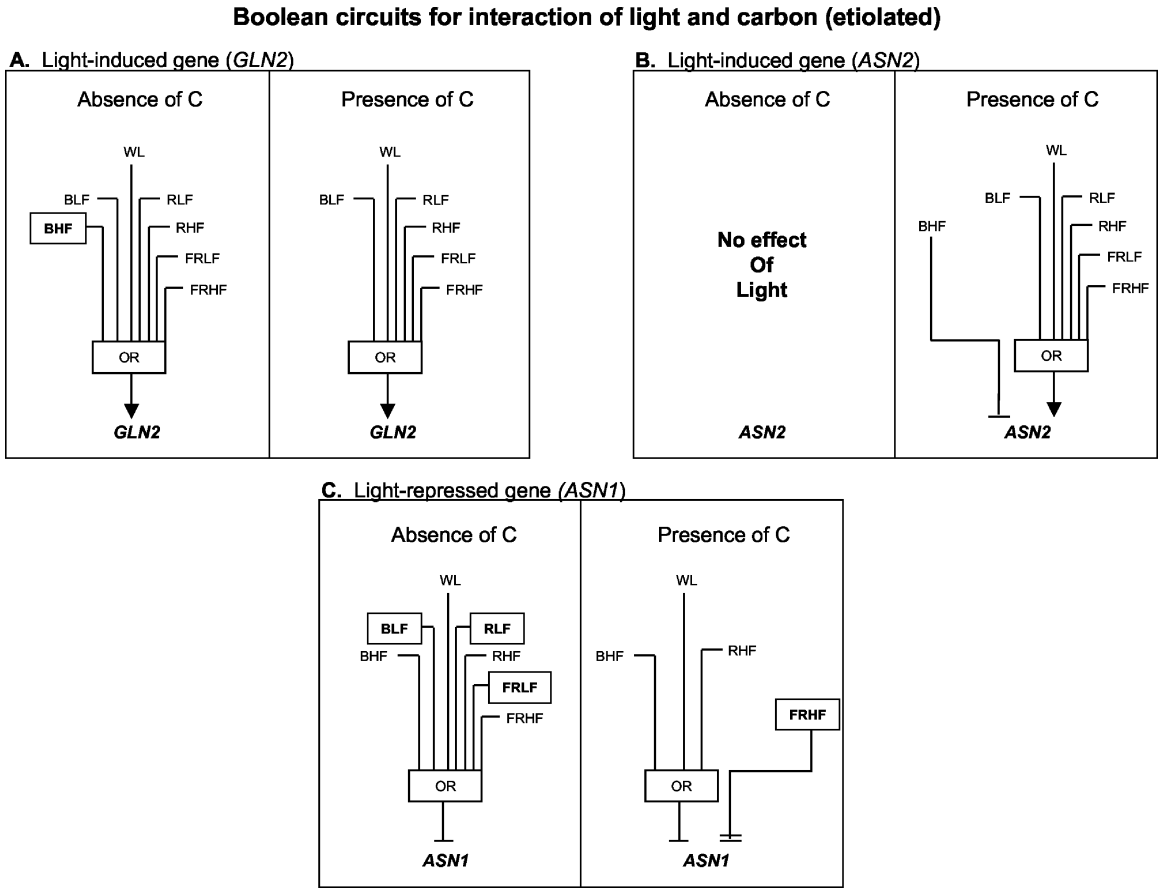

Figure 3. Boolean circuits model *ASN1*, *ASN2*, and *GLN2* regulation by light and carbon in etiolated seedlings. A through C, Boolean circuits based on 16 experiments represented in Table I. A, *GLN2* regulation by WL, blue, red, or far-red light when compared against a base case of no light, no carbon, or no light, carbon (see "Materials and Methods"). B, *ASN2* regulation by WL, blue, red, or far-red light when compared against a base-case of no light, no carbon, or no light, carbon. C, *ASN1* regulation by WL, blue, red, or far-red light when compared against a base case of no light, no carbon, or no light, carbon. The inputs are WL, BLF, BHF, RLF, RHF, FRLF, or FRHF. Low fluence is 2 μE m^{-2} s^{-1}; high fluence is 100 μE m^{-1} s^{-1}. The arrow or barred lines indicate the function of the inputs as either inductive or repressive. Double arrows or double bars denote super-induction or super-repression, respectively. For a Boolean OR, if any one of the inputs is active, the output will also be active. Differences in the input for Boolean circuits when comparing "absence of carbon" to "presence of carbon" are shown by boxed inputs except for *ASN2* where everything is different in the presence versus absence of carbon.

