F-box protein MAX2 has dual roles in karrakin and strigolactone signaling in Arabidopsis thaliana

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Smoke is an important abiotic cue for plant regeneration in postfire landscapes. Karrikins are a class of compounds discovered in smoke that promote seed germination and influence early development of many plants by an unknown mechanism. A genetic screen for karrakin-insensitive mutants in Arabidopsis thaliana revealed that karrakin signaling requires the F-box protein MAX2, which also mediates responses to the structurally-related strigolactone family of phytohormones. Karrikins and the synthetic strigolactone GR24 trigger similar effects on seed germination, seedling development, and inhibition of hypocotyl elongation. These observations suggest that a MAX2-dependent signal transduction mechanism was adapted to mediate responses to two chemical cues with distinct roles in plant ecology and development.

Seed germination is a crucial developmental transition in the plant life cycle. As such it is highly regulated in many plant species by physiological seed dormancy, which prevents germination except under specific environmental conditions (1). Smoke elicits a significant ecological impact on postfire environments, as it is known to stimulate germination of over a thousand plant species (2, 3). A key bioactive signal in smoke was discovered to be the butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one (4, 5), which is now known as karrakinolide or Kar2 (3). Five additional analogous butenolide compounds have since been identified in smoke and comprise a unique family known as karrikins (6). Karrikins trigger germination and promote seedling vigor for a broad range of plant species at concentrations as low as 1 nM (7–9).

Karrikins can strongly enhance germination of primary dormant Arabidopsis thaliana seed, although this response depends upon the depth of seed dormancy and requires both light and synthesis of the phytohormone gibberellin (10). KAR1 and KAR2 (Fig. 1A) were found to be the most active karrikins in Arabidopsis (10). A microarray-based investigation of molecular responses to KAR1 preceding germination revealed a predominance of light-regulated transcripts among karrikin-responsive genes (11). We also discovered that germination of nondormant Arabidopsis seed is triggered at lower fluences of red light when treated with KAR1. Cotyledon expansion and inhibition of hypocotyl elongation are enhanced in the presence of germination-active karrikins in a light-dependent manner, providing further evidence of karrikin crosstalk with light signaling pathways. Indeed, the transcription factor HY5, which is a key player in light signal transduction, is up-regulated by karrikins and has a role in postgerminative karrin responses. We found, however, that HY5 is not required for the induction of early KAR-responsive transcripts in seeds (11). Therefore, central components of the karrin response mechanism remain unknown.

Karrikins share partial structural similarity with strigolactones (Fig. 1A), a family of phytohormones present in root exudates that stimulate germination of parasitic weeds (Orobanchaceae and Strigaceae spp.) (12, 13) and enhance hyphal branching in arbuscular mycorrhizal fungi (14). Additionally, strigolactones or a strigolactone-derived signal repress shoot branching. For the remainder of this article, we will simply refer to the active signal as strigolactone. As demonstrated by an array of mutants in pea, rice, petunia, and A. thaliana, strigolactone deficiency or insensitivity gives rise to increased shoot branching (reviewed in refs. 15, 16). In Arabidopsis, several more auxiliary growth (max) mutants have been characterized. Two carotenoid cleavage dioxygenases, MAX3, MAX7, and MAX4/CCD8, and a cytochrome P450 enzyme, MAX1, are implicated in the formation of strigolactone (17–23). Multiple strigolactones are produced in Arabidopsis, including orobanchol and orobanchyl acetate (24, 25). The ccd7 and ccd8 mutants are strigolactone-deficient, and application of the synthetic strigolactone GR24 restores inhibition of auxillary shoot outgrowth to these mutants in rice, pea, and Arabidopsis (18, 19). Reciprocal grafting experiments demonstrated that the F-box protein MAX2 acts downstream of MAX1/MAX3/MAX4 (26). Consistent with this result, shoot branching in max2 is insensitive to GR24 (18, 19). Recently, max1 and max4 mutants in Arabidopsis, but not max2, have been shown to have significantly reduced levels of orobanchol (25), providing further evidence that the increased branching of max2 is not caused by a defect in strigolactone production.

