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Ethylene and Auxin Control the Arabidopsis Response to Decreased Light Intensity[1]

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Morphological responses of plants to shading have long been studied as a function of light quality, in particular the ratio of red to far red light that affects phytochrome activity. However, changes in light quantity are also expected to be important for the shading response because plants have to adapt to the reduction in overall energy input. Here, we present data on the involvement of auxin and ethylene in the response to low light intensities. Decreased light intensities coincided with increased ethylene production in Arabidopsis rosettes. This response was rapid because the plants reacted within minutes. In addition, ethylene- and auxin-insensitive mutants are impaired in their reaction to shading, which is reflected by a defect in leaf elevation and an aberrant leaf biomass allocation. On the molecular level, several auxin-inducible genes are up-regulated in wild-type Arabidopsis in response to a reduction in light intensity, including the primary auxin response gene *IAA3* and a protein with similarity to *AUX22* and the 1-aminocyclopropane-1-carboxylic acid synthase genes *ACS6*, *ACS8*, and *ACS9* that are involved in ethylene biosynthesis. Taken together, the data show that ethylene and auxin signaling are required for the response to low light intensities.

One of the more important environmental factors for plants is the availability of sufficient light. Shading in nature consists of distinguishable features. First, there are the changes in light quality, most often an increase in far red (FR) light due to neighboring vegetation or end of day effects. Within canopies, other spectral changes are also observed (Holmes, 1983). Second, there is a diminishment of light intensity or photosynthetic photon flux density (PPFD), including blue and red (R) light, which has direct effects on photosynthesis and photomorphogenesis. Although a large amount of data is available on the influence of light intensity on general biomass production and root to shoot partitioning (Blackman and Wilson, 1951; McConnaughay and Coleman, 1999), relatively little is known about more subtle effects of light quantity on plant architecture and morphogenesis. The latter effects include biomass allocation within organs and leaf form.

Many plant species try to avoid shading and adapt their phenotype to reach out for light (Holmes, 1983). Rosette plants can orient their leaves to a more vertical position, sometimes referred to as hyponasty, and redirect accumulation of biomass to stems and petioles rather than leaf blades (Smith, 1992; Hangarter, 1997; Maliakal et al., 1999).

GAs, ethylene, or auxins can induce reorientation of leaves (Brock et al., 1994; Clua et al., 1996; Cox et al., 2003). In leaf blades, hyponasty can result from auxin-induced differential growth (Lippincott and Lippincott, 1971). In Arabidopsis, asymmetric distribution of auxins causes gravitropic and phototropic responses (Friml et al., 2002). A number of Arabidopsis auxin mutants, called *massugu* (*msg1/nph4/arf7*), have constitutive nastic responses, which cause upward or downward orientation of leaves (Watahiki and Yamamoto, 1997; Harper et al., 2000). Auxin is not solely responsible for differential growth but can also interact with ethylene (Lehman et al., 1996; Luschnig et al., 1998; Müller et al., 1998Harper et al., 2000; Friml et al., 2002). In certain cases, as in the development of the apical hook in etiolated Arabidopsis seedlings, the redistribution of auxins is stimulated by ethylene. The *hls1-1* (*hookless1-1*) mutant is impaired in this process (Lehman et al., 1996). In tomato (*Lycopersicon esculentum*), auxin and ethylene also interact in gravitropic and epinastic responses (Ursin and Bradford, 1989; Madlung et al., 1999; Hansen and Grossmann, 2000).

PhyB (*Phytochrome B*) mutants have elevated petioles even in non-shaded conditions (Somers et al., 1991). In addition, their hypocotyls and petioles are elongated, and they display an increased apical dominance, as seen in shaded wild-type plants (Koornneef et al., 1980; Reed et al., 1993; Devlin et al., 1996). These responses also can be induced by auxins (Smalle et al., 1997; Chatfield et al., 2000; Sawa et al.,

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2002). In a screen for suppressors of phytochrome mutants, *shy2*, a mutant defective in the auxin response gene IAA3, was isolated (Kim et al., 1996; Tian and Reed, 1999). Furthermore, a number of AUX/IAA proteins can be phosphorylated by phytochrome A (Colon-Carmona et al., 2000). Thus, the light signaling and auxin pathways are clearly intertwined.

A first indication for a correlation between ethylene action and hyponasty was presented in a study of the submergence response of *Rumex palustris*. In this flooding-tolerant species, ethylene induces hyponasty and extension of petioles, allowing the leaf blades to reach the water surface (Cox et al., 2003).

On the other hand, there is also a relation between light signaling and ethylene. Sorghum (*Sorghum bicolor*) wild-type plants subjected to dim FR-enriched light, and *phyB* mutants produced more ethylene than wild-type plants under white light (Finlayson et al., 1998). Together, these phenotypic similarities indicate a possible role for ethylene in a plant's response to shading.

Data on ethylene and light interactions in vegetative development are rather scarce. It is generally accepted that light inhibits ethylene synthesis by reducing 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) activity (Kao and Yang, 1982; Finlaysonet al., 1998). In leaf segments of oat (*Avena sativa*) and *Begonia hiemalis-Fotsch*, it was demonstrated that light quality also has an influence on ethylene production (Rudnicki et al., 1993; Corbineau et al., 1995).

Considering the phenotypic overlaps of shade, auxin, and ethylene responses, we decided to investigate to which extent these factors work together to determine plant architecture. In the majority of previous studies on shading, the emphasis was put on qualitative differences, i.e. changes in the R to FR ratio. Nevertheless, reacting to mere changes in light intensity can be of considerable importance in pioneer plants such as *Epilobium* species and Arabidopsis (Chapin et al., 1994; Pigliucci and Schmitt, 1999; Al Shehbaz and O'Kane, 2002). A typical strategy for survival in this type of plant species is the production of a large number of small seeds. As a consequence, seedlings are small and often exposed to shading caused by irregularities of the soil surface. Here, we present evidence for the involvement of both auxin and ethylene in the developmental response to decreased light intensity.

RESULTS

Low Light Intensity Is Correlated with an Increase in Ethylene Production

Ethylene production of intact 2-week-old Arabidopsis seedlings was measured using laser-based photo-acoustic detection, able to detect levels as low as 1 nL L^{-1} (Bijnen et al., 1996). After accumulation in a closed cuvette, higher production was found in wild-type plants grown in low light, compared with plants grown in high light (Fig. 1A). *PhyB-9* mutant seedlings showed the same trend but overproduced ethylene in both conditions as compared with wild type (Fig. 1A).