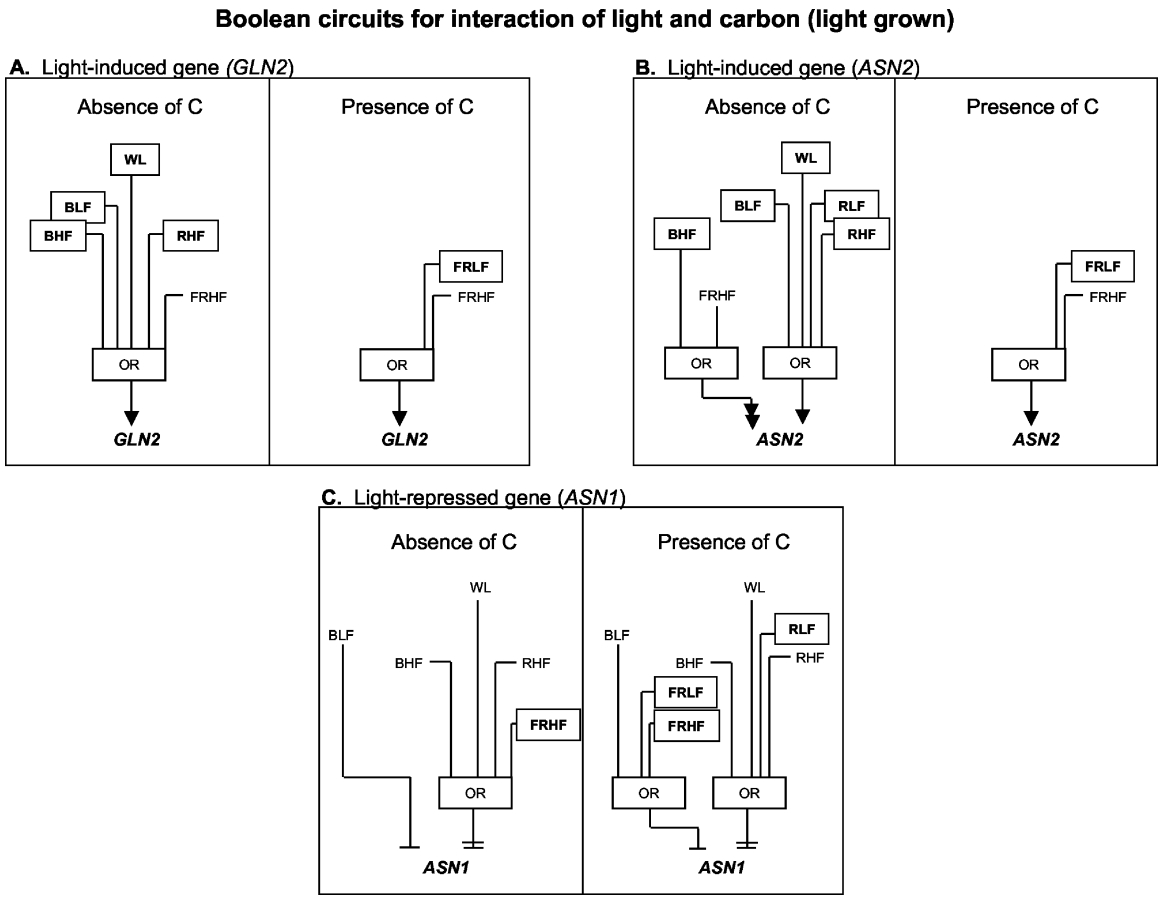

Figure 4. Boolean circuits model *ASN1*, *ASN2*, and *GLN2* regulation by light and carbon in light-grown plants. A through C, Boolean circuits based on 16 experiments represented in Table I. A, *GLN2* regulation by WL, blue, red, or far-red light when compared against a base case of no light, no carbon, or no light, carbon (see "Materials and Methods"). B, *ASN2* regulation by WL, blue, red, or far-red light when compared against a base case of no light, no carbon, or no light, carbon. C, *ASN1* regulation by WL, blue, red, or far-red light when compared against a base case of no light, no carbon, or no light, carbon. The inputs are WL, BLF, BHF, RLF, RHF, FRLF, and FRHF. Low fluence is 2 μE m^{-2} s^{-1}; high fluence is 100 μE m^{-1} s^{-1}. The arrow or barred lines indicate the function of the inputs as either inductive or repressive. Double arrows or double bars denote super-induction or super-repression, respectively. For a Boolean OR, if any one of the inputs is active, the output will also be active. Differences in the input for Boolean circuits when comparing "absence of carbon" to "presence of carbon" are shown as boxed inputs.

BHF, and RHF remains and RLF becomes super-repressive. BLF remains repressive and FRLF and FRHF become repressive for *ASN1* in the presence of carbon.

DISCUSSION

In this study, we employed a systematic approach to investigate and model the interactions between light- and carbon-signaling pathways. Because very little is known about the interactions between these two pathways, all possible combinations of light (WL, BLF, BHF, RLF, RHF, FRLF, and FRHF) and carbon were examined in both etiolated and light-grown seedlings in an attempt to cover a systematic experimental space. The analysis and modeling of these results as Boolean circuits represents a novel method to investigate complex interactions of carbon and light signaling and to identify the major regulatory signals. This analysis revealed interactions between carbon and light that are distinct in etiolated versus green plants, and