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Goldacre Review

# From little things big things grow: karrikins and new directions in plant development

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**Abstract.** Karrikins are a family of compounds generated via the incomplete combustion of plant matter. Since their discovery as seed germination stimulants in 2004, a great deal has been learned about the chemistry and the biological mode of action of karrikins. Much interest and progress have stemmed from the structural similarity of karrikins to that of strigolactones – the shoot branching hormone. This review will provide a historical account of some of the more significant discoveries in this area of plant biology. It will discuss how the study of these abiotic signalling molecules, combined with advances in our understanding of strigolactones, has led us towards the discovery of new mechanisms that regulate plant growth and development.

**Additional keywords:** bioassay, chemical biology, plant development, plant hormone, signalling, strigolactone.

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## Karrikins: chemical germination cues from fire

The evolution of oxygenic photosynthesis and the subsequent emergence of land plants provided the necessary conditions for fire on Earth. Deposits of fossil charcoal indicate that wildfires began in the Silurian some 420 million years ago, and varied in prevalence according to atmospheric oxygen levels and shifts in dominant terrestrial vegetation (Scott and Glasspool 2006). As an ancient and widespread phenomenon, fire has served as a pronounced evolutionary force that has shaped the composition of ecosystems across the globe (Pausas and Keeley 2009). The passage of a fire event presents several opportunities for regeneration as a result of reduced competition for resources. Accordingly, a large number of plant species have evolved the ability to perceive and respond to fire events. A prominent adaptation among species from fire-prone regions is the promotion of seed germination following exposure to smoke, and chemicals therein (Van Staden *et al*. 2000; Nelson *et al*. 2012).

Clues about the chemical nature of smoke compounds with the capacity to stimulate seed germination came from early experiments with 'liquid smoke' or 'smokewater', a complex solution of combustion products generated by bubbling smoke through water (Baldwin *et al*. 1994; Baxter *et al*. 1995; van Staden *et al*. 1995). The presence of active compounds in smoke indicated that they were at least partially volatile, and chromatography-based fractionation experiments revealed that several different compounds are active, depending on the starting material (Baldwin *et al*. 1994; van Staden *et al*. 1995). Despite these promising advances, discovery of an active compound was not made until 2004, using bioassay-guided fractionation of smokewater, followed by gas chromatography-mass spectrometry and nuclear magnetic resonance techniques for structural elucidation. The first report of the butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one was made by chemists at the University of Western Australia using burnt cellulose as a source (Flematti *et al*. 2004). A subsequent report of the same compound was made by a South African team using a similar approach but with burnt plant material (van Staden *et al*. 2004). This compound (Fig. 1*a*) was eventually named 'karrikinolide' or $KAR_1$ (after the word 'karrik', meaning smoke in the language of the Noongar people indigenous to the Australian south-west). Subsequent work identified several synthetic $KAR_1$ analogues with germination-promoting activity, all containing a consistent butenolide moiety but with varying substituents (Flematti *et al*. 2007). Crucially, five of these analogues were also detected at varying levels in smokewater, suggesting that they may each contribute to the bioactivity of smoke and char from burned vegetation (Flematti *et al*. 2009). In keeping with nomenclature for other groups of plant growth regulators (e.g. cytokinins, gibberellins), $KAR_1$ and its analogues $KAR_2$ to $KAR_6$ are collectively known as *karrikins* (Fig. 1*a*).

It is noteworthy that although karrikins were initially isolated from smoke, most of the $KAR_1$ produced during combustion is retained within the char residue, and $KAR_1$ is sparingly soluble in water (Flematti *et al*. 2008). As such, the stimulatory activity of karrikins may not disperse very far from the site of a fire. Furthermore, other bioactive germination stimulants besides karrikins are produced during fires, which may have differing physicochemical properties. These include the cyanohydrin glyceronitrile, which slowly hydrolyses to produce cyanide, and which in turn promotes germination in some smoke-responsive species that are otherwise insensitive to karrikins (Flematti *et al*. 2011).

