KA12- and MAX2-Mediated Responses to Karrikins and Strigolactones Are Largely Independent of HY5 in Arabidopsis Seedlings

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ABSTRACT
Karrikins are butenolide compounds released from burning vegetation that stimulate seed germination and enhance seedling photomorphogenesis. Strigolactones are structurally similar plant hormones that regulate shoot and root development, and promote the germination of parasitic weed seeds. In Arabidopsis, the F-box protein MAX2 is required for responses to karrikins and strigolactones, and the α/β hydrolase KA12 is necessary for responses to karrikins. Both MAX2 and KA12 are essential for normal light-dependent seedling development. The bZIP transcription factor HY5 acts downstream of multiple photoreceptors and promotes photomorphogenesis, but its relationship with MAX2 and KA12 in terms of seedling development and responses to karrikins and strigolactones is poorly defined. Here, we demonstrate that HY5 action is genetically separable from that of MAX2 and KA12. While hy5 mutants have weak hypocotyl elongation responses to karrikins and the artificial strigolactone GR24, they have normal transcriptional responses, suggesting that HY5 is not involved in perception or action of karrikins or strigolactones. Furthermore, we show that overexpression of KA12 is sufficient to enhance responses to both karrikins and GR24 in wild-type seedlings, and that KA12 overexpression partially suppresses the hy5 long hypocotyl phenotype. These results suggest that KA12 and MAX2 define a regulatory pathway that largely operates independently of HY5 to mediate seedling responses to abiotic signals such as smoke and light.

Key words: smoke; karrikin; strigolactone; KA12; HY5; photomorphogenesis.

INTRODUCTION
Successfully transitioning from a germinating seed to an independent, auxotrophic seedling is a fundamental step for angiosperms. Once the decision to germinate has been taken, the seedling must interpret, integrate, and respond to many different abiotic signals. Most prominent among these is light, which initiates a complex cascade of transcriptional and other molecular changes that translate into the light-dependent developmental program known as photomorphogenesis. In epigeal species such as Arabidopsis, photomorphogenesis is characterized by the inhibition of hypocotyl elongation, the opening, expansion and greening of cotyledons, and the promotion of root growth. To bring about these morphological events, plants perceive visible and ultraviolet light through five classes of photoreceptors, of which the most relevant to photomorphogenesis are the red-sensing phytochromes (phyA to E) (Quail, 2010) and the blue/UV-sensing cryptochromes (cry1 to 3) (Chaves et al., 2011). Upon illumination, phytochromes and cryptochromes translocate to the nucleus where they may aggregate in sub-nuclear foci termed photobodies, which are associated with modifying the stability of multiple transcriptional regulators (Chen and Chory, 2011; Van Buskirk et al., 2012). Among the key transcriptional regulators is the basic leucine zipper (bZIP) transcription factor LONG HYPOCOTYL 5 (HY5). In the light, HY5 is stabilized and binds to the G-box, a conserved six base-pair sequence in the upstream promoter regions of light-regulated genes, thus enhancing or repressing transcription (Ang et al., 1998; Chattopadhyay et al., 1998). HY5 operates at a high level in the hierarchy of gene regulation, because it is required for normal seedling development under multiple wavelengths of light (Koornneef et al., 1980), and it regulates
expression of a wide functional range of genes, such as those encoding transcription factors and photosynthesis-related proteins (Lee et al., 2007). In addition, HY5 targets are highly represented among the very earliest transcripts to change in abundance upon exposure to light (Lee et al., 2007). Therefore, HY5 is a pivotal hub in the reprogramming of broad-scale gene expression that brings about the molecular changes associated with photomorphogenesis.

Besides light, other abiotic signals influence seedling development, including temperature and nutrient availability. Fire is a natural phenomenon that dramatically alters the ecological landscape, providing opportunities for plants to exploit newly available resources. Many plants have evolved the capability to sense pyrolysis products from burnt plant material and use them as potent signals to promote seed germination (Light et al., 2009; Nelson et al., 2012). Over the past few years, several bioactive compounds have been isolated from smoke and one, 3-methyl-2H-furo[2,3-c]pyran-2-one or KAR, has defined a class of related compounds known as karrikins (Flematti et al., 2004, 2009). Karrikins (KAR) stimulate seed germination in a wide range of angiosperms including Arabidopsis (Flematti et al., 2004, 2009; Long et al., 2010; Nelson et al., 2012). Structurally, karrikins are characterized by a methyl-butenolide moiety that is also common to strigolactones, a class of endogenous plant hormones originally identified as germination stimulants active on seed of parasitic species within the Orobranchaceae (Cook et al., 1966; Xie et al., 2010). Following the recent identification of strigolactones as the basis for a carotenoid-derived signal that operates via MAX2, the overlapping yet distinct responses of Arabidopsis to these two classes of compounds suggest that the karrikin and strigolactone signal transduction mechanisms might entail both common and separate components.