Plants grown on medium containing the ethylene precursor ACC produced sufficient amounts of ethylene for continuous flow detection. To measure changes in ethylene production in Arabidopsis upon a switch in light intensity, seedlings were grown on a saturating concentration of ACC (50 μM; Smalle et al., 1997). This concentration of ACC was used to prevent interference of varying activities of ACC synthases (ACSs) that control a rate-limiting step for ethylene biosynthesis. In these conditions, having a continuous supply of its substrate ACC, ACO activity limits ethylene production. Switching from low light intensity (30 μmol m^{-2} s^{-1}) to a higher light intensity (130 μmol m^{-2} s^{-1}) inhibited ethylene production within minutes. The change in ethylene production coincided with an increase in CO_2 uptake, indicating that the change in ethylene emanation is not due to stomatal closure (Fig. 1B). Switching from high light to low light intensity caused the opposite effect (Fig. 1C). Addition of extra FR light, without altering the PPFD of 130 μmol m^{-2} s^{-1}, but causing a drop in R:FR ratio from 2.25 to 0.67, did not increase ethylene production, as compared with plants that remained in light with 130 μmol m^{-2} s^{-1} PPFD and a R:FR ratio of 2.25 (data not shown).

Effects of Light Intensity on Leaf Elevation Angles in Ethylene and Auxin Mutants

Auxin-insensitive mutants are affected in numerous developmental processes (for review, see Tian and Reed, 2001; Leyser, 2002; Swarup et al., 2002). Of particular interest are the *massugu1* (*msg1/nph4/ARF7*) mutants. In general, they show leaf epinasty; however, one-half of the plants of the *msg1-3* mutant line have hyponastic leaves (Watahiki and Yamamoto, 1997). We have characterized the *alh1* (*ACC-related long hypocotyl 1*) mutant that shows both ethylene- and auxin-related phenotypes (Vandenbussche et al., 2003). At light intensities below 50 μmol m^{-2} s^{-1} PPFD, leaves of this mutant point upwards, in contrast to wild-type leaves that displayed a more horizontal growth. As a consequence, the *alh1* phenotype in these conditions was highly similar to *phyB* plants (Fig. 2A). This phenotype could also be mimicked by treatment of wild-type seedlings with exogenous ethylene for 6 d (Fig. 2B), preventing horizontal leaf positioning.

We further investigated the role of ethylene and auxin on leaf angle, induced by low light intensity. Elevation angle was defined by Herbert (1983) as the angle between the leaf midrib and the horizontal. In this study, the elevation angle of the petiole of leaf 6, before full expansion, was used as a parameter to

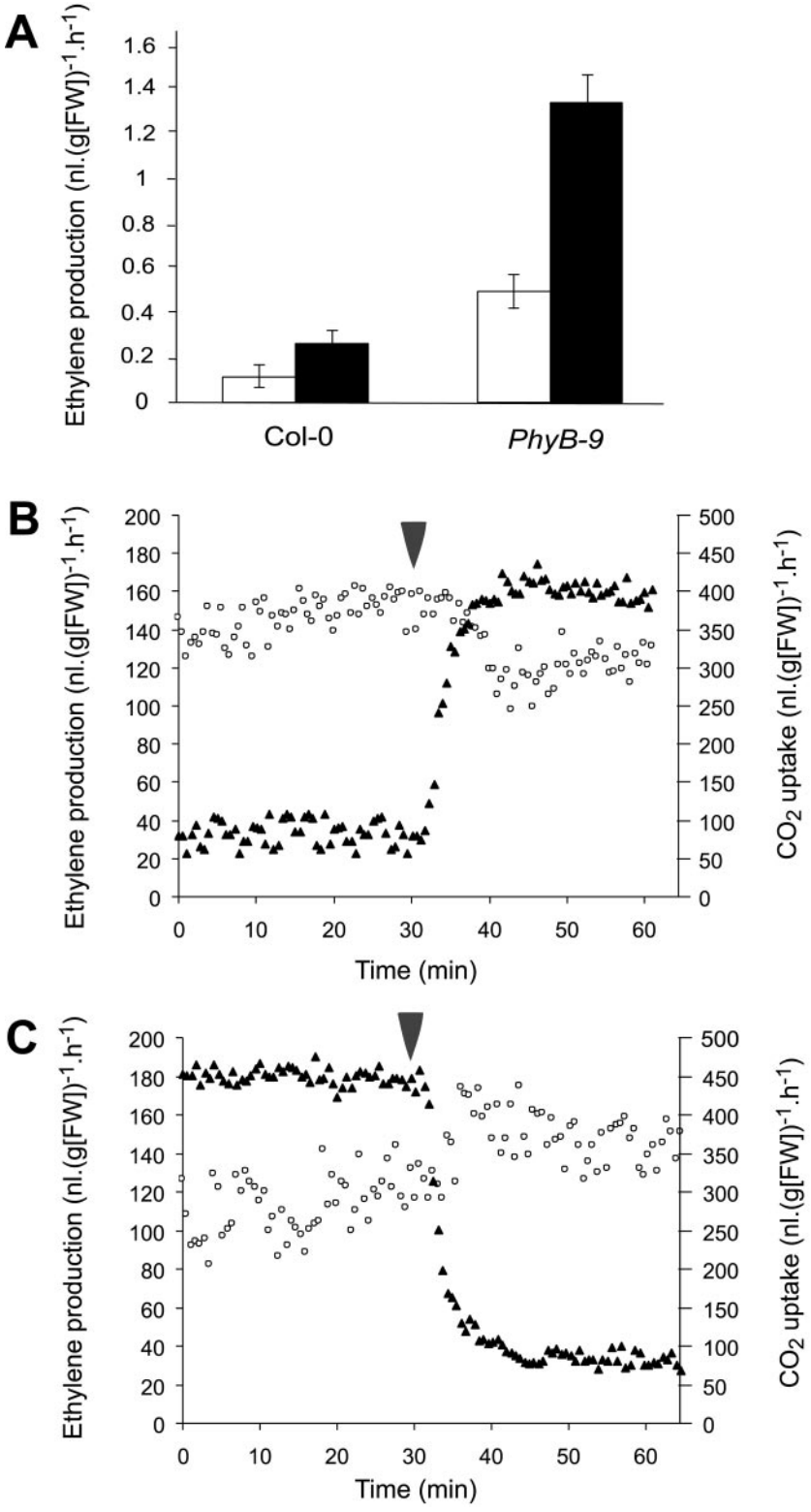

Figure 1. Ethylene production in different light intensities. A, Ethylene production from 2-week-old Arabidopsis wild-type and mutant *phyB-9* rosettes. Plants were kept in gas-tight vials for 2.5 h. Accumulated ethylene was measured using photo-acoustic detection. White bars = 125 μmol m^{-2} s^{-1} PPFD. Black bars = 35 μmol m^{-2} s^{-1} of PPFD. Error bars = SE. B, Changes in ethylene production in ACC-treated rosettes upon a switch in light intensity from 30 to 125 μmol m^{-2} s^{-1} PPFD. Measurements were performed online in 2-week-old plants on medium containing 50 μM ACC. Black triangles, CO_2 uptake; white circles, emanated ethylene. Arrowhead, Time point of light switch. FW, Fresh weight. B and C, Changes in ethylene production in ACC-treated rosettes upon a switch in light intensity from 125 to 30 μmol m^{-2} s^{-1} PPFD. Measurements were performed online in 2-week-old plants on medium containing 50 μM ACC. Black triangles, CO_2 uptake; white circles, emanated ethylene. Arrowhead, Time point of light switch. FW, Fresh weight.

