Karrikins Discovered in Smoke Trigger Arabidopsis Seed Germination by a Mechanism Requiring Gibberellic Acid Synthesis and Light

David C. Nelson, Julie-Anne Riseborough, Gavin R. Flematti, Jason Stevens, Emilio L. Ghisalberti, Kingsley W. Dixon, and Steven M. Smith*

Plant Energy Biology (D.C.N., J.-A.R., S.M.S.), Plant Biology (J.-A.R., K.W.D.), and Biomedical, Biomolecular, and Chemical Sciences (G.R.F., E.L.G., S.M.S.), University of Western Australia, Crawley, Western Australia 6009, Australia; and Kings Park and Botanic Garden, West Perth, Western Australia 6005, Australia (J.S., K.W.D.)

Discovery of the primary seed germination stimulant in smoke, 3-methyl-2H-furo[2,3-c]pyran-2-one (KAR1), has resulted in identification of a family of structurally related plant growth regulators, karrikins. KAR1 acts as a key germination trigger for many species from fire-prone, Mediterranean climates, but a molecular mechanism for this response remains unknown. We demonstrate that Arabidopsis (Arabidopsis thaliana), an ephemeral of the temperate northern hemisphere that has never, to our knowledge, been reported to be responsive to fire or smoke, rapidly and sensitively perceives karrikins. Thus, these signaling molecules may have greater significance among angiosperms than previously realized. Karrikins can trigger germination of primary dormant Arabidopsis seeds far more effectively than known phytohormones or the structurally related strigolactone GR-24. Natural variation and depth of seed dormancy affect the degree of KAR1 stimulation. Analysis of phytohormone primary dormant Arabidopsis seeds far more effectively than known phytohormones or the structurally related strigolactone molecules may have greater significance among angiosperms than previously realized. Karrikins can trigger germination of

Germination by a Mechanism Requiring Gibberellic Acid Synthesis and Light

Germination is a critical event in the plant life cycle, as the timing of emergence from the protective seed coat is crucial for survival and reproductive success. A variety of abiotic stimuli, including light, temperature, and nitrates, provide information about the external environment that affects germination. Seed dormancy gates responses to these factors. Upon maturation, physiologically dormant seeds are in a primary dormant (PD) state, which is lost during afterripening. The transition between a PD and nondormant state is both gradual and reversible and results in relaxation of the set of environmental conditions under which a seed will germinate (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

Despite decades of research, seed dormancy remains a complex physiological state that is not well understood. The plant hormones abscisic acid (ABA) and GA are mutually antagonistic central players in the germination decision (Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008). The role of dormancy establishment and maintenance has been attributed to ABA, while GA has been implicated in the initiation and completion of germination. The ratio of ABA to GA signaling, rather than absolute amounts of the hormones, appears to be critical to dormancy breaking (Finch-Savage and Leubner-Metzger, 2006). Environmental stimuli and phytohormones influence the ABA/GA balance, although the mechanisms of signal integration and hormone cross talk are still largely unknown.

In many biodiverse regions, fire events provide an irregular but important opportunity for seedling establishment by freeing up key resources such as light, space, and nutrients (Van Staden et al., 2000; Dixon et al., 2009). A clear example of this is seen in the flush of new growth in the immediate postfire environment, indicating potent activation of the soil seed bank. Heat...
is not required for the germination response, as cold smoke application induced an up to 48-fold increase in the number of germinating seedlings and approximately 3-fold enrichment in species abundance in field trials (Roche et al., 1997; Rokich et al., 2002). It has now been well established that smoke is a broadly effective stimulant that enhances germination of approximately 1,200 species in more than 80 genera worldwide (Dixon et al., 2009). Attempts to study smoke effects on plant physiology have been confounded by the complex mixture of components within smoke, some of which confer toxicity at high concentrations. Bioassay-guided fractionation of smoke water culminated in the discovery and synthesis of the primary germination stimulant 3-methyl-2H-furo[2,3-c]pyran-2-one (KAR1; Flematti et al., 2004). With the recent identification of three analogous active compounds in smoke water fractions (Fig. 1A; G. Flematti, unpublished data), this family of butenolide molecules have been designated karrikins, after “karrik,” the first recorded Aboriginal Nyungar word for smoke (Dixon et al., 2009).