ones that are specific to a gene or condition. A summary of all results can be found in Table II. In etiolated seedlings, light was generally able to override carbon as a major regulator of *ASN1* and *GLN2* expression. By contrast, in light-grown plants, carbon was shown to override light as the major regulator of *GLN2* and *ASN2* expression. Additionally, carbon was shown to interact with blue, red, or far-red light-signaling pathways in both etiolated and light-grown plants, where carbon was shown to either potentiate or attenuate specific light responses. The significance of these major findings in this study are addressed below. This initial analysis of light and carbon interactions provides the framework for further experiments that we have designed

Table II. *C:L Interactions Discovered by Boolean Analysis*

C, carbon; L, light; WL, white light; BLF, blue light low fluence; BHF, blue light high fluence; RLF, red light low fluence, RHF, red light high fluence; FRLF, far-red low fluence; FRHF, far-red high fluence; E, etiolated; LG, light-grown; NI, no interaction.

A *Overall C:L Interactions*

Developmental stage	Input	Genes Affected
Etiolated seedlings	L > C	*ASN1 / GLN2*
Light-grown plants	C > L	*GLN2 / ASN2*

B *C/Light Quality Interactions*

Light quality	*GLN2*	*ASN2*	*ASN1*
BLF	LG	E / LG	E
BHF	E / LG	E / LG	NI
RLF	NI	E / LG	E / LG
RHF	LG	E / LG	NI
FRLF	LG	E / LG	E / LG
FRHF	NI	E / LG	E / LG

C *Gene-Specific Effects of Carbon on Light Regulation*

	Gene	Etiolated Seedlings	Light-grown plants
Light-induced	*GLN2*	1. C attenuates BHF induction	1. C attenuates WL, BLF, BHF, RHF induction 2. C potentiates FRLF induction
	ASN2	1. C potentiates all light responses	1. C attenuates WL, BLF, BHF, RLF, RHF, FRHF induction 2. C potentiates FRLF induction
Light-repressed	*ASN1*	1. C attenuates BLF, RLF, FRLF repression 2. C enhances FRHF repression	1. C potentiates RLF, FRLF repression 2. C attenuates FRHF repression

using "combinatorial design" to understand how interactions of distinct light qualities may also be affected by interactions with carbon.