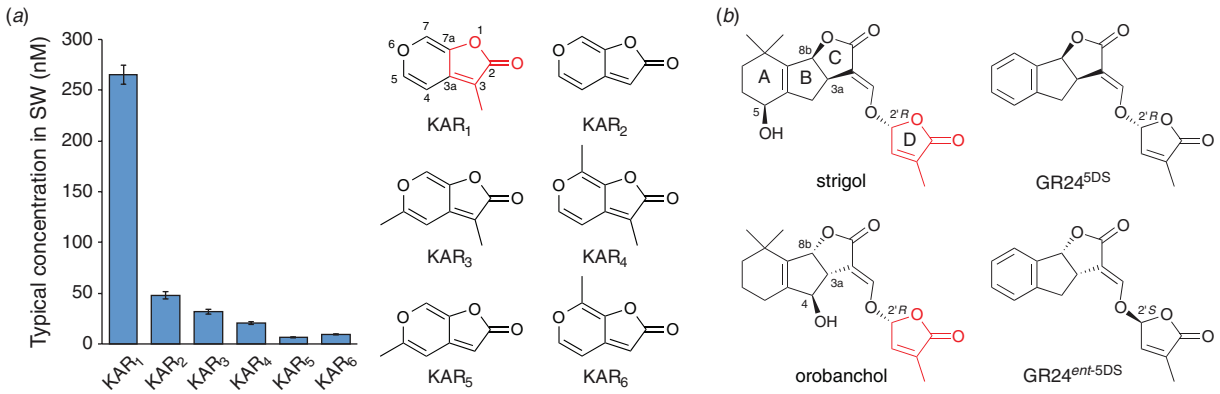


**Fig. 1.** Karrikins and strigolactones. (*a*) Left: Relative abundance of six karrikins in a sample of smokewater (SW) generated from burnt cellulose. Data are means ± s.e. for three technical replicates based on quantitation of original GC-MS data from Flematti *et al*. (2009). Right: chemical structures of $KAR_1$ to $KAR_6$. Three have a methyl group on the butenolide ring (red), whereas three do not. (*b*) Structures of two naturally-occurring strigolactones, strigol and orobanchol, and the synthetic strigolactone analgoue GR24. Note the differing stereochemistry at the 8b and 3a positions between the B and C rings. The butenolide moiety is often referred to as the D ring. All natural strigolactones contain a 2′*R* configuration on the D ring, but racemic GR24 is composed of two enantiomers, one of which has the natural 2′*R* configuration, and one which has the non-natural 2′*S* configuration.

## Seed germination responses to karrikins

Karrikins were isolated by germination-based bioassays using, primarily, seed of lettuce (*Lactuca sativa* cv. Grand Rapids, Asteraceae). This species is exceptionally sensitive to $KAR_1$, responding to concentrations of 1 nM or below (Flematti *et al*. 2004). Additional bioassay species included *Solanum orbiculatum* (Solanaceae; ~10 nM) and *Emmenanthe penduliflora* (Boraginaceae; ~10 nM) (Flematti *et al*. 2007). Since the identification of $KAR_1$, several diverse species have been classified as responding positively to karrikins. These include the arable weeds *Brassica tournefortii* and *Sisymbrium orientale* (Brassicacaeae) (Stevens *et al*. 2007), and *Avena fatua* and *Sorghum halepense* (Poaceae) (Daws *et al*. 2007). A recent study of the effects of smokewater and $KAR_1$ on seed germination of 13 species from South China identified only one (*Aristolochia debilis*; Aristolochiaceae) as responding positively to 1 nM $KAR_1$ or more (Zhou *et al*. 2014). However, classifying a species in a binary fashion as karrikin-responsive or karrikin-non-responsive is fraught with difficulties, because environmental factors (e.g. maternal life history and wet–dry cycling regimes) that influence physiological seed dormancy in turn affect the capacity of seed to respond to karrikins (Long *et al*. 2010, 2011). Indeed, varying degrees of physiological seed dormancy between batches of seed from the same species can lead to apparently opposite responses to karrikins (Stevens *et al*. 2007). It is also possible that 'non-responsive' species have incompatible perception machinery, perhaps because, as discussed below, the relevant receptor protein has undergone selection to detect compounds other than karrikins. Accordingly, it is difficult to estimate the number of karrikin-responsive species based on germination tests alone. However, it is clear that the capacity to respond to smoke, and to karrikins specifically, is taxonomically widespread among angiosperms.

For the few species tested, $KAR_1$ is the generally the most effective germination stimulant among the karrikins, which makes evolutionary sense in light of $KAR_1$ being the most abundant karrikin produced by combustion of cellulose. As a germination stimulant, $KAR_1$ is ~10-fold more effective than $KAR_2$ in Grand Rapids lettuce, and 100-fold more effective in *S. orbiculatum* (Flematti *et al*. 2007). All else being equal, karrikins with a methyl group at the C-3 position on the butenolide ring are generally more active than those without (Flematti *et al*. 2007). Few studies have explored the sensitivity of different species to the various karrikin family members, so it is not yet clear how widespread this pattern is. Nevertheless, a notable reversal of the preference for $KAR_1$ over $KAR_2$ is found in *Arabidopsis thaliana*, as discussed below.