The parent molecule, KAR1, is a potent stimulant that enhances germination in some species at subnanomolar concentrations (Flematti et al., 2004; Stevens et al., 2007). In field trials, KAR1 is effective at less than 5 g ha\(^{-1}\) compared with 10 ton ha\(^{-1}\) smoke water and thus may have practical value in agriculture, conservation, and restoration (Stevens et al., 2007). Smoke water fractions containing KAR1 have been reported to enhance seedling vigor of several weed and crop species, indicating potential use for KAR1 as a seed priming agent to improve germination and seedling establishment (Jain et al., 2006; Jain and Van Staden, 2006; Kulkarni et al., 2006; van Staden et al., 2006; Daws et al., 2007a). Since its discovery, a widespread capacity for KAR1 germination response among angiosperms has been demonstrated (Flematti et al., 2004; van Staden et al., 2004, 2006; Merritt et al., 2006; Daws et al., 2007a; Stevens et al., 2007). Thus, karrikins may be considered a novel class of plant growth regulators with broad impact. To gain a better understanding of the mechanism by which karrikins trigger seed germination and explore their interaction with ABA and GA, we examined KAR1 responses in Arabidopsis (Arabidopsis thaliana).

RESULTS
Karrikins Enhance Germination of Primary Dormant Arabidopsis Seeds

To determine if Arabidopsis is a suitable model system for studies of karrikin action, PD seeds of the Landsberg erecta ecotype (Ler) were tested for germination enhancement by KAR1. While 1 \(\mu M\) KAR1 strongly promoted germination, the phytohormones GA and epibrassinolide (EBR) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) had little or no effect on PD seeds at similar concentrations (Fig. 2, A and B). The KAR1 effect on germination rates was comparable to that of 10 mM KNO3, an effective dormancy-breaking treatment (Alboresi et al., 2005). The combination of these two treatments resulted in a synergistic response.

Karrikins have no distinct structural similarity to known plant hormones, although the A ring of the KAR1 molecule is analogous to the D ring of strigolactones (Fig. 1B; Flematti et al., 2004). Strigolactones are exuded by the roots of host plants and are highly active germination stimulants for a number of parasitic weed species in the Striga, Orobanche, and Alectra genera (Humphrey et al., 2006). Smoke water extracts, in which KAR1 is present, have also been reported to stimulate parasitic weed germination (Bar Nun and Mayer, 2005; Daws et al., 2007b). To test whether karrikins and strigolactones share a similar mechanism of action, we compared the germination response of PD Ler seeds to the four karrikins and the synthetic strigolactone GR-24 (Fig. 2C). KAR4 was the most active analog and clearly enhanced germination at concentrations as low as 10 nM, while KAR1 and KAR3 were slightly less effective. Although KAR4 stimulates germination of Lactuca sativa and Solanum orbiculatum (Flematti et al., 2007), KAR4 was either completely inactive or inhibitory at similar concentrations in Arabidopsis. GR-24 was capable of stimulating PD Ler germination but required approximately 100-fold greater concentrations than the weakest active karrikin, KAR9, to achieve the same effect. Brassica tournefortii, a strongly karrikin-responsive species, also had minimal response to GR-24 (Fig. 2D). Conversely, KAR1 was unable to trigger germination of Orobanche minor, while GR-24 was active at concentrations as low as 1 nM (Fig. 2D). The differences in karrikin and strigolactone efficacy among these species suggest distinct modes of action or a species-specific capacity for response.
KAR1 Effects on Germination Vary with Ecotype and Dormancy Depth

There is substantial natural variation in primary seed dormancy among Arabidopsis ecotypes (van Der Schaar et al., 1997; Alonso-Blanco et al., 2003). PD seeds from seven other ecotypes were tested for germination enhancement by KAR1. A range of responses were observed in which KAR1 had moderate to neg-
ligible effects on germination (Fig. 2E). KAR \textsubscript{1} typically caused an early, limited enhancement of seed germination rates, suggesting that a subset of the seed population had been pushed over a dormancy threshold within an imbibition time window (Supplemental Fig. S1). Notably, the control levels of germination and responses to KNO\textsubscript{3} were also variable across ecotypes. These results may be attributed to natural variation either in the capacity for KAR \textsubscript{1} perception and response or in seed dormancy depth. To address the latter hypothesis, we examined the germination of Ler seeds with different depths of dormancy. Although after 7 d of imbibition the germination enhancement by KAR \textsubscript{1} was apparent and equivalent to 6 weeks of afterripening (Supplemental Fig. S2), KAR \textsubscript{1} was unable to fully stimulate germination of PD seeds within a 4-d period (Fig. 2F). With afterripening, KAR \textsubscript{1} enhanced germination within this time frame at a rate outpacing the rise in control germinability. However, as dormancy was further lost, there was a corresponding reduction in the apparent effects of KAR \textsubscript{1} on germination relative to the control. Thus, in either highly dormant or nondormant states seeds may not show an obvious response to KAR \textsubscript{1} treatment, particularly when assaying germination at a single time point. We found that KAR \textsubscript{1} had a positive effect on seed germination rates even after removal of PD Ler seed dormancy by cold stratification or extended afterripening (Fig. 2G). Karrikins, therefore, display characteristics of dormancy-breaking and germination-stimulating compounds.