Boolean Circuits for Analysis of Complex Interactions

Modeling the cellular activity of a set of genes/proteins as a functional network permits researchers to devise predictive models that may eventually permit intervention in pathways for diagnostic and therapeutic purposes. Two major kinds of network circuits are possible: discrete and continuous. The simplest discrete model is a Boolean network model in which input variables such as light (used here) can be set to one of several values 0/LF/HF, and gene regulation results from a Boolean function, possibly augmented by continuous elements such as amplifiers (Davidson et al., 2002). By contrast, continuous models may be based on stochastic kinetics (Arkin et al., 1998; Goss and Peccoud, 1998) and may include hidden environmental variables (Weaver et al., 1999). Many researchers believe that continuous models are more faithful to nature than discrete ones. For example D'haeseleer et al. (2000) note that Boolean networks do not provide a suitable framework to model feedback and other elements of biological control. Whereas Boolean models are incomplete compared with continuous models, they are more robust to biological noise and are easier to understand. They must be augmented with amplifiers as Davidson et al. (2002) have done (Yuh et al., 1998) enabling one to model, for example, a situation in which input A alone produces no change, input B gives induction, but A and B together give super-induction.

Boolean analysis requires inputs to be described in absolute terms of either having an effect or not having an effect (based on statistical analysis), which may not accurately represent biological systems, as discussed above. In our study, gene responses were deemed significant or not significant based on an unpaired t test at a P value of 0.05, where the presence of a particular Boolean input in the model represents cases where the input(s) had a statistically significant effect on the output (gene expression; Figs. 3 and 4). The absence of a Boolean input is either due to statistical insignificance of the effect or due to no effect of the input on gene expression. To address the biological relevance of this approach, we also carried out Boolean analyses at a lower P value of 0.1 (data not shown). A comparative analysis showed that few minor differences in the Boolean circuits were observed between data analyzed at $P = 0.05$ versus $P = 0.1$.

Boolean analyses for the modeling of plant-signaling networks have previously been described (Genoud and Metraux, 1999; Genoud et al., 2001). Here, we used this type of Boolean analysis to investigate how light and/or carbon interactions affected the regulation of three genes involved in nitrogen assimilation. Figures 3 and 4 give the simplest and

most likely Boolean circuits to explain our results thus far. This analysis indicates that any one of a set of light conditions has the effect shown (either inductive or repressive; Figs. 3 and 4). The "OR" indicates that any of the light qualities tested give similar results. The simplicity of these circuits reflects the fact that, so far, our data do not explore combinations of light quality. We propose methods to test such combinations at the end of this discussion. Further experiments involving multiple inputs can be integrated into these already existing circuits to observe their effect on the output, or on regulation of *GLN2*, *ASN2*, and *ASN1*. Finally, our description of these light and carbon interactions in a binary manner serves as a precursor for the eventual computer modeling of more complex signaling pathways, with dose and kinetic parameters included.

Biological Significance of the Major Conclusions of Light/Carbon Interactions

Light Overrides Carbon as the Major Regulator of ASN1 and GLN2 in Etiolated Plants

In general, light overrides carbon regulation of *ASN1* and *GLN2* expression in etiolated seedlings (Fig. 3, A and C). This regulation may occur because these plants are not yet photosynthetically active, and because light of all wavelengths is required for the induction of genes encoding proteins involved in chloroplast development, metabolism, and the further development of etiolated plants. The primary regulation of *GLN2* expression by light in etiolated seedlings makes sense physiologically, because such pre-induction by light (before photosynthate) will make Gln synthetase available for primary nitrogen assimilation using the energy generated through photosynthesis. Because Asn synthetase (*ASN1*) catalyzes the synthesis of Asn from Gln in the dark when C-skeletons are limiting, the presence of this enzyme in illuminated seedlings is unnecessary, hence the repressive effect of light dominates. However, the repressive effects of some qualities of light on *ASN1* expression are enhanced (FRHF)/or attenuated (BLF, RLF, and FRLF) by Suc, suggesting that some light and carbon interactions exist, requiring further investigation. Interestingly, regulation of *ASN2* expression is an exception to the observed general effect of light overriding C-regulation of *ASN1*/*GLN2*, where in fact, the induction of *ASN2* by light requires a carbon interaction in etiolated seedlings (Fig. 3B).