There are distinct as well as common responses to these two plant-growth regulators. As is the case with karrikins, GR24 has been shown to stimulate seed germination and inhibit hypocotyl elongation in Arabidopsis (10, 11, 27). However, GR24 is typically required at much higher concentrations than karrikins to promote seed germination of Arabidopsis and Brussica tournefortii, and KAR1 is unable to stimulate germination of at least four GR24-responsive parasitic weed species (7, 10). KAR1 is also inactive in assays for induction of hyphal branching in arbuscular mycorrhizal fungi, although strigolactones are extremely effective (14, 28). Karrin molecules have a butenolide moiety in common with the D-ring of strigolactones (Fig. 1A, highlighted in red). The butenolide D-ring of strigolactones is necessary, but not sufficient, to stimulate germination of parasitic weeds and hyphal branching of arbuscular mycorrhizal fungi (28, 29). As several variants of this butenolide ring were also ineffective as Arabidopsis seed-germination stimulants (Fig. S1), the similar responses but distinct biological effects suggest that these compounds may act by different mechanisms.


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Two kai mutants, kai1-1 and kai1-2, shared multiple phenotypes, including increased axillary shoot branching, reduced inflorescence height, delayed senescence, leaf curling, and elongated hypocotyls (Fig. 1 B–D). The kai1 seed had enhanced primary dormancy, which could be partially overcome by 10 mM KNO$_3$ treatment, but was completely unresponsive to promotion of germination by KAR$_1$, KAR$_2$, or GR24 (Fig. 1E).

We noted that kai1-1 and kai1-2 exhibited several phenotypes in common with the Arabidopsis mutant more axillary growth 2 (max2), which has also been identified in genetic screens as oressa9 (ore9) (30), a delayed senescence mutant, and pleiotropic photomorphogenesis (pps) (31), a light hypersensitive mutant. Sequencing of the MAX2 gene in kai1-1 and kai1-2 mutants revealed two unique alleles: a 4-bp net insertion (now called max2-7) and a 4-bp deletion (now called max2-8), resulting in frame-shifts of the 693 aa-encoding MAX2 ORF after 248 and 66 codons, respectively (Fig. 2A).

Germination and Photomorphogenesis Responses to Karrikins and GR24 Are Abolished in max2. To further test the link between kai1 phenotypes and the MAX2 locus, we examined two additional alleles, max2-1 and max2-2, which were previously identified in the Columbia (Col) ecotype (22). Supporting our results with max2-7 and max2-8, these max2 alleles also exhibited highly reduced germination (Fig. 2B). After 92 h in continuous white light conditions, max2-1 and max2-2 seeds attained ~10% to 20%

**Results**

**Two max2 Alleles Are Identified from a Genetic Screen for Karrikin-Insensitive Mutants.** To identify components of the karrikin response mechanism, we took advantage of the ability of KAR$_1$ to promote Arabidopsis seed germination strongly (10). A primary dormant M$_2$ population of $\gamma$-irradiated Ler (Landsberg erecta ecotype) seed was screened for failure to germinate on water-agar supplemented with 1 $\mu$M KAR$_1$. Nongerminating seed were transferred to nitrate-containing media to overcome dormancy and induce germination of viable seed. Primary dormant M$_2$ seed from candidate karrikin-insensitive (kai) mutants were rescreened for germination insensitivity to KAR$_1$. Seven of 245 putative kai lines maintained a reduced germination phenotype in the presence of KAR$_1$.
germination on water-agar control media, compared with ~70% germination for Col wild-type. In addition, germination was not enhanced by KAR, KAR2, or GR24 in either max2 allele (Fig. 2B). It was previously reported that max2 alleles have reduced germination following red or far-red light pulses because of light hyposensitivity (31). However, as light exposure was not a limiting factor in our assays, we propose that the low germination of max2 is also consistent with increased seed dormancy. This max2 phenotype may not have been observed by other researchers, as primary dormancy in Arabidopsis is readily alleviated by the common laboratory practices of after-ripening, sowing seed on nitrate-containing media [e.g., Murashige and Skoog (MS)], and cold stratification (32), procedures which we did not follow.