**GA Biosynthesis Is Required for KAR \textsubscript{1} Promotion of Germination**

To investigate the interaction of karrikins with ABA and GA pathways, we tested the germination responses of several phytohormone mutants (Table I). We first examined mutants with reduced ABA biosynthetic capacity. *aba3-2* had a small response to KAR \textsubscript{1} after 48 h but otherwise exhibited no difference in germination relative to the control (Fig. 3A). We reasoned that if karrikins act through reducing ABA levels or sensitivity, the lack of ABA in this mutant may prevent the detection of a KAR \textsubscript{1} effect. In the presence of exogenous ABA, germination rates were inhibited, although *aba3-2* seeds still showed no response to KAR \textsubscript{1}. Similar results were obtained with

<table>
<thead>
<tr>
<th>Allele</th>
<th>Ecotype</th>
<th>Treatment</th>
<th>KAR \textsubscript{1} Response</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>aba1-3</td>
<td>Ler</td>
<td>−</td>
<td>+</td>
<td>S3B</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>ABA</td>
<td>+</td>
<td>S3B</td>
</tr>
<tr>
<td>aba2-1</td>
<td>Col</td>
<td>−</td>
<td>−</td>
<td>S3C</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>ABA</td>
<td>−</td>
<td>S3C</td>
</tr>
<tr>
<td>aba3-1</td>
<td>Col</td>
<td>−</td>
<td>−</td>
<td>S3D</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>ABA</td>
<td>−</td>
<td>S3D</td>
</tr>
<tr>
<td>aba3-2</td>
<td>Ler</td>
<td>−</td>
<td>?</td>
<td>3A</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>ABA</td>
<td>−</td>
<td>3A</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>PD</td>
<td>−</td>
<td>S3E</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>AR</td>
<td>+</td>
<td>S3F</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>PD</td>
<td>−</td>
<td>S3E</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>AR</td>
<td>+</td>
<td>S3F</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>PD</td>
<td>+</td>
<td>S3E</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>AR</td>
<td>+</td>
<td>S3F</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>−</td>
<td>−</td>
<td>3B</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>GA</td>
<td>−</td>
<td>3C</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>GA</td>
<td>++</td>
<td>S4A</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>−</td>
<td>−</td>
<td>S4C</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>−</td>
<td>++</td>
<td>S4D</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>−</td>
<td>−</td>
<td>S4E</td>
</tr>
<tr>
<td></td>
<td>Col</td>
<td>−</td>
<td>++</td>
<td>S4B</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>−</td>
<td>−</td>
<td>3D</td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>+</td>
<td>3D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ler</td>
<td>+</td>
<td>3D</td>
<td></td>
</tr>
</tbody>
</table>

*Table I. Summary of Arabidopsis phytohormone mutant responses to KAR \textsubscript{1}*

The ecotype for each mutant is indicated. Special conditions (Treatment) are noted (i.e. in the presence [ABA/GA] or absence [−] of exogenous hormone or dormancy state [PD/AR] of the seed). Where not noted, all seeds were of the primary dormancy level within a few days of harvest. KAR \textsubscript{1} response in germination is classified as (0%–10%), + (10%–40%), or ++ (>40%). Col, Ecotype Columbia.
ABA catabolism occurs primarily through an oxidation pathway resulting in phaseic acid via an 8'-hydroxy ABA intermediate (Nambara and Marion-Poll, 2005). The CYP707A family of cytochrome P450s have been characterized as ABA 8'-hydroxylases. Mutations in these genes lead to increased ABA levels in mature seeds and a reduction in germinability (Okamoto et al., 2006). PD cyp707a2 cyp707a3 seeds were responsive to KAR1, while the highly reduced germination of cyp707a1 cyp707a3 and cyp707a1 cyp707a2 showed no enhancement (Supplemental Fig. S3E). With afterripening, however, germination of all three double mutant lines was mildly enhanced by KAR1 (Supplemental Fig. S3F). KAR1 was also unable to overcome the enhanced dormancy of the ABA-hypersensitive era1-2 mutant (Cutler et al., 1996), although KNO3 could eventually induce germination of a ga3ox1 ga3ox2 double mutant was not recovered by KAR1 (Supplemental Fig. S4, D and E). If KAR1 acts primarily through enhancement of GA biosynthesis and not sensitivity to GA, a lack of germination promotion in GA-supplemented ga1-3 seeds would be expected. Indeed, KAR1 produced little to no change in the germination response of ga1-3 to different concentrations of GA3 (Fig. 3C).