measure hyponastic responses in Arabidopsis mutants (Fig. 3A). When grown under low light intensity, leaves of wild-type plants displayed a more vertical orientation. Ethylene-insensitive mutants *etr1-3* and *ein2-1* (Guzmán and Ecker, 1990; Chang et al., 1993) did not show any increase in elevation angles in low light intensity and, thus, had flatter rosettes than wild type, although they reacted

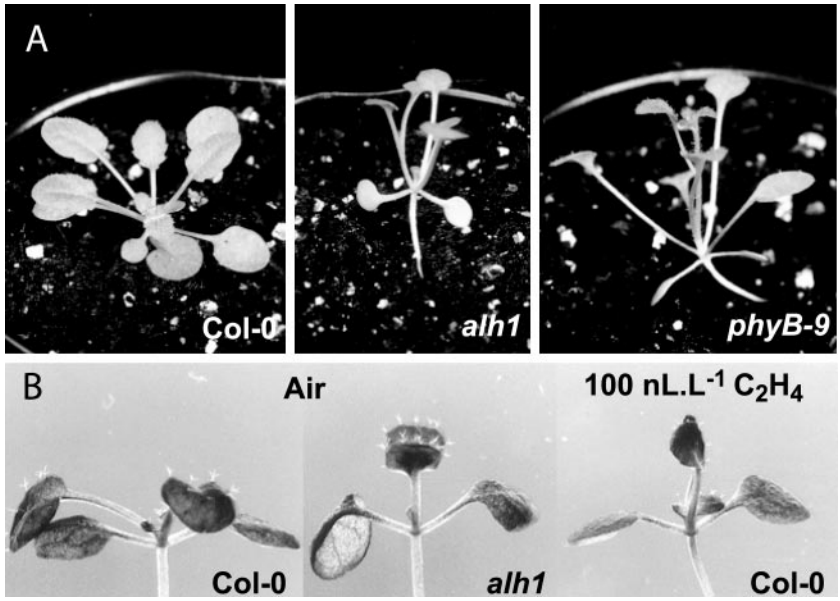

Figure 2. A, *Alh1* has a larger leaf elevation angle. Left to right, Wild-type Col-0, *alh1*, and *PhyB-9*. Plants were grown for 33 d on soil at a PPFD of 30 μmol m^{-2} s^{-1}. B, Treatment of wild-type Arabidopsis with ethylene keeps leaves vertically oriented and phenocopies *alh1*. Left, Air-treated *alh1*; middle, air treated wild type Col-0; right, ethylene-treated wild type. Plants were grown for 4 d on Murashige and Skoog/2 + 1% (w/v) Suc. Subsequently, they were exposed to 100 nL L^{-1} ethylene for 6 d.

strongly to low R to FR ratios as did the wild type in these conditions (Fig. 3). This confirms the suggested separation of R to FR effects and light intensity effects in this kind of response (Hangarter, 1997). In contrast to ethylene-insensitive mutants, the ethylene overproducer *eto2* (Vogel et al., 1998) had larger leaf elevation angles than wild type. In accordance with this observation, *ctr1-1* (*constitutive triple response 1*; Kieber et al., 1993) petioles had large elevation angles under all tested conditions.

Similar to ethylene-insensitive mutants, the effect of light intensities on the elevation angle in auxin mutants was also less pronounced than in the wild type (Fig. 3). *Axr1-3* and *axr2* and did not respond. *Hls1-1*, which is disturbed in differential growth for apical hook formation in dark grown seedlings, had a wild type-like response (Lehman et al., 1996). This indicates that HLS1 does not play a major role in differential growth in petioles. In contrast, *alh1* had a stronger reaction than wild-type Arabidopsis.

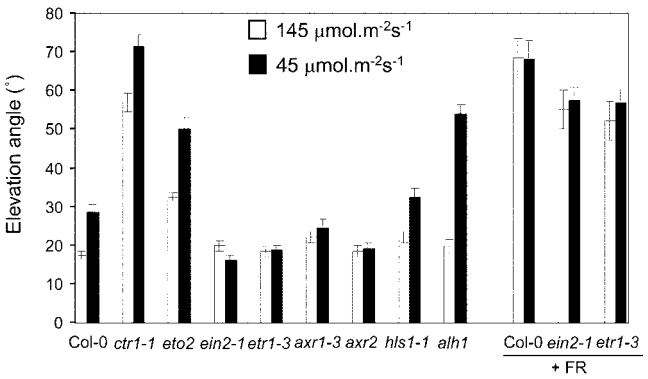

Figure 3. Ethylene and auxin mutants differ from wild type in elevation angle. Plants were grown on soil at a PPFD of 125 μmol m^{-2} s^{-1} (white bars) or 45 μmol m^{-2} s^{-1} (black bars), with or without supplemented FR. Error bars = SE.

To investigate the relation between ethylene and auxin in this process in more detail, we treated auxin mutants with ethylene for 6 d. The effect was severely attenuated in *axr2* and was still very obvious in *axr1-3* (Fig. 4). Thus, intact auxin signaling is needed for the ethylene-induced phenotype to occur.

Together, these data suggest that low light-induced leaf elevation is dependent of both ethylene and auxin signals and that the ethylene response requires a functional auxin signaling pathway.