Seed dormancy state has been demonstrated to restrict the germination response to karrikins or smoke (9, 10). In our hands, Col seed has low primary dormancy and its germination is not strongly promoted by karrikin (Fig. 2B and ref. 10). Thus, it could be argued that this assay is not sufficiently amenable to test for karrikin responses in max2 seed. However, seedling photomorphogenesis is consistently enhanced by karrikins in multiple Arabidopsis ecotypes, including Col, even after alleviation of seed dormancy (11). One microarray Kar1, Kar2, and GR24 inhibited hypocotyl elongation of wild-type Col under continuous red light by ~25%, 45%, and 65%, respectively, and similar degrees of inhibition were observed in max1, max3, and max4 seedlings (Fig. 2C). In contrast, hypocotyl elongation of max2 was unaffected by treatment with karrikins or GR24 (Fig. 2C). The max2 hypocotyls were also more elongated compared with wild-type (10.4 mm vs. 8.4 mm, P < 0.001), consistent with prior reports (22, 31). We conclude that MAX2 regulates seed dormancy, and it is also required for early developmental responses to both karrikins and strigolactones.

Unlike max2, the max1, max3, and max4 mutants exhibited normal seed germination and hypocotyl length under control conditions, and maintained wild-type growth responses to both karrikin and GR24 (Fig. 2 B and C). Thus, other components of the MAX pathway, which are involved in the biosynthesis of strigolactones, are not required for karrikin activity. This finding also demonstrates that although Arabidopsis plants are competent to respond to exogenous strigolactone application during early developmental stages in a MAX-dependent manner, strigolactone deficiency does not appear to affect normal seed dormancy or hypocotyl elongation.

In contrast with our results, Tsuchiya et al. (27) found that max1-1 seed has reduced germination compared with wild-type under a specific light condition. As only a single strigolactone-deficient allele was reported, and details with regard to seed dormancy, light intensities, and growth media are not described, it is difficult to address whether this observation does, in fact, indicate a role for endogenous strigolactones in promoting seed germination in particular circumstances.

**MAX2 is Required for Induction of Early Transcriptional Markers of Karrikin Response.** We previously demonstrated that the ga1-3 and hy5-1 mutants maintain transcriptional responses to karrikin despite displaying karrikin hyposensitivity in germination or hypocotyl elongation assays, respectively (11). To determine if MAX2 has a central role in karrikin signaling, we examined the effects of KAR, KAR2, and GR24 on early transcriptional markers of karrikin response in the max2 mutant. The most quickly and strongly karrikin-induced transcripts in primary dormant Ler seed include a double B-box domain transcription factor, STH7 (At4g39070), an oxidoreductase, KUOX1 (At1g70740), and another F-box protein, KUF1 (At1g31350) (11). We found that GR24 was as effective as karrikins in up-regulating these three marker genes after 24 h of seed imbibition, providing molecular evidence that strigolactones and karrikins may share some signaling mechanisms (Fig. 3). However, no induction of these markers by either karrikin or GR24 was observed in imbibed max2-7 or max2-8 seed, demonstrating that this is a MAX2-dependent response (Fig. 3). In seedlings of max2-7 and max2-8, a similar lack of STH7 and KUF1 transcriptional responses to karrikin or GR24 was observed (Fig. S2). These findings are further supported by a recent report that STH7 is induced by GR24 application within 3 h in max1, but not max2, seedlings (33).