GA signaling is mediated by SLEEPY1 (SLY1), an F-box protein that targets the DELLA repressor proteins for degradation in a GA-dependent manner (Dill et al., 2004; Finkelstein et al., 2008). Interestingly, KAR1 treatment partially restored germination of the GA signaling-deficient sly1-2 and sly1-10 alleles, while GA is typically required for germination and can overcome dormancy or promote germination in restrictive conditions. Alleles of GA3ox1 (ga4-1) and GA20ox1 (ga5-1) have reduced GA biosynthetic capacity but were both strongly responsive to KAR1 (Supplemental Fig. S4, A and B). Germination is blocked in the GA-deficient mutant ga1-3, but this can be overcome by other hormones, such as brassinosteroid and ethylene, or by reduced ABA synthesis during seed maturation (Karssen et al., 1989; Debeaujon and Koornneef, 2000; Steber and McCourt, 2001). In our hands, very limited ga1-3 germination was achieved with either ACC or EBR, even with the inclusion of nitrates in the medium and a stratification treatment (Fig. 3B). However the ga1-3 mutant had no germination in the presence of KAR1. Similarly, while a ga3ox2 allele was KAR1 responsive, the highly reduced germination of a ga3ox1 ga3ox2 double mutant was not recovered by KAR1 (Supplemental Fig. S4, D and E). If KAR1 acts primarily through enhancement of GA biosynthesis and not sensitivity to GA, a lack of germination promotion in GA-supplemented ga1-3 seeds would be expected. Indeed, KAR1 produced little to no change in the germination response of ga1-3 to different concentrations of GA3 (Fig. 3C).
KNO₃ was less effective (Fig. 3D). This suggests that nitrate and karrikin have distinct modes of action and that KAR₁ may partially influence germination in a DELLA-independent manner.

Transcription of GA 3-Oxidases and CPI Is Induced by Active Karrikins

To gain further insight into KAR₁ effects on hormone metabolism during PD Ler seed imbibition, we performed quantitative reverse transcription (qRT)-PCR analysis for a set of genes involved in the synthesis, catabolism, or response of ABA and GA. Transcriptional changes were assessed during the first 48 h of imbibition, before KAR₁-treated seeds begin to germinate (Fig. 2B), in order to identify early regulatory events that may result in a germination decision.

No transcript differences were observed for the major ABA biosynthetic enzymes ABA₁, ABA₂, ABA₃, NCED₆, or AAO₃ in response to KAR₁ (Supplemental Fig. S5A). AAO₄, which can have a role in ABA biosynthesis in the absence of the major isoforms AAO₁-AAO₃ (Seo et al., 2004), was up-regulated after 24 h by KAR₁. In a comparison of dormant and nondormant Arabidopsis seeds, expression of the ABA 8’–hydroxylase CYP707A2 was 4-fold up-regulated in nondormant seeds at 6 h of imbibition (Millar et al., 2006). However, CYP707A2 and CYP707A3 showed no change in expression in response to KAR₁ treatment during imbibition (Supplemental Fig. S5B). ABA can also be inactivated through the formation of an ABA-Glc ester conjugate by the glucosyltransferase UGT71B6 (Priet et al., 2005). UGT71B6 transcript abundance was unaffected by KAR₁ (Supplemental Fig. S5B).

ABI genes were identified as components of ABA signal transduction pathways through genetic screens for ABA-insensitive mutants (Finkelstein et al., 2002). Of the five ABI genes tested, only ABI₄ transcripts were affected by KAR₁ (Supplemental Fig. S5C). At 48 h, ABI₄ expression was up-regulated by KAR₁. ABI₄ encodes an AP2 domain transcription factor (Finkelstein et al., 1998) and confers sensitivity to sugar and salt stress (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Quesada et al., 2000). The implications of ABI₄ induction by KAR₁ are currently unclear, but they may reflect preparation for seedling emergence. FRY1/SAL1 encodes an inositol 1,4,5-triphosphate phosphatase and acts as a negative regulator of ABA signaling (Xiong et al., 2001). We observed up-regulation of FRY1 in KAR₁-treated seeds after 48 h of imbibition (Supplemental Fig. S5C).

GAs are derived from geranylgeranyl diphosphate through the action of a series of terpene cyclases, cytochrome P450s, and 2OG-dependent dioxygenases (Olszewski et al., 2002). No transcript changes were observed for GA2/KS1, GA3/KO1, KAO1, or KAO2 (Supplemental Fig. S6A). Among the GA 20-oxidases, which catalyze the penultimate step in GA biosynthesis, expression of GA20ox2, GA20ox3, and GA20ox4 was relatively unaffected by KAR₁, but GA20ox1 was initially suppressed by KAR₁ and then induced at 48 h (Supplemental Fig. S6A). GA 3-oxidases catalyze the final step of GA biosynthesis to produce active GA₄ and GA₃. GA3ox₁/GA44 and GA3ox₂ are the primary genes encoding GA 3-oxidases (Yamaguchi et al., 2001). GA3ox₁ and GA3ox₂ are light responsive, and GA3ox₁ is also regulated by stratification and GA feedback inhibition (Yamaguchi et al., 1998; Yamauchi et al., 2004). KAR₁ induced significant up-regulation of both GA3ox₁ and GA3ox₂. GA3ox₁ transcripts showed 4-fold up-regulation by KAR₁, as early as 6 h of imbibition, while GA3ox₂ was differentially expressed after 12 h (Fig. 4A). The GA 2-oxidases are responsible for GA catabolism (Thomas et al., 1999). No KAR₁-induced expression changes were observed for GA2ox₂ or GA2ox₃ (Supplemental Fig. S6B).