## Other plant responses to karrikins

Karrikins were discovered on the basis of their ability to promote seed germination and overcome seed dormancy, but they are widely reported also to enhance seedling survival and seedling vigour. Both smokewater (1 : 500 dilution) and $KAR_1$ (100 nM) led to increased shoot and root growth in tomato, okra, maize and rice (Kulkarni *et al*. 2006; van Staden *et al*. 2006). In maize, a single 1 h soak of kernels in 100 nM $KAR_1$ enhanced post-germination seedling growth relative to untreated controls when measured 30 days after germination (although effects at earlier or later growth stages were not reported). The same treatment also led to significantly improved seedling survival rates (van Staden *et al*. 2006). $KAR_1$ was also shown to improve germination of tomato seed and subsequent seedling vigour when grown at low (10°C) or high (40°C) temperatures (Jain *et al*. 2006). These findings suggest that karrikins might be powerful agents for seed preconditioning to improve crop productivity under stressful conditions.

Recently, it was demonstrated that biochars – charcoal-like material produced by pyrolysis of organic material under oxygen-limiting conditions – contain readily detectable amounts of $KAR_1$ (Kochanek *et al*. 2016). The yield of $KAR_1$ varied greatly depending on the pyrolysis technology used, but reached 82 ng per 100 g (~1 ppb) of biochar. Liquid by-products of one biochar process contained over 400 nM $KAR_1$, which compares

favourably with smokewater (Fig. 1*a*). Pertinently, the shoot length of both tomato and lettuce plants grown in soil containing up to 10% biochar was significantly greater than untreated controls after two weeks of growth (Kochanek *et al*. 2016). In addition, the $KAR_1$ content of biochar correlated positively with the effects on plant growth, tempting one to conclude that karrikins are responsible for the benefits of biochars. However, the physiological changes that karrikins and biochar bring about to stimulate plant growth remain to be investigated in any detail, and such studies would benefit greatly from the inclusion of genetic resources.

## Karrikin studies in *Arabidopsis*

A great deal of our knowledge on the effects and mechanisms of karrikins on plant growth come from studies on the genetic workhorse *A. thaliana*. The seed of this species exhibits variable degrees of primary seed dormancy, defined as the tendency for freshly harvested seed not to germinate under otherwise favourable conditions. The depth of primary dormancy depends heavily on the ecotype in question: Cape Verde Islands (Cvi-0) is highly dormant, whereas Landsberg *erecta* (L*er*) is less so (Alonso-Blanco *et al*. 2003). Columbia-0 (Col-0) meanwhile exhibits little primary dormancy at all (van der Schaar *et al*. 1997). Therefore, as with other species, applying karrikins to stimulate seed germination has variable effect on the ecotypes of *Arabidopsis*. Nevertheless, primary dormant L*er* seeds demonstrate a robust response to 10 nM $KAR_2$, and to 100 nM $KAR_1$ and $KAR_3$ (Nelson *et al*. 2009). Further experiments showed that $KAR_1$ cannot overcome the requirement of germination for gibberellin (GA) biosynthesis, and indeed that $KAR_1$ might act in part by stimulating the expression of the GA biosynthetic genes *GA3ox1* and *GA3ox2*, which would, in turn, stimulate germination (Nelson *et al*. 2009).

The developmental response of a seedling to light is known as photomorphogenesis. In epigeal seedlings like *Arabidopsis* – where the cotyledons emerge above ground and become photosynthetic – photomorphogenesis is characterised by greening and expansion of the cotyledons, reduction or cessation of hypocotyl elongation, and increased root growth relative to dark-grown seedlings. Treatment of wild-type *Arabidopsis* seedlings with 100 nM $KAR_1$ or $KAR_2$ led to an enhancement of these traits, especially cotyledon expansion and inhibition of hypocotyl elongation (Nelson *et al*. 2010). As with seed germination, *Arabidopsis* seedlings were more responsive to $KAR_2$ than to $KAR_1$. Karrikins also reduced the minimum amount of red light required to trigger germination after an inhibitory far-red light pulse; however, karrikins were insufficient to overcome the requirement for light altogether, suggesting that they act downstream of phytochrome B in the control of germination and photomorphogenesis (Nelson *et al*. 2010). Nevertheless, karrikins do induce transcriptional changes in darkness, suggesting that some karrikin responses are light-independent (Waters and Smith 2013). Together, these findings indicate that karrikins enhance the sensitivity of seedlings and seed to light, and act as general positive regulators of light-dependent development.

As a northern hemisphere temperate species with no ecological requirement for fire regimes, the response of *A. thaliana* to karrikins was a critical finding. First, it provided a powerful means to investigate, using genetics, the molecular basis for the perception and response of plants to karrikins. In addition, it indicated that these mechanisms are likely to be conserved among angiosperms, aiding the transfer of knowledge gleaned from one species to others of ecological or economic significance. Most importantly, the evolutionary conservation of a trait that is presumably not under selection indicates that the mechanisms for karrikin perception and response may have other functions in plant development beyond the detection of compounds released by fire.