Notably, STH7 and KUF1 transcripts were respectively reduced by ~25- and 3.5-fold in untreated max2 seed (Fig. 3) and ~4- and 1.5-fold in max2 seedlings (Fig. S2), indicating that MAX2 promotes their expression even in the absence of exogenous treatments. In contrast to the strong, early markers of karrikin response, HY5 is only mildly induced by karrikin after ~12 to 24 h of seed imbibition (11). Although HY5 transcript abundance was not decreased in max2 seed relative to wild-type, as it was for the early markers, max2 also lacked the small but significant up-regulation of HY5 by karrikin or GR24 treatment (Fig. 3). As early transcriptional markers as well as developmental responses to karrikins and strigolactone are abolished in max2, we conclude that MAX2 is an essential part of the signal transduction mechanism for both of these classes of plant-growth regulators.

**MAX4 and IAA1 Are Suppressed by Karrikins in a MAX2-Dependent Manner.** Negative feedback regulation of transcripts involved in strigolactone biosynthesis (e.g., MAX3/CCD7 and MAX4/CCD8) has been previously observed in several species (23, 33–38). In the Arabidopsis max1, max2, max3, and max4 mutants, MAX3 and MAX4 as well as auxin-responsive IAA1 transcripts are all elevated (34). Complementing this observation, application of GR24 to max1 seedlings suppressed the overexpression of MAX3 and MAX4 (33). The feedback inhibition mechanism appears to be MAX2-dependent (33). To test whether karrikins can mimic strigolactone feedback, we examined the expression of MAX4 and IAA1 in treated wild-type and max2 seedlings (in our hands, MAX3 expression was too low for reliable detection). KAR1 and KAR2 reduced MAX4 and IAA1 expression by approximately twofold in wild-type seedlings (Fig. 4). These responses were similar to those observed in GR24-treated seedlings, except that although karrikins and GR24 have the same effects on IAA1 expression, karrikins were less effective than GR24 as inhibitors of MAX4 expression. As expected, MAX4 and IAA1 were up-
regulated in max2 seedlings and unresponsive to either karrkin or GR24 treatment (Fig. 4).

**Karrkins Do Not Suppress Shoot Branching in Arabidopsis or Pea.**

The above data suggest that karrkins and strigolactones share a common MAX2-dependent signal transduction mechanism in Arabidopsis. However, as there are multiple examples that indicate these signals are not interchangeable (see Introduction), distinct functional roles for karrkins and strigolactones in other aspects of plant development may be expected. To determine whether karrkins can compensate for the effect of strigolactone deficiency in adult plants, we tested the response of max3 and max4 axillary shoot development to KAR1 and KAR2. Shoot branching in both mutants was clearly suppressed by GR24 delivered hydroponically through roots, but was insensitive to an equivalent karrkin application (Fig. 5 A and B). The lack of a max4 branching response to karrkins was confirmed independently by two of us in a second laboratory (E.A.D. and C.A.B.) using an alternative semihydroponic application method (Fig. S3). As expected, the increased shoot branching phenotype of max2 mutants was insensitive to any treatments (Fig. S3). Thus, strigolactones regulate shoot branching by a MAX2-dependent mechanism that does not recognize karrkins.

To address the possibility that karrkins only appear to be ineffective shoot branching inhibitors in our assays because they are not taken up by roots and delivered to shoots, we examined transport of [3H]-KAR1 in hydroponically grown Arabidopsis plants. Radioactivity was detectable in the lower stem within 3 h of addition to hydroponic media, and continued to increase in abundance throughout the plant over the 96-h time course (Fig. 5C). Although transport was initially observed in stem tissue, by 96 h the radiolabel was present in all aerial tissues and most highly concentrated in the siliques (Fig. S4). Scintillation counting of HPLC-fractionated (6) plant extracts confirmed that [3H]-KAR1 was present in each part of the plant. This finding demonstrates that karrkins is readily taken up by roots and distributed throughout the aerial tissue of adult plants.