Genetic screens for karrikin insensitive (kai) mutants have revealed the role of an F-box protein, MAX2, and an αβ hydrolase, KAI2, in seed and seedling responses to karrikins and strigolactones (Nelson et al., 2011; Waters et al., 2012). max2 mutants are fully insensitive to both KAR and GR24 at the seed and seedling stages. In contrast, while kai2 seeds respond to neither KAR nor GR24, kai2 seedlings are insensitive to KAR, but remain sensitive to GR24. Both max2 and kai2 mutants exhibit delayed germination, and both share the same strong seedling phenotype of increased hypocotyl length and epinastic cotyledons (Nelson et al., 2011; Waters et al., 2012). MAX2 and KAI2 likely act in the same signaling pathway, because max2 kai2 double mutant seedlings are phenotypically identical to each single mutant, with the exception that max2 suppresses the GR24 sensitivity of kai2 (Waters et al., 2012). In addition, while max2 exhibits an increased shoot branching phenotype associated with strigolactone insensitivity, the shoot branching phenotype of kai2 is normal, indicating that strigolactone signaling is unperturbed in kai2. Accordingly, we have concluded that KAI2 is necessary for the strigolactone-independent functions of MAX2. Strikingly, a paralog of KAI2, AtD14, is required for normal responses to strigolactones, but not to karrikins (Waters et al., 2012). AtD14 mutants share the same leaf morphology and branching phenotypes as max2 mutants, suggesting that AtD14 is specifically involved in mediating the strigolactone-independent roles of MAX2. Interestingly, AtD14 mutants do not exhibit the same seedling phenotype as max2 and kai2, suggesting that AtD14 is not essential for normal seedling development. KAI2 and AtD14 therefore represent a means of discriminating between similar butenolide compounds that operate via MAX2.

Characterization of kai mutants has revealed a signaling pathway necessary for normal seed germination and seedling development, but how this pathway relates to other known processes is unknown. Intriguingly, both KAI2 and MAX2 have been identified independently in mutant screens for impaired photomorphogenesis. Like hy5 mutants, kai2 and max2 exhibit long hypocotyls under red, far-red, blue, and white light, and the mutations have thus been assigned as lesions in general light signaling (Shen et al., 2007; Sun and Ni, 2011). This has prompted a number of authors to investigate possible interactions between the signaling systems defined by KAI2, MAX2, and HY5. First, HY5 has been implicated in strigolactone signaling, based on the reported insensitivity of hy5 seedlings to GR24 (Tsuchiya et al., 2010). If this is the case, then HY5 and MAX2 are both required for strigolactone signaling; however, hy5 mutants do not exhibit an increased shoot branching phenotype that would normally indicate a strigolactone-related defect. Tsuchiya et al. (2010) also reported that MAX2- and HY5-dependent regulation of hypocotyl growth are genetically separable, making the relative relationships between MAX2 and HY5 in strigolactone signaling difficult to resolve. Previously, Nelson et al. (2010) showed that hy5 mutants were responsive to kai2 in the germination level and, while HY5 was not required for transcriptional responses to karrikins in seed, some 54% of KAR-induced transcripts were also putative HY5
targets, demonstrating a potentially large degree of crosstalk between HY5 and karrikin signaling. At the seedling stage, hy5 was responsive to karrikins but less so than wild-type, implying that HY5 might be necessary for mediating only specific aspects of the karrikin response. However, responses of hy5 to GR24 under similar conditions were not examined, so it remains a formal possibility that hy5 seedlings are sensitive to karrikins, and not to strigolactones. Finally, KAI2 itself has been identified as a likely transcriptional target of HY5 (Sun and Ni, 2011), implying that HY5 might be a direct regulator of the karrikin signaling pathway. Overall, these previous findings demonstrate that the relative roles and interactions of HY5, KAI2, and MAX2 in the regulation of seedling development are unclear.

As KAI2 and MAX2 operate in the same genetic pathway, we set out to resolve how this pathway relates to HY5-dependent development during seedling photomorphogenesis. Here, we report that HY5 is sensitive to both karrikins and strigolactones, and that signaling via HY5 is largely separate from that of KAI2 and MAX2.

RESULTS

hy5 Seedlings Are Sensitive to Both Karrikins and Strigolactones

As demonstrated previously, both KAR and the synthetic strigolactone analog GR24 inhibit hypocotyl elongation in Arabidopsis seedlings, and this fact has been used to identify and characterize mutants with decreased sensitivity to both classes of compound (Nelson et al., 2011; Waters et al., 2012). To clarify the role of HY5 in mediating seedling responses to KAR and strigolactones, we grew wild-type Landsberg erecta (Ler) and hy5-1 (Ler) seedlings in the presence of a 10-fold concentration range of KAR2 and GR24, and measured the hypocotyl elongation response. Under all conditions tested, hy5-1 seedlings showed a consistently longer hypocotyl than Ler. However, while the inhibition of hypocotyl elongation by KAR2 and GR24 was proportionately much stronger in Ler, a significant and dose-dependent inhibition was visible in hy5-1 seedlings (Figure 1A and B). Notably, the trend of decreasing hypocotyl length with increasing KAR and GR24 concentration was the same for both genotypes. Thus, while hy5-1 seedlings respond on a morphological level less prominently to KAR and GR24 than wild-type, these data demonstrate that HY5 is not essential for perception of these compounds.