Allocation of Leaf Biomass in Ethylene and Auxin Mutants

Another characteristic typical of wild-type Arabidopsis plants grown in low R to FR ratios is the increase in elongation of petioles and a reduction of leaf blade surface (Robson et al., 1993). As shown in Figure 5, this was also the case when light intensity was diminished without changing the R to FR ratio. To test whether ethylene and auxin influence the allocation of leaf biomass, we have grown mutants defective in their response to these hormones in different light intensities. Under low light conditions, the ethylene-insensitive mutants (*etr1-3* and *ein2-1*) had larger leaf blades than wild-type plants (Table I). Wild-type plants grown in low light (15 μmol m^{-2} s^{-1}) had a 30% reduction in leaf blade surface area compared with plants grown in high light intensity (125 μmol m^{-2} s^{-1}), whereas the blades of ethylene-insensitive mutants even showed a slight increase in size (i.e. 16% for *ein2-1*; data not shown). Mutants with a constitutive ethylene phenotype, such as *eto2* and *ctr1-1*, and wild-type plants treated with ACC had smaller leaf blades than untreated wild-type

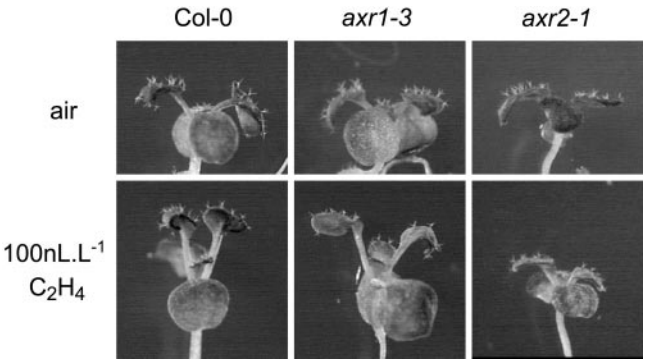

Figure 4. The auxin-insensitive mutants *axr1-3* and *axr2-1* are attenuated in the leaf elevation response induced by ethylene. Upper row, Untreated plants; lower row, plants treated with 100 nL L^{-1} of ethylene for 6 d. Left to right, Col-0, *axr1-3*, and *axr2-1*.

plants (Table I). In general, constitutive ethylene responses result in dwarfism (Kieber et al., 1993; Rodrigues-Pousada et al., 1993; Hirayama et al., 1999). Thus, the petiole length is also affected (Table I). However, *eto2* was less affected in petiole growth than *ctr1-1* or ACC-treated wild type (Fig. 6A; Table I). To correct for the global effect of the mutations on growth, petiole length and leaf blade area have to be taken into account. A parameter that expresses the DSA was defined: DSA = (petiole length)^2 × (leaf blade surface)$^{-1}$. *Eto2*, an ACS mutant expressing a hyperstable ACS5 protein, is oversensitive to decrease in light intensities, exemplified by higher DSA values than the wild type (Fig. 6; Vogel et al., 1998; Chae et al., 2003). This implies that disturbing ethylene biosynthesis alters the responses to shading. Moreover, light may control ethylene biosynthesis *ACS5*. In contrast to *eto2*, ethylene-insensitive mutants had smaller DSA values than wild type (Fig. 6A).

The auxin-insensitive mutants (*axr2* and *axr1-3*) were also impaired in the redistribution of biomass (Fig. 6B). In comparison with wild type, they had short petioles, especially in low light (Table I; Fig. 5). *Axr2* had the most severe phenotype. In this mutant, no change was observed. This confirms the necessity for a normal auxin response in the reaction to shading. Continuous supply of 50 μM ACC could not revert the phenotype of the auxin-insensitive mutant *axr2* (Fig. 6C).

Regulation of Transcription by Different Light Intensities

To investigate to what degree changes in gene expression are involved in the above-described responses, we used a modified cDNA-AFLP method to analyze part of the transcriptome (Breyne et al., 2002). Of 800 tags, we isolated 18 fragments of genes that were differentially regulated by low light intensities in wild type but not in the *axr2* mutant (Fig. 7A). Five tags were homologous to genes with a known function, whereas four others were not. Nine tags could not be sequenced. Among those that were sequenced, we found *CAT3* (*catalase 3*), a gene known to be negatively regulated by light (McClung, 1997). We also found a steroid sulfotransferase-like gene and *RPN1* (regulatory subunit of the 26S proteasome) clustering with *CAT3*, although *RPN1* did not appear to be strongly light regulated. Further, we detected two AUX/IAA-related genes. *IAA3* was predominantly expressed in petioles, whereas transcription in leaf blades of a gene with similarity to *AUX22d* of mung bean (*Vigna radiata*) was similar to that in petioles. In *eto2*, its expression was lower than in wild type. Subsequently, we investigated auxin induced ethylene biosynthesis in low light intensities. To that end, we used a reverse transcriptase (RT)-PCR approach for the analysis of ethylene biosynthesis genes. A number of ACSs are known to be auxin induced. Among them are, in ascending order of inducibility, *ACS6*, *ACS4*, and *ACS8* (Abel et al., 1995; Tian et al., 2002). Steady-state messenger levels of *ACS6*, *ACS8*, *ACS9*, and *ACS11* genes were clearly higher in low light intensities (Fig. 8). Other ACSs, *ACS2*, *ACS7*, *ACS10*, and *ACS12*, were expressed

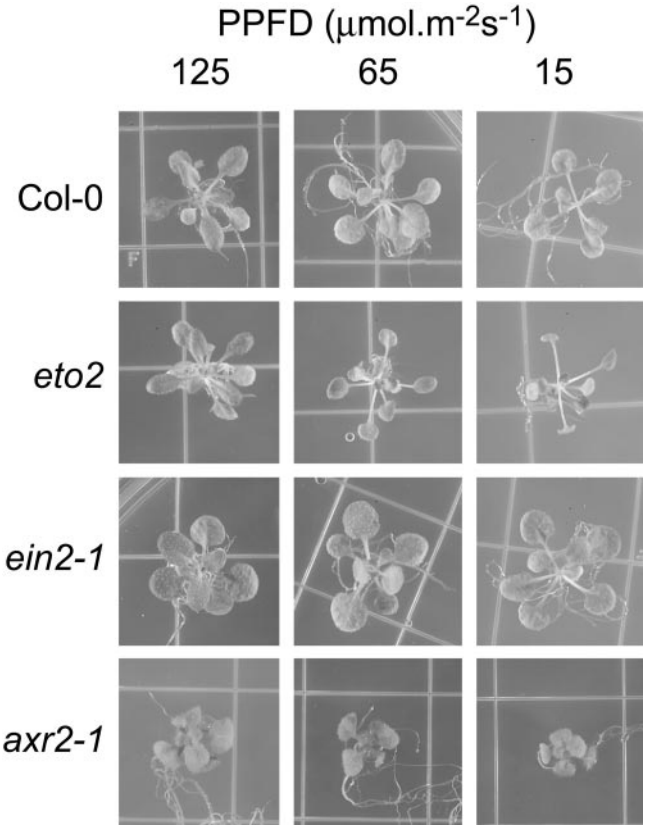

Figure 5. Ethylene and auxin mutants differ from wild type in allocation of biomass in low light intensities. Plants were grown for 1 week in 45 μmol m^{-2} s^{-1} PPFD. Photographs were taken 1.5 weeks after transfer to the indicated light conditions.