Carbon Overrides Light as the Major Regulator of GLN2 and ASN2 in Light-Grown Plants

Carbon appears to attenuate the light regulation of *GLN2* and *ASN2* expression in light-grown plants. In the absence of carbon, light has a major influence on regulation of *ASN2* and *GLN2* expression. By contrast, in the presence of carbon, the effect of light becomes negligible (Fig. 4, A and B). The ability of a plant to sense and respond to light in the absence of carbon assures that at the onset of photosynthesis, the assimilation of nitrogen onto C-skeletons will be immediate via *GLN2*/*ASN2* activation. At the onset of photosynthesis, however, the predominant regulation of these genes by carbon enables the plants to regulate nitrogen assimilation in response to levels of photosynthate.

By contrast to *GLN2*/*ASN2*, the expression of *ASN1*, appears to be equally regulated by both light and carbon at this stage of development. Because *ASN1* is most likely involved in the dark synthesis of Asn, the preferred amino acid for the transport of nitrogen in dark-adapted plants, repression by both light and carbon guarantees the absence of this enzyme in plants in the light whether or not they are photosynthesizing. The different regulation of these genes in etiolated versus light-grown plants may be due to different regulatory pathways or that some of the signaling components regulating these genes in light-grown plants are not yet present in etiolated seedlings.

Gene-Specific Interactions of Light and Carbon

Carbon Attenuates Blue-Light Induction of GLN2 in Etiolated Seedlings

Boolean analysis of the data from Table I showed that BHF was able to induce *GLN2* expression in the absence of Suc, but not in the presence of Suc. This indicates that BHF perception or signaling may be antagonized by carbon in etiolated seedlings (Fig. 3A). These results suggest a fluence-rate dependence of the carbon/blue-light interaction, because carbon affected the BHF response and not the BLF response. A detailed fluence rate study needs to be carried out for blue light in the presence or absence of carbon to confirm these preliminary findings.

ASN2 Is Repressed by BHF in the Presence of Carbon in Etiolated Seedlings

Interactions between blue-light and carbon signaling were further observed to regulate expression of the *ASN2* gene. We found that repression of *ASN2* by blue light occurs only in the presence of Suc (Fig. 3B). This suggests a Suc dependence of blue-light signaling. Because the presence of Suc potentiates all light responses for *ASN2* (Fig. 3B), the interaction between BHF and Suc may be a general effect of carbon. However, the finding that BHF represses *ASN2* expression is surprising because, as shown in this study, all other light qualities are inductive for *ASN2*. Thus, this appears to be a specific interaction of BHF light and Suc.

Carbon Affects Far-Red Light Repression of ASN1 in Etiolated Seedlings

ASN1 expression is repressed by FRHF and FRLF in the absence of carbon, whereas FRHF becomes super-repressive in the presence of carbon (Fig. 3C). These results suggest that the fluence rate of far-red light influences its interaction with carbon. Carbon attenuates the repression of *ASN1* by FRLF and yet it enhances repression of *ASN1* by FRHF. The additive effect of FRHF and carbon results in super-repression of *ASN1*, suggesting that they are two separate pathways converging on this gene. Alternatively, carbon and far-red-light may affect the same pathway, where the level of either signal by itself was not high enough to maximize the effect. Carbon and phyA interactions have been documented, where carbon antagonizes a phyA pathway in etiolated seedlings (Barnes et al., 1996). PhyA is the predominant red/far-red light-absorbing phytochrome present in etiolated seedlings where it most likely plays a role in the repression of *ASN1*.

For *ASN1* repression, carbon seems to antagonize only FRLF but not FRHF (Fig. 3C). Carbon may interfere with FRLF repression, or because this is observed for light at all low fluences in this study, it is more likely a general effect and not specific to any wavelength of light. This suggests that it may be the number but not wavelength of photons that is important in carbon/light interactions. Carbon attenuation of *ASN1* repression by FRLF may be due to carbon overriding or masking the repression of low fluences of light, or it could be that the differences between low- and high-fluence light at any wavelength are not large enough to distinguish between the two effects on *ASN1* repression.