KAI2 and HY5 Are Independent Regulators of Hypocotyl Development

KAI2, MAX2, and HY5 are necessary for normal seedling development, because kai2, max2, and hy5 mutants all exhibit elongated hypocotyls in the light. Since KAI2 has been shown to be a likely transcriptional target of HY5, it is possible that KAI2 and HY5 operate in the same genetic network. Likewise, as KAI2 and MAX2 regulate hypocotyl development via the same pathway, we might expect HY5 and MAX2 to interact in the same genetic manner as HY5 and KAI2. To test these hypotheses, we generated hy5 kai2 and hy5 max2 double mutants and assayed their hypocotyl responses to KAR2, KAR20, 10 µM GR24, and 10 µM GR24. Data are means ± SE, n = 3 biological replicates, 15–23 seedlings per sample.

Figure 1. hy5 Seedlings Respond to Both KAR2 and GR24 in a Dose-Dependent Manner.
(A) Hypocotyl elongation responses in Ler and hy5-1 seedlings grown for 4 d under continuous red light on 0.5 MS medium plus 1 µM KAR2, 10 µM KAR2, 1 µM GR24, or 10 µM GR24. Data are means ± SE, n = 3 biological replicates, 15–23 seedlings per sample.
(B) Ler and hy5-1 seedlings grown as described in (A). Scale bar = 10 mm.
DOUBLE MUTANTS HAD SIGNIFICANTLY LONGER HYPOCOTYLS THAN ANY OF THE SINGLE MUTANTS (ANOVA, P < 0.01; Figure 2). Untreated hy5 kai2 and hy5 max2 double mutants had similar hypocotyl lengths to one another and, except for the lack of a response to GR24, the kai2 max2 double mutant resembled both parental single mutants. Both of these observations are consistent with KAI2 and MAX2 acting in the same genetic pathway. These findings suggest that HY5 acts largely independently from KAI2 and MAX2. Furthermore, hy5 kai2 retained sensitivity to GR24, while hy5 max2 did not, further indicating that responses to butenolide compounds derive from a process that is KAI2- and MAX2-dependent but HY5-independent.

**hy5 Seedlings Exhibit Normal Transcript Responses to Karrikins and Strigolactones**

While HY5 is not essential for responses to KAR and GR24, we wondered whether the relatively weak hypocotyl response might also be reflected in other seedling responses. To investigate whether HY5 might mediate responses to KAR and GR24 on a molecular level, we assayed the accumulation of several marker transcripts known to change in abundance in seedlings treated with these two compounds. Levels of STH7, KUF1, and DLK2 transcripts respond positively to KAR and GR24, while the auxin-inducible transcript IAA1 decreases in abundance; these effects are all dependent on MAX2 and KAI2 (Nelson et al., 2011; Waters et al., 2012). Under identical conditions to those used to assay hypocotyl responses, the changes of these transcripts in response to KAR and GR24 were essentially identical in both Ler and hy5-1 seedlings (Figure 3). In kai2-2, STH7, KUF1, and DLK2 transcripts were repressed and IAA1 levels were enhanced relative to wild-type, but these transcripts were unchanged in hy5-1. Furthermore, the hy5 kai2 double mutant responded in a similar manner to kai2-2, suggesting that there was no substantial enhancement or suppression of the kai2-2 phenotype, at least on the basis of these marker transcripts. In contrast, transcripts corresponding to CHALCONE SYNTHASE (CHS; a well-characterized transcriptional target of HY5 involved in the biosynthesis of anthocyanins and defense compounds) were substantially depressed in hy5-1 and hy5 kai2, and were no longer induced by GR24. This observation demonstrates that a subset of GR24 responses may be HY5-dependent. Importantly, induction of CHS by GR24 is still MAX2-dependent, as max2 mutants are fully insensitive to GR24 in terms of CHS expression, and show reduced expression of CHS overall (Supplemental Figure 1). However, and unlike in max2, levels of CHS transcripts in kai2-2 seedlings grown on unmodified medium were not affected relative to Ler, suggesting that CHS is not subject to regulation by the KAI2-dependent pathway to the same degree as STH7, KUF1, and DLK2 (Figure 3). Indeed, both KAI2 and AtD14 are required for induction of CHS transcripts by GR24, as the kai2 atd14 double mutant lacks this response entirely (Supplemental Figure 1). Overall, these findings corroborate the morphological data by showing that HY5 activity is separable from that of KAI2 and MAX2. Therefore, with the exception of the induction of direct transcriptional targets such as CHS, HY5 is not required for seedling developmental responses to KAR and GR24.

**Only Short-Term Responses of KAI2 Transcripts to Light Require HY5**

Previously, we showed that transcripts of both KAI2 and its paralog AtD14 are positively regulated by light in seedlings (Waters et al., 2012). In addition, work by others has further shown that the light-dependent induction of KAI2 transcripts requires HY5, consistent with KAI2 being a transcriptional target of HY5 (Sun and Ni, 2011). If HY5 is strictly required for normal levels of KAI2 transcription, then the long hypocotyl phenotype of hy5-1 might in part result from improper expression of KAI2. However, such a direct link between HY5 and KAI2 conflicts with the genetic data presented above, which suggest that the two signaling systems are largely...
independent. To investigate this matter further, we asked whether KAI2 transcripts are misregulated in hy5-1 in a similar manner to other HY5 targets.