Table I. *Leaf surface and petiole length relative to the wild type in specific light conditions*

Values are fractions of 1.

Investigated Trait	Columbia (Col)-0	5 μM ACC	50 μM ACC	*ctr1-1*	*ein2-1*	*eto2*	*etr1-3*	*phyB-9*	*alh1*	*axr1-3*	*axr2*	*hls1-1*
Area 125[a]	1.00	0.54	0.21	0.29	0.98	0.57	1.00	0.86	0.98	1.52	1.05	1.11
Area 15[a]	1.00	0.64	0.19	0.24	1.63	0.59	1.79	0.65	1.19	1.09	0.94	0.97
Petiole length 125[a]	1.00	0.92	0.52	0.58	0.71	0.90	0.66	2.18	1.22	1.18	0.32	1.02
Petiole length 15[a]	1.00	0.89	0.48	0.50	0.95	1.25	1.20	1.64	1.54	0.38	0.03	1.00

[a] No. indicates light intensity (in μmol m^{-2} s^{-1}).

more constitutively. Moreover, in *phyB-9* leaf blades, the expression level of *ACS6*, *ACS8*, and *ACS11* was higher than in wild type in high light intensities. A similar feature was found for *ACS4* and *ACS9* in *phyB-9* petioles. This suggests that these genes may be involved in the constitutive shading phenotype in the absence of active phytochrome. *ACS5* also had a higher expression in low light intensities and appeared more petiole specific. This confirms earlier observations of significantly higher ethylene production in dark-grown compared with light-grown *eto2* mutants, which have a hyperactive ACS5 (Vogel et al., 1998). The most obvious way to explain the exaggerated shading response of *eto2* might at least partly be in this low light (or dark)-specific accumulation of *ACS5* combined with additional stabilization of the protein (Chae et al., 2003). As a consequence, a higher ACC level is available for the ACOs in shaded *eto2* plants.

To assess the effect on the final step in ethylene biosynthesis, the expression of six ACO genes was studied. Transcript regulation was similar to that of the *ACS* genes. A number of *ACO* genes, with *At1g5010* (*ACO1*), *At1g62380* (*ACO2*), and *At1g04350* among them, were rather constitutively expressed. In contrast, the putative ACOs, *At2g19590* and *At5g63600*, were clearly induced in leaf blades in low light intensities. This was also the case for *At5g43450*, albeit to a lesser extent. ACO mRNA levels were never down-regulated in low light intensity.

DISCUSSION

Quality versus Quantity Shading. Can They Be Uncoupled?

Plants react to shading of canopies by detecting changes in light quality, i.e. R to FR ratio (Smith and Whitelam, 1997). Recently, it has become clear that diminishing this ratio triggers a number of auxin transport-dependent responses, including hypocotyl elongation (Steindler et al., 1999). Other studies also have linked photomorphogenesis to auxin responses (for review, see Swarup et al., 2002). We studied the effects of light quantity and found that auxin and ethylene-insensitive mutants have a reduced response to low light intensities. In addition, the induction of auxin up-regulated genes in plants in lower light intensities indicates that phenotypic adaptations upon quantity shading are auxin mediated. One of those genes was *IAA3/SHY2*. This gene was already shown to be necessary for elongation growth in PHYB-deficient plants (Tian et al., 1999). The role for *IAA3/SHY2* in elongation matches its lower expression in wild-type plants in white light compared with dark (Tian et al., 2002). *Iaa3/shy2* mutants have a reduction of expression of the *IAA7/AXR2* gene (Tian et al., 2002). The reverse relation is also true. *Iaa7/axr2* mutants are defective in the expression of *IAA3/SHY2* (Fig. 6; Tian et al., 2002). Tian et al. (2002) have suggested that *IAA7/AXR2* is positively controlled by *IAA3/SHY2* in shoots. Our data suggest a crucial role for both genes in shading. The mutations in *iaa7/shy2* and *iaa3/axr2* cause defects in auxin-induced hypocotyl elongation (Kim et al., 1996; Vandenbussche et al., 2003). Because they are dominant gain-of-function mutations, a functional redundancy of other *IAA* genes cannot be ruled out. Nevertheless, *IAA3/SHY2* and *IAA7/AXR2* remain good indicators for a role for auxin in the elongated shoot phenotype during shading.

At4g32285 is a homolog of the auxin inducible *AUX22* genes of mung bean hypocotyls (Yamamoto et al., 1992). The first *AUX22* gene was isolated from dark-grown elongating hypocotyl tissues of soybean (*Glycine max*; Ainley et al., 1988). This again corroborates the role of auxin in the response to diminished light intensities.

Moreover, auxin-inducible ethylene biosynthesis genes had a higher transcript level in *phyB-9* mutants (Fig. 7). These plants have a constitutive shade avoidance phenotype, typical for plants grown under low R to FR ratios. Therefore, it is likely that the responses to quantity shading have a similar underlying auxin-dependent mechanism as those upon growth in quality shading, i.e. low R to FR ratios. However, as to the ethylene production rates, quantity and quality shading can be uncoupled. Upon FR enrichment without altering PPFD levels, the ethylene signal is of minor importance because ethylene-insensitive mutants do react to the addition of FR. Moreover, we could not detect any increase in ethylene production. In contrast, lowering light intensity coincided with increased ethylene production, and ethylene-insensitive mutants have a defect in leaf hyponasty.