Carbon Attenuates Light Induction of GLN2 and ASN2 in Light-Grown Plants

The induction of *GLN2* in the absence of carbon requires WL, BLF, BHF, or RHF (Fig. 4A). By contrast, in the presence of Suc, these wavelengths of light no longer induce *GLN2* expression in light-grown plants. This regulation of *GLN2* is similar to that observed for *ASN2* (Fig. 4B). Carbon may attenuate the WL, blue-, or red-light induction of *ASN2* and *GLN2*, or it is possible that carbon overrides the induction of these wavelengths of light. Some physiological responses require co-action between light qualities, specifically between red and blue light (Chon and Briggs, 1966; Mohr, 1994). Because *GLN2* and *ASN2* are not modulated by blue or red monochromatic illumination in the presence of Suc, it would be interesting to investigate dichromatic illumination in the presence of Suc to see whether these wavelengths together are able to significantly induce the expression of *ASN2* and *GLN2*. Because the co-action between wavelengths of light should be observed by illumination with WL, it is possible that the WL used in this study did not encompass the appropriate fluence rates of blue and red light to observe this interaction.

Carbon Potentiates Far-Red-Light Induction of GLN2 and ASN2 in Light-Grown Plants

FRLF induces *GLN2* and *ASN2* in the presence of carbon and has no effect on the expression of these genes in the absence of carbon (Fig. 4, A and B). This suggests that the presence of carbon permits regulation by far-red light of these genes. The fact that in the presence of Suc, far-red is the only wavelength of light able to induce the expression of *ASN2* and *GLN2* suggests that phytochrome is involved in the regulation of these genes in photosynthetically active plants. Exploitation of different phytochrome mutants in this response will be useful to identify the photoreceptor (s) involved in this response. Additionally, it is interesting that *GLN2* and *ASN2* both retain the same regulation by far-red light in the presence of Suc. It is possible that these genes share a similar pathway for far-red-light regulation in the presence of Suc in light-grown plants.

Carbon Affects Far-Red-Light Repression of ASN1 in Light-Grown Plants

ASN1 is super-repressed by FRHF in the absence of carbon, whereas in the presence of carbon, FRHF is only repressive (Fig. 4C). This suggests that carbon may antagonize the far-red super-repression of *ASN1*. FRLF is only repressive in the presence of carbon, indicating that carbon may potentiate the far-red repression of *ASN1*. The interaction between carbon and far-red light suggests carbon interacts with a phytochrome-signaling pathway. By contrast, blue-light repression of *ASN1* occurs in the presence or absence of carbon (Fig. 4C), indicating the involvement of a blue-light photoreceptor or signaling pathway that is carbon independent.

Interactions between Distinct Light Qualities and Carbon

Previous studies have shown interactions between carbon and phytochrome signaling (Barnes et al., 1996; Dijkwel et al., 1997; Short, 1999). Our studies extend this analysis to describe models for specific interactions of carbon and red- versus far-red-light signaling. In addition, we discovered that blue-light perception or signaling is influenced by the carbon status of the plants. This is of new and particular interest, suggesting an interaction between blue-light and sugar signaling. The photoreceptor, cryptochrome 1 is primarily involved in BHF-light responses (Cashmore, 1997; Cashmore et al., 1999), phototropin is involved in BLF-light responses (Liscum and Briggs, 1995), and cryptochrome 2 plays a role in both BHF- and BLF-light responses (Lin,

2000). Experiments are under way in our lab to identify the blue-light-signaling pathway affected by Suc through the analysis of these blue-light photoreceptor mutants.