When dark-grown Ler seedlings were exposed to 3 h of continuous red light, KAI2 transcripts increased fivefold relative to dark-incubated controls (Figure 4). As anticipated, this induction was limited to just twofold in hy5-1, with KAI2 transcripts reaching only 40% of the level in Ler. However, we also noticed that levels of KAI2 transcripts in dark-grown seedlings were no different between Ler and hy5-1. As a marker for known HY5 targets, we also measured transcripts of CHS and ELIP1. CHS transcripts were enhanced very strongly (40-fold) by light in Ler, but only 20-fold in hy5-1, reaching just 13% of the levels in Ler (Figure 4). An even more disparate pattern was observed for ELIP1, which was induced 500-fold in Ler but just eightfold in hy5-1 (Figure 4). Moreover, levels of CHS and ELIP1 transcripts in dark-grown hy5-1 seedlings were just 26% and 51% that of Ler, respectively (Figure 4), implying that HY5 is required for normal CHS and ELIP1 transcription even in the absence of light, unlike for KAI2. In contrast to KAI2, CHS, and ELIP1, AtD14 transcripts showed a similar induction by light in both Ler and hy5-1, implying that HY5 is not required for this particular light-dependent response.

The relatively small HY5-dependent effect on induction of KAI2 transcripts by light, compared to those of CHS and ELIP1, suggested to us that the influence of HY5 on KAI2 transcription might only be transient. Therefore, we examined gene expression in Ler and hy5-1 seedlings that were exposed to continuous red light for 4 d from germination. In contrast to hy5-1 seedlings given a short exposure to light, hy5-1 seedlings exposed to constant light expressed the same level of KAI2 transcripts as identically treated Ler seedlings (Figure 4). However, both CHS and ELIP1 transcripts remained lower in hy5-1 seedlings, and were present at just 25% and 19%, respectively, of the levels in Ler (Figure 4).

Together, these findings demonstrate that, while HY5 is necessary for initial light-dependent induction of KAI2 transcription, it is not required for long-term maintenance of KAI2 transcript levels, either in the light or in the dark. Furthermore, KAI2 does not exhibit the same HY5-dependent behavior as more robustly light-regulated genes such as CHS and ELIP1.
Overexpression of KAI2 Is Sufficient to Enhance Hypocotyl Responses to Karrikins and Strigolactones

As KAI2 is a key regulator of KAR and GR24 responses in seedlings, we postulated that overexpression of KAI2 would enhance the extent of seedling responses to these compounds. To test this hypothesis, we generated stably transformed lines carrying a 35S:KAI2 transgene in the Ler background, and subjected them to a 10-fold concentration range of KAR2 and GR24. In two independent transgenic lines, we found that inhibition of hypocotyl elongation was significantly enhanced relative to Ler controls treated with the same concentration of KAR2 and GR24 (Figure 5A and B). Notably, we found that 0.1 µM KAR2 and 0.1 µM GR24 triggered a similar degree of hypocotyl growth inhibition in the 35S:KAI2 lines, as did 1 µM KAR2 and 1 µM GR24 in Ler (Figure 5A). Importantly, hypocotyl lengths of untreated 35S:KAI2 seedlings did not differ significantly from untreated Ler controls, suggesting that overexpression of KAI2 does not lead to a constitutive short hypocotyl phenotype under these growth conditions. These results suggest that increased levels of KAI2 enhance both the sensitivity and extent of hypocotyl growth inhibition by exogenous karrikins, and also by GR24.

To establish whether the enhanced hypocotyl responses to KAR2 in 35S:KAI2 seedlings were paralleled on the transcriptional level, we assayed STH7, KUF1, DLK2, and IAA1 transcripts in response to 0.1 µM and 1 µM KAR2. Surprisingly, we found that only DLK2 transcripts accumulated differentially in response to KAR in 35S:KAI2 seedlings compared to Ler. While 1 µM KAR2 triggered a 9.8-fold increase in DLK2 transcripts in Ler relative to untreated seedlings, this fold change was 15.5 and 16.9-fold for the two 35S:KAI2 lines (Figure 5C). A similar enhancement occurred on 0.1 µM KAR2 and, again, 0.1 µM KAR2 was about as effective in 35S:KAI2 lines as 1 µM KAR2 in Ler. By contrast, STH7 and KUF1 transcripts responded in a similar manner to both concentrations of KAR2 in all three genotypes (Figure 5C). While the response of IAA1 transcripts to KAR2 did not differ between Ler and 35S:KAI2 seedlings, these transcripts did show a consistent dose-dependent response, mirroring the dose response of DLK2. On average, KAI2 transcripts were 25–30-fold more abundant in the two independent 35S:KAI2 lines, accounting for the similar responses of both lines. Moreover, neither the endogenous nor 35S promoter-driven KAI2 transcripts themselves changed in response to KAR2 (Figure 5C), suggesting that KAR responses are not modulated by feedback regulation of KAI2 transcription and/or transcript stability.