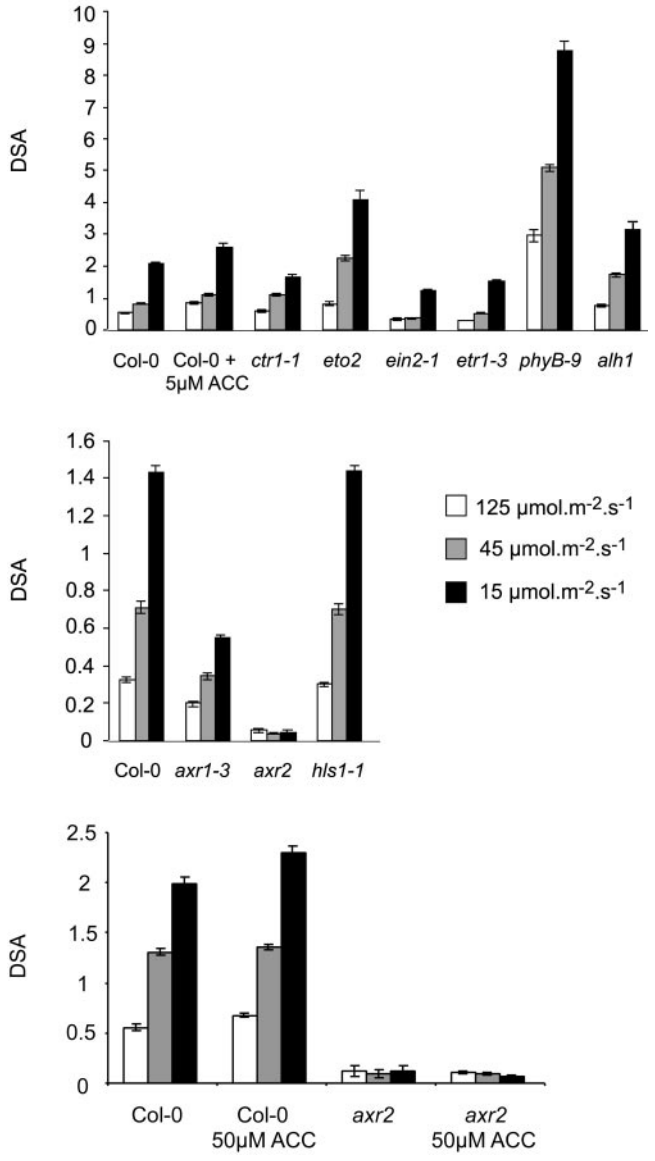

Figure 6. Comparison of biomass allocation in ethylene mutants (A), auxin mutants (B), and the effect of exogenous ACC (C), using a defined parameter: degree of similarity to shade avoidance (DSA). DSA = (petiole length)^2 × (leaf blade surface area)$^{-1}$. Plants were grown for 1 week in 45 μmol m^{-2} s^{-1} PPFD. After transfer (1.5 weeks) to 125 μmol m^{-2} s^{-1} (white bars), 45 μmol m^{-2} s^{-1} (gray bars), or 15 μmol m^{-2} s^{-1} (black bars), leaf 4 was analyzed for leaf blade surface and petiole length. Error bars = SE.

Biological Significance for Ethylene Biosynthesis in Shading Responses

In Arabidopsis, elevation of leaves has been described as a function of gravity and is influenced by R to FR ratio and light intensity (Fig. 3; Hangarter, 1997). In many species, ethylene can promote negative gravitropism in stems (Wheeler et al., 1986). In Arabidopsis, ethylene stimulates leaves to grow in a vertical position (Fig. 2). This is also an effective mechanism to reach out for light. Prolonged treatments during leaf development were needed to retain the more vertical position. Therefore, it is unlikely that this response is based on the same mechanisms found in the hyponastic response of *R. palustris* petioles, which is a much faster response, occurring within less than 1 h (Cox et al., 2003).

One of the best known ethylene responses is the inhibition of cell expansion and consequent dwarfism. This has been shown for roots, dark-grown hypocotyls, and light-grown mature plants. Previous work indicated that as leaves expand, *ACS1* mRNA levels decrease (Rodrigues-Pousada et al., 1993; renamed *ACS2* in The Arabidopsis Information Resource database). We found that *ACS6* and *ACS8* were up-regulated in shaded plants and that their expression was the highest in leaf blades. These genes are also strongly induced by auxins (Tian et al., 2002). Gene expression of ACSs is regulated by a number of different factors, and, in most cases, a subset is induced by auxins (Yi et al., 1999).

The respective partial and complete failure of ethylene- and auxin-insensitive mutants to react to shading suggests that both hormones may be involved in the same cascade. However, auxin-insensitive mutants could not be rescued by continuously applying exogenous ACC or ethylene. This may not reflect the natural conditions, in which diurnal fluctuations of ethylene production can occur (F. Vandenbussche and D. Van Der Straeten, unpublished data). Thus, timing of the signals may be important. Nonetheless, it is also possible that light intensity exerts its regulation of ethylene synthesis independent of the auxin signal.

Transcription of putative ACO genes, although shade induced, appeared independent of phytochrome B signaling with the exception of *At2g19590*, which had a lower expression level in *phyB-9*. However, it remains to be determined whether *At2g19590* is a true ACO. Other factors probably exert an effect on expression of ACOs upon shading. One possibility is regulation by other stable phytochromes (Clack et al., 1994). In addition, other photoreceptors, such as cryptochromes or chlorophyll, may mediate the signaling pathway that leads to their transcription.

Apart from regulation of ethylene biosynthesis genes on the transcriptional level, light also has an influence on ethylene production by the modification of ACO. The fast decrease in ethylene production, which we measured during the change from low to high light intensities, might indicate a rapid change of enzyme activity due to a depletion of catalytic CO_2 caused by an increase in photosynthetic activity. This confirms previous reports in which light was found to have an inhibitory effect on ethylene biosynthesis in green tissues (Yang and Hoffman, 1984).

PhyB mutants have a higher ethylene production (Fig. 1A; Finlayson et al., 1998). It is known that the amount of chlorophyll in these plants is lower (Reed