Combinatorial Design for Further Analysis of Complex Interactions

This initial, systematic investigation into the interactions of light and/or carbon signaling was investigated by looking at monochromatic light of different wavelengths and fluences independent of each other, in the absence or presence of carbon. This work sets the stage for further investigation into light and carbon signaling using photoreceptor mutants and downstream light-signaling mutants. It is also known that complex interactions exist between different qualities of light, where some physiological responses require dichromatic wavelengths of lights to achieve their maximum effects (Chon and Briggs, 1966; Mohr, 1994). These interactions of light qualities and the possible influence of Suc on these interactions are of interest to us. Table III shows additional experiments designed using combinatorial design to investigate how combinations of multiple light quality inputs, and their interactions with each another and with carbon can ultimately affect gene expression (Shasha et al., 2001; L.V. Lejay, D.E. Shasha, A.Y. Kouranov, P.M. Palenchar, A.A. Cruikshank, M. Chou, and G.M. Coruzzi, unpublished data). Combinatorial design allows a minimal number of experiments to be designed that cover a large experimental space of treatment conditions (Shasha et al., 2001; L.V. Lejay, D.E. Shasha, A.Y. Kouranov, P.M. Palenchar, A.A. Cruikshank, M. Chou, and G.M. Coruzzi, unpublished data). The design of experiments in this manner should allow us to study and model a large web of light and carbon interactions, using a minimal number of samples, amenable to genome scale analyses. Furthermore, such studies that include the analysis of genome-scale data should allow us to model networks of genes that are the downstream targets of converging light- and carbon-signaling pathways in plants. Such information should enable us to predict how changes in light quality, and photosynthesis will affect many processes involved in metabolism and plant development.

Table III. *Prediction of additional experiments using combinatorial design (Shasha et al., 2001) to further investigate interactions between light qualities alone and their interaction with sugar*

Input variables are indicated as present (Y) or absent (N) or present at a low fluence (LF) or high fluence (HF).

Experiment No.	Carbon	Blue Light	Red Light	Far-red Light
1	N	LF	HF	HF
2	N	HF	N	HF
3	N	N	LF	HF
4	N	LF	LF	N
5	N	HF	LF	LF
6	N	N	HF	LF
7	N	N	N	N
8	N	LF	N	LF
9	N	HF	HF	N
10	Y	LF	HF	HF
11	Y	HF	N	HF
12	Y	N	LF	HF
13	Y	LF	LF	N
14	Y	HF	LF	LF
15	Y	N	HF	LF
16	Y	N	N	N
17	Y	LF	N	LF
18	Y	HF	HF	N

MATERIALS AND METHODS

Plant Growth and Treatment for Analysis

All experiments were carried out at least five times using the ecotype Columbia of Arabidopsis. Seeds were surface-sterilized, plated on designated media, and vernalized for 48 h at 8°C. For studies on etiolated seedlings, approximately 150 seeds plate^{-1} were grown on media containing 1× basal Murashige and Skoog (Invitrogen, Carlsbad, CA) and 0.9% (w/v) bactoagar, pH adjusted to 5.7 with KOH, supplemented with 2 mM KNO_3, and either 0% or 1% (w/v) Suc. Plants were grown vertically in the dark at 23°C for 7 d, after which seedlings grown on 0% or 1% (w/v) Suc-containing media were maintained in the darkness or illuminated with either red (2 or 100 μE m^{-2} s^{-1}), blue (2 or 100 μE m^{-2} s^{-1}), far-red (2 or 100 μE m^{-2} s^{-1}), or WL (70 μE m^{-2} s^{-1}) for an additional 3 h. For experiments carried out on light-grown plants, approximately 30 seeds plate^{-1} were grown on the same media used for etiolated seedlings, except the media contained 0.5% (w/v) Suc. Plants were grown vertically under 16-h-light (70 μE m^{-2} s^{-1})/8-h-dark cycles at a constant temperature of 23°C. After growth for 14 d, all plants were transferred to fresh media containing either 0% or 1% (w/v) Suc and dark-adapted for 48 h, after which the plants were treated with different light treatments as described for etiolated seedlings. After light treatments, whole plants were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until further use.

Light Sources

Photon fluence rates of WL, red, and blue light were measured with a quantum photometer (LI-1800, LI-COR, Lincoln, NE). WL was obtained from fluorescent light tubes (F72T12/CW; Philips, Eindhoven, The Netherlands). Blue light was obtained using actinic blue-light tubes (peak at 420 nm, Coralife, Pembroke Pines, FL). Red and far-red light was obtained using an SNAP-LITE light-emitting diode array from Quantum Devices (Barneveld, WI). All light experiments were carried out in light-tight boxes maintained in a dark, temperature-controlled environmental growth chamber.