Taken together, these results imply that increased levels of KAI2 are sufficient to enhance both the sensitivity and absolute response of Arabidopsis seedlings to KAR and GR24. In addition, these data suggest that the response of some transcripts to exogenous butenolides is already saturated at wild-type levels of KAI2.

Figure 4. HY5 Is Required for Early Induction of KAI2 Transcripts by Light But Not for Longer-Term Maintenance of KAI2 Expression.
Levels of KAI2, AtD14, CHS, and ELIP1 transcripts in Ler and hy5-1 seedlings grown on 0.5 MS medium and treated as follows: incubated for 4 d in the dark and then treated either with 3 h red light or 3 h further darkness (left column), and incubated for 4 days under continuous red light (right column). Transcript levels are normalized to CACS reference transcript and scaled to the value of Ler treated with 3 h red light (left column) or Ler (right column). Note the logarithmic scale for CHS and ELIP1. Data are means ± SE, n = 3 biological replicates, greater than 50 seedlings per sample.
Overexpression of KAI2 Weakly Complements the hy5 Long Hypocotyl Phenotype

Considering that KAI2 and HY5 are largely independent at the level of regulating hypocotyl growth, we surmised that KAI2 overexpression might influence the long hypocotyl phenotype of hy5-1, and enhance the responses of hy5-1 to KAR and GR24. Again, we generated two independent lines carrying the 35S:KAI2 transgene in the hy5-1 background, and assessed their hypocotyl growth phenotypes. Relative to untransformed seedlings, overexpression of KAI2 in hy5-1 led to a general decrease in hypocotyl length under all treatments (Figure 6A and B). However, these effects were only consistently statistically significant in one of the two transformed lines, in which expression of KAI2 was several-fold higher than in the other (Figure 6C). Thus, while the trend for the effect of KAI2 overexpression in hy5-1 is consistent, the magnitude of this effect is dependent on KAI2 expression level. Furthermore, while the absolute hypocotyl lengths of 35S:KAI2 seedlings were shorter than untransformed hy5-1 seedlings under each treatment, the proportional inhibition of hypocotyl growth
by KAR$_2$ and GR24 was similar for all lines (Figure 6A). This contrasts with the effect of KAI2 overexpression in wild-type, where KAR$_2$ and GR24 were proportionally more effective in the 35S:KAI2 lines than in Ler controls (Figure 5A). Therefore, the morphological effects of KAR and GR24 are restricted in the absence of functional HY5, consistent with the weak morphological responses of hy5-1 seedlings (Figure 1 and 2).

Taken together, these findings suggest that reduced levels of KAI2 transcripts in hy5-1 may, to a limited degree, account for the long hypocotyl phenotype of hy5-1. However, the weak hypocotyl response to KAR$_2$ and GR24, even in the presence of elevated KAI2 expression, suggests that HY5-dependent signaling dominates over the KAI2- and MAX2-dependent pathway in the regulation of hypocotyl growth. These data are consistent with the interpretation that KAI2 and MAX2 are largely independent of HY5 in regulating light-dependent seedling development.

**DISCUSSION**

Using a combination of molecular and genetic analyses, we have shown the substantially independent nature of two signaling systems that orchestrate light-dependent seedling development. In contrast to a previous report (Tsuchiya et al., 2010), our results show conclusively that HY5 is not necessary for either perception or response to karrikins and strigolactones, but rather that the broad light-hypsensitive phenotype of hy5 masks much of the visible effects of these compounds on seedling growth. More importantly, the data from KAI2 overexpression suggest that changes in KAI2 levels alone are sufficient to modulate signaling in response to exogenous karrikins and strigolactones, at least with respect to seedling hypocotyl development.

Three main lines of evidence demonstrate that KAI2–MAX2 and HY5 define two genetically distinct signaling systems regulating hypocotyl growth. First, hy5-1 enhances the long hypocotyl phenotype of both kai2-2 and max2-8, while kai2-2 and max2-8 do not enhance one another (Figure 2). Second, with the exception of CHS as discussed below, hy5-1 does not influence known responses to KAR and GR24 on the transcript level (Figure 3). Third, overexpression of KAI2 is sufficient to enhance KAR and GR24 responses and can thereby partially suppress the hy5-1 phenotype (Figures 5 and 6). Nevertheless, KAI2–MAX2 and HY5 signaling have the capacity to interact on at least two levels. One possibility is by the direct binding of HY5 to the G-box in the KAI2 promoter, as has been shown in vitro (Sun and Ni, 2011). However, our results suggest that the functional relevance of this interaction is limited, because HY5 appears to regulate only the initial, short-term response of KAI2 transcripts to light (Figure 4). As overexpression of KAI2 only weakly complements hy5-1, it seems that misregulation of KAI2 is not a primary cause of the long hypocotyl phenotype. Indeed, KAI2 is not among those target genes classified as misregulated in the hy5 mutant (Lee et al., 2007).
Therefore, we propose that HY5 is important for coordinating the timing of KAI2 induction in parallel with numerous other light-responsive components, but is not critical for sustaining the KAI2–MAX2 signaling process.