Among the five DELLA proteins, RGL2 is considered the main repressor in seed germination (Lee et al., 2002; Tyler et al., 2004; Cao et al., 2005). We detected no KAR₁-induced changes in transcript abundance during imbibition (Supplemental Fig. S6C). The putative Cys proteinase CPI is a useful marker for GA signaling, as it is up-regulated by GA₄ in a concentration-dependent manner and also induced by GA-sensitizing treatments such as stratification (Yamauchi et al., 2004). CPI exhibited a very similar pattern of expression as GA3ox₂ and was up-regulated by KAR₁ after 24 h (Supplemental Fig. S6C). This suggested that a rise in GA levels consistent with GA3ox expression was occurring in KAR₁-treated seeds.

The relatively few changes in gene expression we observed implied that KAR₁ enhances GA synthesis via GA3ox₁ and GA3ox₂ but does not directly affect ABA pathways. To assess these predictions, we quantitated changes in the levels of ABA and GAs during imbibition of PD seeds in response to KAR₁ (Fig. 4B). ABA levels declined substantially during the first 48 h of imbibition but were unaffected by KAR₁. Interestingly, we did not detect a dramatic rise in GA₄ levels predicted by GA3ox and CPI expression levels at 48 h. At 24 h, GA₄ levels were the same in control and KAR₁-treated seeds, and at 48 h, there was a statistically significant (Student’s paired t test, P < 0.02), although small (10%), increase in GA₄ induced by KAR₁.

We examined the transcript levels of GA3ox₁, GA3ox₂, and CPI in PD seeds imbibed for 24 h to compare the expression changes observed with KAR₁ and other germination stimulants. KAR₁, KAR₂, and KAR₅, but not KAR₃, induced expression of GA3ox₁, GA3ox₂, and CPI. The degree of up-regulation of these genes corresponded to the effectiveness of each treatment on stimulating germination (Fig. 4C). GR-24 produced a slight enhancement of GA3ox expression but did not result in CPI up-regulation, at least at this time point. Exogenous GA₄ treatments produced the expected transcriptional effects: GA3ox₁, which is feedback inhibited, was down-regulated, GA3ox₂ was unaffected, and GA-responsive CPI was strongly induced. KNO₃ was particularly effective at inducing GA3ox and CPI transcripts, which may explain its broad
Karrikins Overcome Arabidopsis Seed Dormancy

KAR1 Requires Light to Enhance Arabidopsis Germination

Moisture and light are minimal requirements for normal Arabidopsis seed germination. We found that karrick could not replace the light requirement (Fig. 5A). Under continuous light, KAR1 induced nearly complete germination of PD Ler seeds within 7 d (Fig. 2A) but caused minimal germination (2%) of PD seeds incubated in the dark after a 2-h initial white light exposure. AR seeds were much more responsive than PD seeds to the early light exposure but did not achieve maximal germination without KAR1. When the early light treatment was reversed by a far-red (FR) light pulse prior to dark incubation, no germination was observed for KAR1-treated seeds regardless of seed dormancy state. Light induces GA synthesis in seeds, and exogenous GA is sufficient to induce germination of Arabidopsis seeds in the dark. To determine whether KAR1 action requires light because GA synthesis is not triggered, we tested the germination of GA-treated seeds in the presence and absence of KAR1 during dark incubation. GA supplements were not sufficient to restore KAR1 promotive effects, and KAR1 had little or no effect on seed sensitivity to exogenous GA (Fig. 5A). It is also interesting that PD and AR seeds had nearly equivalent responses to FR reversal of the early light exposure. Thus, light, but not dormancy loss through afterripening, enhances seed sensitivity to GA.

To further examine the relationship of light to KAR1 effects on germination, we tested the expression of the KAR1-responsive genes GA3ox1 and CP1 under the same conditions. Interestingly, KAR1 stimulated GA3ox1 expression in AR seeds in the dark even after FR exposure (Fig. 5B). However, the levels of GA3ox1 expression were dramatically enhanced by the combination of light and KAR1 in AR seeds. CP1 expression showed a similar trend (data not shown). Notably, the strong induction of GA3ox1 occurred only in the treatment producing maximum dark germination. Thus, while KAR1 can up-regulate GA3ox1 expression independently of light, its effects are insufficient to induce germination in the absence of light.