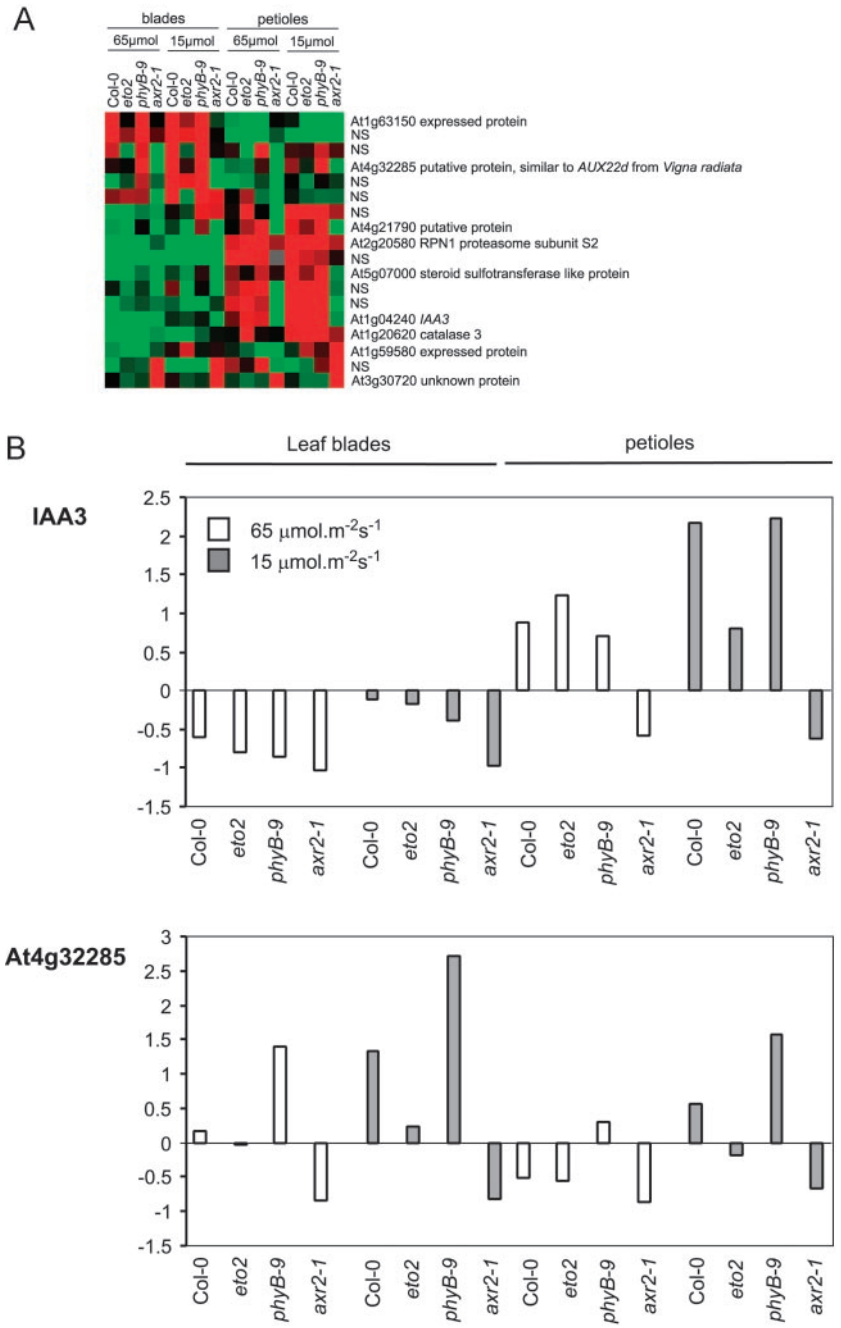

Figure 7. A, Cluster of shade-regulated fragments derived from cDNA-AFLP transcript profiling analysis. Colored squares, Relative transcript expression values of the genes in leaf blades and petioles of rosettes that were grown either in 65 or 15 μmol m^{-2} s^{-1} PPFD. Red, Up-regulated; green, down-regulated; black, neutral; gray, missing value. B, Histograms of the expression pattern in leaf blades and petioles of auxin-related genes *IAA3* and *At4g32285* derived from the semiquantitative analysis of cDNA-AFLP fragments, represented in A. Expression of the genes at a higher light intensity (65 μmol m^{-2} s^{-1}, white bars) and a lower light intensity (15 μmol m^{-2} s^{-1}, gray bars) are compared. Values are normalized and express the induction or repression compared with the mean of all samples, which is set as 0. The following formula was used: normalized value = (sample value − mean value of all samples) × (SD of all samples)$^{-1}$. A value lower than 0 represents a repression and higher than 0 an induction relative to the mean value of all tested samples.

et al., 1993). Previous work indicated that the effect of light on ethylene production was coupled to the photosynthetic electron transport (De Laat et al., 1981). It also was shown that ethylene decreases electron transport but not Rubisco activity (Wullschleger et al., 1992). Thus, an increase in ethylene production under low light might be due to insufficient capacity to deplete CO_2 from the cellular environment, which would leave ACO in a more active state. However, a second possibility is that the effect on ethylene production is directly connected to Rubisco because it was shown in Arabidopsis that *RBCS* genes and Rubisco activity are up-regulated in higher light intensities (Dedonder et al., 1993; Zhang et al., 2002). Thus, there could be a competition for CO_2 among ACOs and Rubisco, with the activity of one enzyme being balanced by the other. However, the extent of control at the level of ACC remains to be investigated because the predominant regulation of ethylene production resides at the level of ACC synthesis, which is a function of protein stability of ACSs (Chae et al., 2003). Nonetheless, it can be concluded that the light control over ethylene production is exerted at different levels, ranging from transcriptional regula-

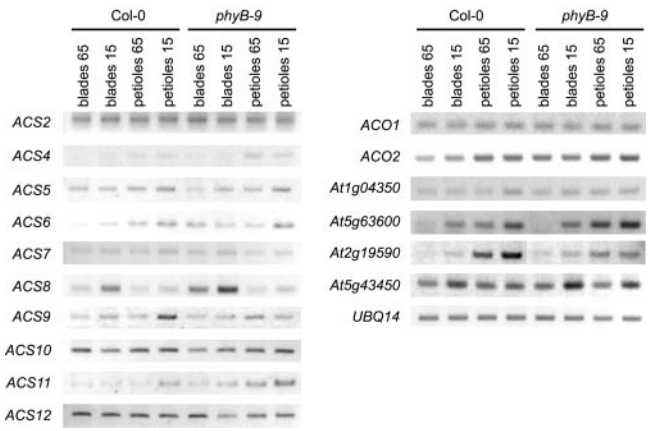

Figure 8. RT-PCR analysis of ethylene biosynthesis enzymes in wild type and *phyB-9* mutants. RNA was prepared from leave blades and petioles from plants grown in 65 μmol m^{-2} s^{-1} (65) or 15 μmol m^{-2} s^{-1} (15).

tion to posttranslational modification and regulated proteolysis.

The reaction of Arabidopsis plants to shading, caused by spectral changes (i.e. FR enrichment) or low light intensity, probably relies to some extent on the same mechanism. This involves a precise control of auxin and ethylene signals and determines the architecture of the leaves.

MATERIALS AND METHODS

Plant Material and Biometrics

Col-0, *phyB-9*, *axr1-3*, *axr2-1*, *hls1-1*, *eto2*, *ein2-1*, and *etr1-3* seeds were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). All mutants are of the Col-0 background. Seeds were sown and plants were grown under sterile conditions as described (Smalle et al., 1997). The plants were subjected to a light intensity of 65 μmol m^{-2} s^{-1} for 1 week and then transferred to light conditions as indicated. Variety in light intensities was obtained by adding or removing cool-white light tubes or by shading with layers of Miracloth (Calbiochem Biosciences, La Jolla, CA), which did not alter the light quality, as assessed by measuring the spectrum with a portable spectroradiometer, Li-1800 (LI-COR, Lincoln, NE).

For the leaf biomass distribution experiments, plants were grown on a medium containing one-half-strength Murashige and Skoog salts and 1% (w/v) Suc in cool-white light (Lumilux Plus, Osram, Germany) under a photoperiod of 16 h of light/8 h of dark at 22°C. Three days after the emergence of leaves 5 and 6, corresponding to approximately 2.5 weeks at midday, leaves 3 and 4 were harvested and stuck to paper with tape. An image was acquired using a flatbed scanner. Analysis of the petiole length and leaf blade surfaces was done with ScionImage software (Scion Corp., Frederick, MD).