A second route for limited crosstalk between HY5 and KAI2–MAX2 signaling is at the point of transcription of common targets. While the KAR- and GR24-responsive transcripts STH7, KUF1, DLK2, and IAA1 are unaffected by loss of HY5, GR24 induces CHS in a HY5-dependent manner (Figure 3). CHS is a direct transcriptional target of HY5, a conclusion based on chromatin immunoprecipitation assays, in planta promoter analysis, and electromobility shift assays (Ang et al., 1998; Lee et al., 2007). The induction of CHS by GR24 is dependent on MAX2 as well as HY5, implying that MAX2 and HY5 both contribute to normal CHS expression; this conclusion is also supported by the reduced levels of CHS transcripts in max2 seedlings (Supplemental Figure 1). Importantly, the MAX2-dependent nature of CHS induction suggests that the GR24 treatment does not simply trigger a non-specific stress response that is commonly associated with increased anthocyanin levels (Winkel-Shirley, 2002). Mechanistically, GR24 may promote transcription of CHS indirectly via an increase in HY5 protein levels, possibly by modulating COP1 activity as has been reported (Tsuchiya et al., 2010). COP1 is a ring domain-containing WD40 repeat protein that functions as an E3 ubiquitin ligase, and which targets positive regulators of photomorphogenesis (such as HY5) for proteasomal degradation in the dark (Osterlund et al., 2000; Seo et al., 2003). Our observations imply that this increase in HY5 activity is achieved via MAX2, because max2 (but not hy5-1) is truly insensitive to GR24, and GR24 cannot promote CHS transcription in the absence of functional MAX2. Curiously, we found that CHS transcript levels in untreated seedlings grown under continuous red light were unaltered by the kai2-2 mutation, which is consistent with KAR, having only a weak effect on CHS expression (Figure 3 and Supplemental Figure 1). This is contrary to a previous report stating that htl-1 mutants (synonymous with kai2) exhibited reduced CHS expression and lower anthocyanin levels (Sun and Ni, 2011). This discrepancy may reflect slight differences in growth conditions, especially temperature, which in turn may affect anthocyanin production. Alternatively, the discrepancy may result from ecotype-specific differences (Ws-4 for htl-1 versus Ler for kai2-2). Nevertheless, it is clear that KAI2 is involved in mediating changes in CHS expression induced by GR24; more specifically, KAI2 acts redundantly with its paralog AtD14 in this process (Supplemental Figure 1). It should be noted that AtD14 likely plays a minor role during seedling development, as AtD14 mutant seedlings resemble wild-type, and the seedling phenotype of kai2 AtD14 mutants is not noticeably enhanced over that of the kai2 single mutant (Waters et al., 2012). Nevertheless, together, these observations provide strong evidence for the MAX2 pathway mediating primary responses to karrikins and strigolactones, via KAI2 and AtD14, respectively, while HY5 acts largely in parallel (Figure 7). However, considering that light perception is necessary for morphological karrikin responses and that karrikins enhance light sensitivity (Nelson et al., 2010), a certain degree of overlap between the transcriptional outputs of MAX2 and HY5 signaling, as exemplified by CHS, is to be expected.

The discovery that the KAI2–MAX2 pathway is distinct from that of HY5 raises the question of how the former relates to other known light signaling pathways. Both the HY5- and KAI2–MAX2-dependent signaling pathways operate...
downstream of multiple photoreceptors, because the respective mutant phenotypes are not specific to any wavelength of light (Koornneef et al., 1980; Shen et al., 2007; Sun and Ni, 2011). PhyB binds at least four different phytochrome-interacting proteins (PIFs), which suppress photomorphogenesis in the dark by binding to the G-box motif in the promoters of light-regulated genes and acting as negative transcriptional regulators (Leivar et al., 2008; Shin et al., 2009; Stephenson et al., 2009). Upon illumination, activated PhyB translocates to the nucleus, binds to PIFs, triggers their phosphorylation and proteosomal degradation, and thus relieves transcriptional repression (Bauer et al., 2004; Shen et al., 2008). Consistently with the role of PIFs as negative regulators of light signaling, pif1 pif3 pif4 pif5 quadruple mutants exhibit enhanced seed germination in the dark and short hypocotyls, both in the dark and in red light (Shin et al., 2009). These phenotypes are opposite to those shown by max2 and ka2 mutants. Recently, the relationship between MAX2 and PIF1 was assessed directly by epistasis analysis, which showed that pif1 could not suppress the max2 long hypocotyl phenotype in red light (Shen et al., 2012). However, because PIF1 does not play a major role in inhibition of hypocotyl elongation under red light (Shin et al., 2009), the interpretation of the relationship between MAX2 and PIF1 is unclear on the basis of these results. Instead, pif1 max2 double mutants exhibited a germination phenotype intermediate between that of the pif1 and max2 parents, suggesting that PIF1 and MAX2 act in opposite directions and in separate, parallel pathways to regulate seed germination and seedling de-etiolation (Shen et al., 2012). Among other regulatory components of light signaling, COP1 activity is down-regulated by both phytochrome- and cryptochrome-dependent light signaling (Yang et al., 2001; Chen and Chory, 2011; Liu et al., 2011). Thus, loss-of-function cop1 mutants exhibit photomorphogenic seedling development in the dark and exaggerated, hypersensitive responses to light (Deng and Quail, 1992). Interestingly, max2 was shown to partially suppress the cop1 de-etiolation phenotype, and vice versa; the intermediate phenotype of cop1 max2 mutants suggests that MAX2 likely operates in parallel with COP1 (Shen et al., 2012). By extension, it is therefore reasonable to assume that KAI2 and MAX2 define a developmental pathway that is distinguishable from, but interacts with, HY5, PIF, and COP1 signaling during seedling development.