DISCUSSION

Karrikins are a novel family of plant growth regulators that affect key processes for a broad range of angiosperms. However, very little is known concerning the potential mechanisms of karrikin action in germination or seedling establishment. We provide, to our knowledge, the first demonstration that three karrikins, KAR1, KAR2, and KAR3, promote germination of Arabidopsis seeds. At high concentrations, the structurally related synthetic strigolactone GR-24 enhanced germination of two karrikin-responsive species. However, KAR1 was completely inactive on the smoke water and the strigolactone-responsive parasitic weed O. minor, suggesting that another component of smoke water is responsible for triggering Orobanche germination. These results indicate that karrikins and strigolactones are not interchangeable and may act via different mechanisms. The level of GA3ox effectiveness in Arabidopsis as a dormancy-breaking treatment.

KAR1 Requires Light to Enhance Arabidopsis Germination

Moisture and light are minimal requirements for normal Arabidopsis seed germination. We found that

Figure 4. Karrikins induce GA 3-oxidase and CP1 transcripts. A, Relative expression of GA3ox1 and GA3ox2 during a 48-h time course of PD Ler seed imbibition on water (white symbols) or 1 μM KAR1 (black symbols) in the light at 20°C. Inset bar graph indicates fold change in expression (KAR1/water) at the indicated time points. B, Quantification of ABA and GA, in dry, 24-h, and 48-h KAR1-imbibed (≤1 μM) seeds. The y axis indicates ng g−1 preimbibition seed weight. C, Expression of GA3ox1, GA3ox2, and CP1 in PD Ler seeds after 24 h of imbibition in the light at 20°C on water, 1 μM KAR1, KAR2, KAR3, or KAR4, 10 μM GR-24, 10 μM GA4, or 10 mM KNO3. Relative expression values for each gene in seeds imbibed in water were set to 1, and other expression values were scaled accordingly.
induction corresponded with the efficacy of these germination stimulants (KAR2 > KAR1 > KAR3 > GR-24). However, it cannot be assessed from GA3ox and CP1 expression alone whether karrikin and strigolactone signals are perceived and transduced via common pathways, as both stimulants may ultimately enhance GA synthesis as part of the germination process.

KAR1 was not equally effective across all tested Arabidopsis ecotypes. Different depths of primary seed dormancy in these lines may provide at least a partial explanation for this observation. A progressive receptiveness to germination-promoting factors (nitrates, stratification) has been observed during afterripening of Arabidopsis Cvi seeds, indicating dynamic capacities for seed response to stimuli (Finch-Savage et al., 2007). Similarly, KAR1 may be an ineffective stimulant under certain dormancy states. As an example, B. tournefortii germination is particularly sensitive to KAR1, but some seed collections require several months of afterripening to become responsive (Stevens et al., 2007). As seeds in the soil seed bank cycle between dormancy states in response to afterripening or environmental stimuli, limited windows of opportunity for karrikin action may be created. In support of this, smoke treatments to enhance seed bank germination have marked differences in effectiveness at specific times of the year (Rokich and Dixon, 2007). Natural variation provides another likely explanation for variable KAR1 responses. Here, we do not simply refer to variation in the depth of established seed dormancy but in the mechanisms by which dormancy is broken. Light, cold, and nitrate commonly promote germination across Arabidopsis ecotypes, but the contribution of each signal toward germination commitment can vary. For instance, polymorphisms in the photoreceptor phytochrome B have been identified as a source of natural variation in light responses (Filiault et al., 2008). In consideration of the range of KAR1 effectiveness even within a single species, it would not be surprising to discover that karrikins activate germination under specific conditions for many more taxa than are currently known to be responsive.

Uncovering cross talk between karrikins and phytohormones is important for determining a mechanism for karrikin promotion of germination. KAR1 was ineffective at overcoming inhibition of germination by exogenous ABA or high endogenous ABA signaling. While high concentrations of GA3 are often effective at stimulating germination of KAR1-responsive species, there has been no direct evidence for GA-mediated KAR1 signaling (Merritt et al., 2006; Daws et al., 2007a; Stevens et al., 2007). We demonstrate that KAR1 requires GA synthesis to induce germination, as both ga1-3 and ga3ox1 ga3ox2 mutants were unresponsive. KAR1 did not affect seed sensitivity to GA, either in ga1-3 or in dark-incubated wild-type plants.