We performed an additional test for karrkin influence on bud outgrowth in pea, which is responsive to GR24 at concentrations as low as 10 nM (18). As previously demonstrated, bud elongation of rms1 (equivalent to Arabidopsis max4) plants was strongly suppressed by direct application of 1 μM GR24, but ms4 (equivalent to Arabidopsis max2) buds had no significant response to the treatment (Fig. 6). In contrast, even at a 10-μM concentration neither KAR1 nor KAR2 reduced bud elongation in the rms1 mutant. Furthermore, ms4 was similarly unresponsive to karrkins. Thus, karrkins appear to be ineffective inhibitors of axillary shoot outgrowth in both pea and Arabidopsis, and cannot compensate for strigolactone deficiency in this aspect of development.

**Discussion**

As structurally related molecules, it is perhaps not altogether surprising that karrkins and strigolactones can similarly promote seed germination, inhibit hypocotyl elongation, and up-regulate expression of a common subset of genes in Arabidopsis. Even more suggestive of a common perception mechanism is the feedback effect of karrkins on MAX4 and IAA1 expression.
However, these signaling molecules are also quite distinct in terms of their known sources (i.e., in planta vs. smoke), as well as the effects they produce on seed from different species, arbuscular mycorrhizal association, and shoot branching. Thus, the extent of the relationship between karrikins and strigolactones has in the past remained unclear. Through a forward genetic screen we have now clearly linked the signal transduction for these two families of plant-growth regulators and identified previously unexplored roles for MAX2 as a key regulator of karrikin signaling and seed dormancy.

With the revelation that karrikin and strigolactone signaling must converge at or before MAX2, we wished to address whether these ligands are metabolically linked to produce a common signal transduced by MAX2. For example, karrikins may not have restored branching suppression in the max3 or max4 mutants if they must be converted to a strigolactone-related signal in a MAX3/MAX4-dependent manner. However, as max3 and max4 were fully responsive to karrikin during germination and seedling development, this contradicts a MAX3/MAX4-dependent metabolism model. One possible explanation for this discrepancy is that conversion of karrikin to an active strigolactone-related signal may be specifically lacking in adult tissues. However, we alternatively propose that karrikins converge with strigolactones at the point of signal transduction rather than through metabolism. MAX2 encodes an F-box protein with 18 leucine-rich repeats (30). F-box proteins act as components of the SCF (Skp1, Cul1, RBX1, F-box protein) complex, a class of E3 ligases that ubiquitinate specific target proteins. Depending on the degree of ubiquitination, modified proteins may have altered function or be targeted for degradation by the 26S proteasome. An F-box protein acts as an adapter protein for the SCF complex; the N-terminal F-box domain associates with Skp1 and the C terminus confers substrate specificity (39). MAX2 was shown to interact with the Arabidopsis homolog of Skp1, ASK1, in yeast two-hybrid and in vitro binding assays (30).

F-box proteins have been implicated in the direct perception or early signal transduction of several phytohormones, including auxin (40), jasmonic acid (41), and gibberellin (42). For each of these hormones, the F-box protein targets negative regulators of transcription factors that carry out the hormone responses. In the case of auxin, association of the F-box protein TIR1 with an Aux/IAA negative regulator is promoted by auxin-binding in a TIR1 pocket, a nonallosteric interaction in which the ligand acts as “molecular glue” (43). In the current model of gibberelin signaling, however, the receptor GID1 undergoes allosteric activation after gibberelin-binding, which enhances its association with the DELLA negative regulators and promotes their recognition by the F-box protein SLY1 (44).

As max2 is insensitive to the synthetic strigolactone GR24, it has been hypothesized that MAX2 may either be the strigolactone receptor or associate with a receptor complex (18, 39). Whatever the case may be, we now propose that MAX2 also manages karrikin signal transduction in a manner similar to that for strigolactones. In a dual-role scenario, how might MAX2 distinguish between karrikin and strigolactone signals during regulation of shoot branching, but not during seed germination and seedling establishment? The d14/hd1/hd8 mutant in rice exhibits strigolactone-insensitive, enhanced tillering, as well as feedback up-regulation of strigolactone biosynthesis genes (45–47). D14 is classified as an α/β-fold hydrolase superfamily member, as is the gibberelin receptor GID1, and has been proposed to be a potential component of strigolactone signaling (45). In a model based upon the gibberelin signaling mechanism, MAX2 could target strigolactone or karrikin pathway repressors for proteasomal degradation upon their specific association with a respective ligand-activated D14 homolog (Fig. S5). The spatiotemporal availability of either the receptors or signaling pathway repressors could then restrict the effects of these two families of plant-growth regulators during different stages of development. As future studies reveal additional components of the MAX2-dependent pathway, it will be interesting to determine how an abiotic smoke signal and a phytohormone came to share a common signal integration point.