Considering what we know about the role of MAX2 and KAI2 in strigolactone and karrikin signaling, the interpretation of max2 and ka2 as light signaling mutants may be overly simplistic. Rather than primarily exhibiting light hypersensitivity, we propose that the morphological phenotypes of max2 and ka2 mutants result in part from impaired auxin signaling. First, treatment of wild-type seedlings with exogenous auxin leads to elongated hypocotyls and epinastic cotyledons, effects that can be phenocopied in high auxin mutants such as yucca (Zhao et al., 2001) and superroot (Boerjan et al., 1995). Second, both max2 and ka2 mutants show elevated levels of IAA1 expression (Hayward et al., 2009; Nelson et al., 2011; Waters et al., 2012), consistent with increased auxin levels and/or signaling (Park et al., 2002; Yang et al., 2004). Notably, this change in IAA1 transcripts is not observed in hy5-1 mutants (Figure 3), demonstrating that an elongated hypocotyl is not necessarily correlated with broadly impaired auxin signaling. Third, max2 hypocotyls are disproportionately sensitive to the auxin transport inhibitor NPA (Shen et al., 2012). Fourth, max2 mutants show several auxin-related defects such as increased adventitious rooting (Rasmussen et al., 2012), increased stem auxin transport (Bennett et al., 2006), and increased lateral root density (Kapulnik et al., 2011). The extent to which these defects are shared by ka2 mutants is currently under investigation. Finally, strigolactones and auxins are intricately linked in feedback loops that underpin the regulation of shoot branching. Auxin up-regulates the expression of strigolactone biosynthetic genes in Arabidopsis, pea, and rice (Foo et al., 2005; Arite et al., 2007; Hayward et al., 2009), and strigolactones inhibit auxin transport from axillary buds in a MAX2-dependent manner (Crawford et al., 2010). Endogenous strigolactones are not critical for Arabidopsis seedling development, because strigolactone biosynthesis mutants appear normal in this respect (Nelson et al., 2011; Shen et al., 2012), raising the question of the functional relevance of the KAI2–MAX2 signaling pathway. We propose that the KAR-insensitive phenotypes of ka2 and max2 mutants reflect the existence of an endogenous butenolide-based signaling pathway that does not involve canonical strigolactones (Waters et al., 2012), and which interacts at some level with auxin and light signaling to regulate growth and development.

Interestingly, aberrant auxin signaling also appears to be partially responsible for several phenotypes of hy5 seedlings (Cluis et al., 2004; Sibout et al., 2006). This link is particularly apparent in mutants lacking activity of both HY5 and its homolog HYH. hy5 hyh double mutants display enhanced auxin-related defects such as reduced primary root length, impaired vascular patterning, and altered auxin-induced gene expression (Sibout et al., 2006). Overexpression of HYH complements the hy5 seedling phenotypes, suggesting that HY5 and HYH act largely redundantly to regulate various aspects of seedling development, including auxin response (Sibout et al., 2006). Inappropriate auxin responses could explain, in part, the weak morphological response of hy5 seedlings to KAR and GR24. Accordingly, any relationship between the KAI2–MAX2 pathway and HY5 signaling should consider HYH as well, which, together with HY5, could potentially modulate some of the auxin-related changes induced by KAR and GR24. From this point of view, while HY5 and KAI2–MAX2 generally operate in separate and parallel signaling processes during early light-dependent seedling development, they may subsequently converge downstream via auxin-dependent growth processes.

In summary, we have shown that HY5 is not essential for the perception of strigolactones or karrikins. Instead, our data are consistent with the current position that all known SL and KAR responses in Arabidopsis are mediated by MAX2, and different butenolides are functionally discriminated by
KAI2 and AtD14. Understanding how the KAI2–MAX2 pathway relates to other hormone signaling processes will further help to clarify how these butenolide compounds act.

METHODS

Plant Material and Growth Conditions

Plants were grown in peat, vermiculite, and perlite mixture (6:1:1) under white fluorescent lamps emitting 100–120 µmol photons s⁻¹ m⁻² with a 16-h light/8-h dark photoperiod, and a 22°C light/16°C dark temperature cycle. For seedlings grown on 0.5 MS medium, karrikins and GR24 were added from 1000 stock solutions in acetone (0.1, 1, or 10 mM); an equivalent volume of acetone was added to untreated controls. The hy5-1 mutant was obtained from the European Arabidopsis Stock Centre (NASC ID: N71). The kai2-2, AtD14-1, and max2-8 mutants, and the double mutant combinations, were described previously (Nelson et al., 2011; Waters et al., 2012). To isolate hy5-1 kai2-2 double mutants, F₂ seed were sown on 0.5 MS medium containing 1 µM KAR, and grown under conditions for hypocotyl elongation assays. Seedlings with long hypocotyls (putative kai2-2 homozygotes) were selected and transferred to soil. kai2-2 homozygotes were confirmed by genomic PCR (Waters et al., 2012), and the F₃ progeny of kai2-2 mutants were further screened for the agavitropic lateral root phenotype of hy5 (Oyama et al., 1997). The double mutant F₂ seed was used for experiments. To generate hy5-1 max2-8 double mutants, the procedure was identical, except that F₂ max2-8 homozygotes were identified by the increased auxillary shoot branching phenotype.