In support of the germination phenotypes, KAR1 did not affect transcript abundance of the majority of genes involved in ABA and GA biosynthesis and catabolism but did induce GA3ox1 and GA3ox2. It is difficult to assess whether GA3ox induction by KAR1 is a cause or a result of the seed’s commitment to germination. As GA3ox1 expression is influenced by cold, light, and GA levels, it may serve as a signal integration point with a direct effect on germination. While KAR1 enhancement of germination requires both light and GA biosynthetic capacity, it cannot be concluded that KAR1 acts solely or directly through enhancement of light-induced GA3ox transcription.

The abundance of ABA during imbibition was unaffected by KAR1, while GA4 levels were only slightly increased. As dormancy loss has been reported to result in a faster decline of ABA levels during seed imbibition (Ali-Rachedi et al., 2004; Millar et al., 2006) and afterripening leads to enhanced GA sensitivity (Karssen et al., 1989), KAR1 does not overcome seed dormancy in a similar manner. Although we had anticipated changes in hormone levels prior to initiation of germination, these results correspond with a
similar experiment in which strong induction of GA3ox transcripts preceded germination but a rise in GA4 levels was concomitant only with radicle emergence (Ogawa et al., 2003). Therefore, a substantial rise in total seed GA4 levels may be a result of a seed’s commitment to germination, rather than a cause. As there is evidence for spatial separation of transcripts for early and late phases of the GA biosynthetic pathway in embryonic tissues (Yamaguchi et al., 2001), it would be interesting if production of active GAs is restricted to a small “decision” sector of the seed prior to the initiation of germination and thus initially contributes little to the overall seed GA4 abundance. Alternatively, the initial suppression of GA20ox1 by KAR1 may restrict the GA precursor supply during the first 24 h of imbibition.

The broad conservation of karrakin perception, even among taxa not obviously subject to selective pressures from a “fire-prone” environment, suggests several intriguing hypotheses. First, karramins may be generated via other mechanisms than fire. A biotic source such as bacteria or fungi, or the slow chemical breakdown of organic matter at the soil surface, could provide alternative sources of karrakin. Second, there may be a strong selective advantage for species that have maintained a karrakin signaling system even in ecosystems with rare fire events. Third, karramins may be endogenous plant hormones that await identification. Our demonstration of a karrakin response in Arabidopsis seed germination opens the door for a genetic approach to addressing these possibilities.

**MATERIALS AND METHODS**

**Plant Growth and Germination Assays**

Arabidopsis (*Arabidopsis thaliana*) plants were grown in soil under continuous white light, 22°C, 60% relative humidity conditions. Harvested plants were dried for 4 to 7 d under ambient conditions in paper bags. Seeds pooled from multiple parent plants (>10) were then cleaned and stored at −80°C to preserve primary dormancy. Cryostorage had no obvious effect on seed viability or germination. AR seeds were maintained in the dark at room temperature. For germination assays, seeds were surface sterilized for 5 min with immersion in 70% ethanol and 0.05% Triton X-100, rinsed with 70% ethanol, rinsed again with 95% ethanol, and rapidly dried on filter papers in a sterile laminar flow cabinet. Sterilization treatment had no effect on germination. Sterilized seeds were sprinkled onto 0.8% Bacto-agar plates supplemented with 0.5 mL of water for 2 weeks at 20°C in the dark. A second filter paper was placed on top of the agar to which brine (10 mL) and 0.1% AA water (20 mL) were added. The aqueous solution was partitioned three times with 15% methanol/0.1% AA water (10 mL), and the retained material was rinsed with 15% methanol/0.1% AA water (20 mL) and dissolved in the original extract. The extract was reduced to an aqueous solution under reduced pressure at 35°C to which brine (10 mL) and 0.1% AA water (20 mL) were added. The aqueous solution was partitioned three times with equal volumes of ethyl acetate, and the combined organic phase was evaporated to dryness under reduced pressure at 35°C. The ethyl acetate extract was dissolved in methanol/0.1% AA water (10 mL) and was applied directly to a preconditioned C18 Sep-Pak (Waters; 1 g cartridge). The retained material was rinsed with 15% methanol/0.1% AA water (10 mL), and the GAs and ABA were eluted with 80% methanol/0.1% AA water (10 mL). The 80% methanol fraction was evaporated to dryness under reduced pressure at 35°C, and the sample was dissolved in methanol (2 mL) and methylated with excess ethereal diazomethane. The sample was dried and dissolved in 20% methanol/0.1% AA water (1 mL) and separated by HPLC (Hewlett-Packard 1050 apparatus). The sample was injected (1 mL) onto a C18 reversed-phase column (Grace-Davison Apollo; 250 × 10 mm, 5 μm) and eluted with 0.041 acetone/0.1% AA water, which increased to 100% acetone/0.1% AA over 40 min. Fractions were collected every minute.