Table II. *RT-PCR primers, cycle no., and hybridization temperatures*

Gene	Primer Set	No. of Cycles	Hybridization Temperature
ACS2 (At1g01480)	5′AGATCGTCGAGAAAGCATCTG3′ 5′GAAGAGGTGAGTGTGGTGAC3′	30	56°C
ACS4	5′GTTTACGAAGTGAAGCTCAAC3′ 5′GTCTCATCAATCATGTTCGCG3′	35	56°C
ACS5	5′GCGGCAAGTCTCAAGAGGA3′ 5′TTCTGGGCTTGTTGGTAAGC3′	28	54°C
ACS6	5′CTGAATCTATTGTCTAAAATCGC3′ 5′ACGCATCAAATCTCCACAAAG3′	30	55°C
ACS7	5′TATTTTGTTGGATGAATTTGGGT3′ 5′TTCTCTTCAACGCAATCTCC3′	30	56°C
ACS8	5′GTCCAGTTTCGGTCTAATCTC3′ 5′ATAGGTGTCTCATGTCAACCC3′	28	55°C
ACS9	5′TCGGTTTACCAGGTTTTCGC3′ 5′ACACGAGTTTCTTCTGACGAA3′	30	55°C
ACS10	5′ACAGGCAGAGATTGCAGAG3′ 5′ACTGAAACAGATACGGAACC3′	30	55°C
ACS11	5′CAGTTGTTTGAAGAGTAACGC3′ 5′TAACAGGAAAGCTTGGAGA3′	30	55°C
ACS12	5′AGAGCTGGAGTCATCTACTCC3′ 5′GCAAGCTGTCTGATTCGTTCC3′	30	55°C
ACO (At5g63600)	5′CCTGTCTACTGAAAACCCTC3′ 5′GTCTCCTTGAACAATTCATCA3′	30	56°C
ACO (At1g04350)	5′GCATTCACTAAAATTATACA3′ 5′CAAATAAGTAAACCATTTCCT3′	28	56°C
ACO (At1g05010)	5′GATCTGCTGTGCGAGAATCTC3′ 5′TAAATAACCCTTCTCTAAACC3′	28	56°C
ACO (At1g62380)	5′CCAGCTACTTCGCTTGTCGAG3′ 5′GTCTCTACGGCTGCTGTAGGA3′	27	56°C
ACO (At2g19590)	5′CAGAGGAACTCAGCAAGACG3′ 5′ATCCGTATGTTCTCTCAGCC3′	28	56°C
ACO (At5g43450)	5′GTACAAAGATATCACCATACCAG3′ 5′TGGTTGAGGAACTCTATAGC3′	28	56°C
UBQ14	5′GATCCAGGACAAGGAAGGTATC3′ 5′AGCCTTAGCACCAAGTGAAGG3′	25	55°C

For the study of leaf angles, plants were grown on soil in cool-white light (Lumilux Plus) under the same photoperiod as above. When the fourth leaf pair emerged, plants were taken from the soil at midday and meticulously dissected such that only the hypocotyl, the apical meristem, the petiole, and the midvein of leaf 6 were left. These were very carefully stuck onto paper without disturbing the original inclination. An image was acquired using a flatbed scanner. Analysis of the elevation angles (this is the angle between petiole and the horizontal) was done with ScionImage software (Scion Corp.).

Gas Measurements

Plants were grown on Murashige and Skoog/2 medium containing 1% (w/v) Suc and 50 μM ACC in 16 h of light/8 h of dark at 22°C and 60% relative humidity. To compare the effect of low light and higher light intensities on ethylene biosynthesis, 2-week-old Col-0 and *phyB-9* plants were grown in 120 or 30 μmol m^{-2} s^{-1} PPFD, respectively, for 3 d to minimize effects of circadian rhythms. After that, 12 plants were put in air-tight 100-mL glass vials while remaining on the same medium. Ethylene production of wild-type Arabidopsis plants was too low for continuous flow measurements. Therefore, we measured ethylene after accumulation in a closed vial. Every 2 h, the vials were flushed at a flow rate of 1 L h^{-1}, and ethylene was measured.

For the light switch experiment, plants were essentially in the same conditions as mentioned above. Variety in light intensity was achieved by adding or removing cool-white light tubes. Changes in R to FR ratio were obtained by adding filtered incandescent light from a 60-W bulb. The R to FR and PPFD values were determined using an Li-1800 (LI-COR) portable spectroradiometer. After 2 weeks, they were put in glass jars for gas measurements while still on the same medium. The jars were fit into a continuous flow system, connected to a photo-acoustic detector for measuring ethylene (Bijnen et al., 1996). CO_2 was measured using a URAS 14 apparatus (Hartmann & Braun, Frankfurt, Germany).

Transcript Analysis

Leaf blades and petioles of 2.5-week-old plants, grown on Murashige and Skoog/2 + 1% (w/v) Suc in 16 h of light/8 h of dark at 22°C, were harvested at midday and frozen in liquid nitrogen. RNA was prepared using QIAGEN RNeasy (QIAGEN GmbH, Hilden, Germany). RNA was treated with Dnase amplification grade (Life Technologies/Gibco-BRL, Cleveland).

To get an overview of gene expression, we used a modified cDNA-AFLP technique (Breyne et al., 2002). Twenty primer combinations were tested. A total of 800 fragments were analyzed corresponding to 4% to 5% of the total genome. Cluster analysis was performed using the Eisen software (http://rana.lbl.gov/EisenSoftware.htm) performing hierarchical clustering. Two biological repeats were done. Fragments that had a cutoff value (SD on mean/mean of all samples) smaller than 0.3 or that were not repeatable in clustering were discarded. The remaining fragments that fell into a cluster of interest were sequenced. Only fragments that produced a sequence with an "expect value" of less than 10^{-5} in a BLAST search were retained.

RT-PCR with gene-specific primers was also performed. For *ACS4*, *ACS6*, *ACS8*, all *ACO* genes, and *UBQ 14*, the pre-amplification reactions of the cDNA-AFLP were used as template material for the gene-specific PCR. For all other genes, the gene-specific PCRs were done directly on cDNA that was obtained by a classical RT reaction according to protocol (Invitrogen, Carlsbad, CA). All PCRs were done in a Mastercycler (Eppendorf, Hamburg, Germany). Cycles had 30 sec at 95°C, 35 sec at hybridization temperature, and 30" at 72°C. A list of gene-specific primers and reaction conditions is given in Table II. Separation was done on a 1% (w/v) agarose gel. DNA was stained with ethidium bromide in the gel.

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