RNA Isolation and Quantitative PCR

RNA was isolated from whole plants according to Kim et al. (1993). cDNA synthesis from total RNA was carried out according to Invitrogen (catalog no. 11146-024). Subsequent real-time quantitative PCR was carried out with a LightCycler (Roche Diagnostics, Mannheim, Germany). PCR amplification in a 20-μL reaction volume consisted of a master mixture containing *Taq* DNA polymerase, dNTP mixture and buffer (LightCycler DNA Master Hybridization probes, Roche Diagnostics), 4 mM $MgCl_2$, 0.9 μM of each primer, 0.2 μM of each hybridization probe, and cDNA in a glass capillary tube. Primers and hybridization probes spanned at least one intron for each gene analyzed and were designed using the LightCycler probe design software (Roche Diagnostics). The primers were synthesized at Invitrogen and the fluorescent-labeled hybridization probes were synthesized and HPLC purified by TIB Molbiol LLC (Adelphia, NJ). Anchor probes were labeled at the 3′ end with fluorescein, and sensor probes were labeled at the 5′ end with LC-Red 640 and phosphorylated at the 3′ end. The following primers and probes were used for amplification and detection: *ASN1*, 5′-TCACGCTGCTCAAAATGT-3′

(forward primer), 5′-AGCTTGCATCCCACTC-3′ (reverse primer), 5′-AGAACTCTGCGAGACTAACGG-3′ (anchor probe), and 5′-CCTGGAGGTGCCACCG-3′ (sensor probe); *ASN2*, 5′-GAGCGACTGTACCAGG-3′ (forward primer), 5′-ACAACGTGTATCACTTGC-3′ (reverse primer), 5′-ATGGGATGCAACTTGGTCAAAG-3′ (anchor probe), and 5′-TCTTGATCCGTCAGGCCGT-3′ (sensor probe); *GLN2*, 5′-AGCTAGTATTGACCAGTTCT-3′ (forward primer), 5′-GCTGCAAGGGCTTCAG-3′ (reverse primer), 5′-AACCGTGGATGCTCTATTCGT-3′ (anchor probe), and 5′-GGGACGTGACACCGAGG-3′ (sensor probe); and At4g24550 (putative clathrin coat assembly protein), 5′-ATACACTGCGTGCAAAG-3′ (forward primer), 5′-TTCGCCTGTGTCACAT-3′ (reverse primer), 5′-AAGGAAGCAGGGCCAGT-3′ (anchor probe), and 5′-AAGGAAGCAGGGCCAGT-3′ (sensor probe). Thermal cycling was performed as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 0 s, annealing at 55°C for 5 s (*GLN2*) or 10 s (*ASN1*, *ASN2*, and At4g24550), and extension at 72°C for 10 s (*GLN2*) or 15 s (*ASN1*, *ASN2*, and At4g24550). Standards were prepared with a 10-fold serial dilution (10^{-4} to 10 pg) of the PCR products and were run under the same PCR conditions used for the samples. The amount of *ASN1*, *ASN2*, and *GLN2* was corrected/normalized according to the amount of At4g24550.

Boolean Analysis

For Boolean analysis (Nelson and Nagle, 1995), the two base conditions (a) no carbon, no light and (b) carbon, no light were used as a comparison against non-base conditions. For every non-base condition, the expression of the target gene was compared with the expression of that target gene in the base condition. If the expression was significantly different based on an unpaired *t* test at the 5% level, the values of all input variables for that non-base condition and the expression level relative to the base condition were recorded. For the experiments in this study, the input variables are carbon, WL, BLF, BHF, RLF, RHF, FRLF, and FRHF. The expression annotation relative to the base condition was (a) super-inductive if the average expression value was more than 10 times greater than the level for the base condition; (b) inductive if the average expression value was less than or equal to 10 times greater than the level for the base condition but remains significantly inductive; (c) super-repressive if the average expression value was more than 10 times less than the level for the base condition; or (d) repressive if the average expression value was less than or equal to 10 times less than the level for the base condition but still significantly repressive. The set of all recorded input values at a certain annotation level constitutes a Boolean conjunction, where Boolean circuit reduction techniques reduced this set to fewer conjunctions having "don't care" elements.

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