Generation of Transgenic Plants

Total RNA was extracted from 7-day-old Arabidopsis seedlings using the RNeasy (Qiagen; www.qiagen.com) procedure. cDNA was generated from 1 µg of total RNA in a 20-µl reverse transcription reaction (Superscript III, Life Technologies; www.lifetechnologies.com). The KAI2 coding region was amplified with oligonucleotides MW311 5′-GGGACCAAGTTGTACAAAAAAGCAGGCTTGTTGAGAAAGAAAGCTGTCAT-GGTGTGGTAGAAGAA-3′ and MW312 5′-GGGACCACTTTGTACAAGAAAGCTGTCAT-GGTGTGGTAGAAGAA-3′ (translation initiation and stop codons, respectively, in boldface; Gateway attB combination sequences underlined) using a proofreading DNA polymerase (Phusion, New England Biolabs; www.neb.com). The PCR product was cloned into pDONR207 via Gateway-mediated recombination, and positive clones were confirmed by sequencing. To generate a binary vector driving expression of KAI2 by the 35S promoter, the coding sequence was transferred to pMDC32 (Curtis and Grossniklaus, 2003) via Gateway-mediated recombination. Ler and hy5-1 plants were independently transformed using the floral dip method. Transgenic seedlings were selected by growth in continuous light for 7 d on 0.5 MS medium containing 25 µg ml⁻¹ hygromycin B. At least 15 and 22 T₁, primary transformants were obtained for Ler and hy5-1, respectively, and propagated on soil. The T₂ progeny of primary transformants were screened for segregation of the transgene based on a 3:1 ratio of resistant to sensitive seedlings. Of these, at least two lines in each background were propagated to homozygosity. Experiments were performed on T₃ seedlings.

Hypocotyl Elongation Assays

Hypocotyl elongation assays were performed as described previously (Waters et al., 2012).

RNA Isolation and Transcript Analysis

Surface-sterilized seed, sown on 6-cm plates containing solidified 0.5 MS medium (pH 5.9), was stratified in the dark at 4°C for 3 d. The plates were then exposed to white light (100–120 µmol photons s⁻¹ m⁻²) for 3 h at 20°C, darkened by covering in foil for a further 21 h, and then exposed to continuous red light from LEDs (λ_max = 652 nm, 20 µmol photons s⁻¹ m⁻²) for 4 d in a light-proof growth cabinet maintained at 19–20°C. Pools of seedlings grown on separate plates were harvested under red light and snap-frozen in liquid nitrogen. Dark-grown seedlings were grown in parallel under otherwise identical conditions, and harvested under green safe light. Total RNA was extracted using the RNeasy (Qiagen) procedure coupled with on-column DNase digestion as recommended by the manufacturer, and the RNA was quantified with a NanoDrop 1000 spectrophotometer. cDNA was generated from 0.5 µg total RNA in a 10-µl reaction using the iScript cDNA Synthesis kit (Bio-Rad; www.bio-rad.com). The cDNA was then diluted with 30 µl water. Quantitative RT-PCR was performed on a Roche LC480 (Roche; www.roche-applied-science.com) using 0.5 µl cDNA (12.5 ng µl⁻¹), 0.2 µM each primer, and 2.5 µl 2x LightCycler 480 SYBR Green Master Mix (Roche) in a final volume of 5 µl per reaction. Cycle conditions were: 95°C for 10 min; then 45 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, followed by melt curve analysis. Crossing point (Cp) values were calculated under high confidence. For each biological replicate, two technical replicates of each real-time PCR were examined, and the mean Cp value was used to calculate expression relative to an internal reference gene using the formula (1+ΔCp)²–Cp, where E is the primer efficiency. Primer efficiencies (0.9 or greater) were determined in separate runs using serial dilutions of pooled cDNA. Primer sequences for quantitative PCR are listed in Supplemental Table 1.

Statistical Analysis

To compare hypocotyl responses between genotypes within a single treatment, one-way, two-sided ANOVA (Bonferroni t-test) was performed. P-values were derived from post-hoc tests using Tukey’s correction for multiple pairwise comparisons. Statistical computation was performed with SAS Enterprise Guide 4.3 (SAS; www.sas.com).

Accession Numbers

The AGI identifiers for the Arabidopsis genes described in this work are: KAI2, At4g37470; MAX2, At2g42620; HYS,
At5g11260; AtD14, At3g03990; STH7, At4g39070; KLF1, At1g31350; DLK2, At3g24420; IAA1, At4g14560; CHS, At5g13930; ELIP1, At3g22840; and CACS, At5g46630.

**SUPPLEMENTARY DATA**

Supplementary Data are available at *Molecular Plant* Online.

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**REFERENCES**