We finally note that the max2 mutants exhibit enhanced seed dormancy, elongated hypocotyls, and gene expression (e.g., STH7, KUF1, KUXO1) phenotypes that are opposite to the effects of karrikin or GR24 application on wild-type plants (summarized in Fig. S6). As these phenotypes are not present in the strigolactone-deficient max1, max3, or max4 mutants, it may indicate overaccumulation of a constitutive karrikin pathway repressor in max2. Alternatively, this observation may suggest the existence of an endogenous nonstrigolactone-derived signal at these growth stages to which max2 mutants can no longer respond. If such a signal were structurally similar to karrikins and strigolactones, it could explain the broad conservation of a karrikin-response mechanism in plant species from nonfire-prone environments, such as A. thaliana, in the absence of an obvious selective pressure.

Materials and Methods

Plant-Growth Conditions, Germination Tests, Hypocotyl Elongation Assays, and qRT-PCR. Plant-growth conditions, germination tests, hypocotyl elongation assays, and qRT-PCR were performed as previously described (10, 11). MAX4 and IAA7 primer sequences were the same as in Hayward et al. (34). Karrikin and GR24 stock (1,000x) solutions were prepared in acetone. In each experiment, the control contained an equivalent amount of acetone.

Arabidopsis Shoot Branching Assays. Seedlings were grown in 16-h light/8-h dark for 7 d and transferred to perlite substrate in clear, sterile pots with 0.3x MS + Gamborg vitamins (Sigma), 2 mM Mes, pH 5.7 liquid media. Media supplemented with 5 μM KAR, 5 μM KAR, or 5 μM GR24 was exchanged weekly. All solutions contained 0.05% (vol/vol) aceton. Rosette shoot branches ≥ 5 mm were assayed when plants were 42 d old. Semihydroponic phyttary assays shown in Fig. S3 were performed as previously described (48), except growth was at 24 °C and rosette branches longer than 5 mm were counted when plants were 35 d old.

Pea Bud Outgrowth Assays. Pea bud outgrowth assays were performed as previously described (49), except solutions contained 0.1% (vol/vol) aceton.

[1H1]-KAR, Transport Assays. For a more detailed description of the experimental setup, please see SI Materials and Methods. In brief, A. thaliana (Ler) plants were grown on a rockwool substrate plug in the lid of 15-mL tubes. Roots were fed liquid 0.5x MS + Gamborg vitamins, 2 mM Mes, pH 5.7 media. Before addition of [1H1]-KAR, an air gap was made between the media and rockwool to prevent direct moistening of the plug. [1H1]-KAR, was added to a 10-ppm concentration to the media of 5-wk-old flowering plants, and plant tissue was harvested over a 96-h period. Scintillation activity of tissue in Beckman Coulter Ready-Safe Liquid Scintillation Mixture was determined over a 10-min interval using a Beckman Coulter LS6500 Multipurpose Scintillation Counter.

Synthesis of Karrikins, GR24, and Butenolides. KAR, (50), [7,1H1]-KAR, (51), KAR, (50), GR24 (52), butenolide B1, 3,4,5-trimethylfurany-2(5H)-one (53), B2, 3-methylbutany-2(5H)-one 3-methyl (54), B3, 4-[methyl(furan-2(5H)-one (54), and B4, 3,4-dimethylfurany-2(5H)-one (55) were prepared as previously described.

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