**Quantitation of ABA and GA**

Per time point, three replicates of 300 mg of seeds (preimbibition weight) from independent PD Ler seed batches were pulverized in liquid nitrogen using a ball mill. Powder was extracted with 5 mL of 80% (v/v) methanol-water (with 0.1% acetic acid [AA]) with the following deuterated internal standards added: 10 ng g⁻¹ [17,17-D2]GA4 and [17,17-D2]GA3 (from L.N. Mander) and 20 ng g⁻¹ [1H7]ABA (from L.I. Zaharia, National Research Council, Canada). The suspension was stored at 4°C for 24 h and filtered. The residue was rinsed with a further 50 mL of 80% methanol/0.1% AA water and combined with the original extract. The extract was reduced to an aqueous solution under reduced pressure at 35°C to which brine (10 mL) and 0.1% AA water (20 mL) were added. The aqueous solution was partitioned three times with equal volumes of ethyl acetate, and the combined organic phase was evaporated to dryness under reduced pressure at 35°C. The ethyl acetate extract was dissolved in methanol/0.1% AA water (10 mL) and was applied directly to a preconditioned C18 Sep-Pak (Waters; 1 g cartridge). The retained material was rinsed with 15% methanol/0.1% AA water (10 mL), and the GAs and ABA were eluted with 80% methanol/0.1% AA water (10 mL). The 80% methanol fraction was evaporated to dryness under reduced pressure at 35°C, and the sample was dissolved in methanol (2 mL) and methylated with excess ethereal diazomethane. The sample was dried and dissolved in 20% methanol/0.1% AA water (1 mL) and separated by HPLC (Hewlett-Packard 1050 apparatus). The sample was injected (1 mL) onto a C18 reversed-phase column (Grace-Davison Apollo; 250 × 10 mm, 5 μm) and eluted with 0.041 acetone/0.1% AA water, which increased to 100% acetone/0.1% AA over 40 min. Fractions were collected every minute.
for 40 min. After all of the extracts had been separated, retention times of the GAs and ABA were established by running authentic samples and monitoring for UV_{254}, GA_{x} and GA_{y} eluted at 11.24 and 25.35 min, respectively, and ABA eluted at 20.06 min. Individual fractions containing these hormones plus one fraction on either side were combined and dried under reduced pressure. The ABA fraction was dissolved in dry acetonitrile (12 μL) and analyzed by gas chromatography-mass spectrometry (GC-MS; Shimadzu QP2010). The GA fractions were treated with dry pyridine (50 μL) and BSTFA (Sigma-Aldrich; 50 μL) at 60°C for 20 min before drying under a stream of nitrogen. The GA fractions were dissolved in dry acetonitrile (12 μL) and analyzed by GC-MS. For GC-MS, the samples (1 μL) were injected onto a BFX-5 capillary column (SGE 30 μm × 0.25 mm, 0.25 μm) using helium as the carrier gas (1 mL min⁻¹), and the inlet temperature was 280°C. The initial oven temperature was set at 50°C and held for 1 min before increasing to 280°C at 15°C min⁻¹. The temperature was increased at 3°C min⁻¹ up to 270°C followed by 15°C min⁻¹ to 320°C and held for 5 min. The transfer line was set at 280°C, the ion source was 200°C, and the ionization potential was 70 eV. The analyses were performed in selected ion monitoring mode, monitoring for the following ions: 190 and 194 for ABA/d6ABA, 284 and 286 for GA4/d2GA4, 506 and 508 for GA_{x} and GA_{y}. Amounts were calculated based on the ratio of these ions with corrections made to account for contributions to the area of the deuterated authentic samples to provide an accurate measurement of the endogenous hormone levels.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Time course of germination for PD Arabidopsis ecotypes.
Supplemental Figure S2. Seven-day germination of afterripening Ler seed.
Supplemental Figure S3. KAR_{ε} effects on ABA mutant seed germination.
Supplemental Figure S4. KAR_{ε} effects on GA mutant seed germination.
Supplemental Figure S5. Transcriptional changes of ABA-related genes during imbibition.
Supplemental Figure S6. Transcriptional changes of GA-related genes during imbibition.
Supplemental Table S1. Primer sequences for qRT-PCR analysis.

ACKNOWLEDGMENTS
We gratefully acknowledge Dr. Eiji Nambara for cyp707a double mutants, Dr. Camille Steber for slf1 mutants, Dr. Tai-ping Sun for gai3;1 single and double mutants, Dr. Peter McCourt for erat-1, and the Arabidopsis Biological Resource Center for all other seed stocks. Thanks to Dave Merritt for discussion.

Received October 21, 2008; accepted December 5, 2008; published December 12, 2008.

LITERATURE CITED
Flematti GR, Goddard-Borger ED, Merritt DJ, Ghisalberti EL, Dixon KW, Trengove RD (2007) Preparation of 2H-furo[2,3-c]pyran-2-one deriva-