## Karrikins are structurally related to strigolactones

Like karrikins, strigolactones are also butenolide compounds (Fig. 1*b*), and the structural similarity between these compounds was quickly recognised (Flematti *et al*. 2004). Strigolactones were first discovered in the 1960s and '70s as compounds exuded from plant roots that induced the germination of seed of root parasitic weeds in the Orobanchaceae (reviewed by Ruyter-Spira *et al*. 2013). But why should a plant synthesise and secrete compounds that might cause self-harm through parasitism? In 2005, Akiyama *et al*. (2005) demonstrated that natural strigolactones (such as 5-deoxystrigol), and the synthetic strigolactone analogue GR24, could stimulate germination and hyphal branching in fungal spores that form arbuscular mycorrhizal (AM) symbioses. These associations, in which the fungi assist the plant in mineral uptake in exchange for organic carbon, are ubiquitous throughout the plant kingdom, even though a few families (such as the Brassicaceae) have lost the ability to form them (Bouwmeester *et al*. 2007; Bravo *et al*. 2016). Therefore strigolactones were shown to constitute the elusive 'branching factor' that enabled host plants to recruit AM fungi, especially under nutrient-limiting conditions. The biosynthetic source of strigolactones was unclear, but carotenoids were considered a likely possibility based on inhibitor studies (Matusova *et al*. 2005).

In parallel, plant developmental biologists and geneticists were seeking to understand the mechanistic basis for controlling shoot architecture – specifically the regulation of shoot branching. Several studies of mutants with increased shoot branching phenotypes in pea (*Pisum sativum*), rice (*Oryza sativa*), petunia (*Petunia hybrida*) and *Arabidopsis* had indicated that a mobile substance, synthesised in and/or transported from the roots, could inhibit branching in the shoot and thus promote apical dominance (Beveridge *et al*. 1996; Napoli 1996; Beveridge *et al*. 1997; Stirnberg *et al*. 2002; Ishikawa *et al*. 2005). Two of the affected genes in these mutants were found to encode carotenoid cleavage dioxygenase (CCD) enzymes, thus implicating carotenoids as a possible source (Sorefan *et al*. 2003; Booker *et al*. 2004; Johnson *et al*. 2006; Arite *et al*. 2007; Drummond *et al*. 2009). In 2008, these lines of evidence were brought together to show that strigolactones were endogenous plant hormones responsible for controlling shoot architecture (Gomez-Roldan *et al*. 2008; Umehara *et al*. 2008). Since this major step forward was made, strigolactones have been implicated in numerous other processes, including leaf senescence (Snowden *et al*. 2005; Yamada *et al*. 2014), adventitious rooting (Rasmussen

*et al*. 2012), secondary thickening (Agusti *et al*. 2011), and regulation of root architecture under changing nutrient conditions (Ruyter-Spira *et al*. 2011; Mayzlish-Gati *et al*. 2012; reviewed by Al-Babili and Bouwmeester 2015; Waters *et al*. 2017).

At the time of the discovery of karrikins in 2004, little more about the function of strigolactones was known, and the significance of the structural similarities was not clear. As both were butenolide germination stimulants, it was reasonably hypothesised that karrikins might be abiotic mimics of strigolactones with similar modes of action (Flematti *et al*. 2004). However, it was soon shown that the relationship was not so straightforward. First, although karrikins were potent germination stimulants of *Brassica tournefortii*, the synthetic strigolactone GR24 was not (Nelson *et al*. 2009). Conversely, seed of the parasitic weed *Orobanche minor* responded strongly to GR24, but not at all to karrikins; this observation has since been extended to several other parasitic species (Conn *et al*. 2015). These observations may simply reflect species-specific adaptation towards different germination stimulants. However, karrikins were also inactive as inhibitors of shoot branching, unlike GR24 (Nelson *et al*. 2011). Thus, physiological evidence was building that karrikins and strigolactones have distinct bioactivities and, therefore, different perception mechanisms. More direct molecular–genetic evidence has subsequently solidified this position, but we now know that the parallels between karrikins and strigolactones extend far beyond their superficially similar chemical structures.

## Genetic analysis of karrikin perception and signalling

### *2011:* MAX2

Genetic screens for *Arabidopsis* mutants deficient in karrikin signalling have been central to building our knowledge of the function of karrikins. The first screen for *karrikin insensitive* (*kai*) mutants involved the selection of mutagenised, primary dormant seed that failed to germinate on water-agar medium supplemented with karrikins. This screen identified two allelic mutants called *kai1*, which, besides impaired seed and seedling responses to karrikins, also exhibited several other phenotypes (Nelson *et al*. 2011). These included abnormal seedling development, curled, rounded leaves, increased shoot branching, and reduced stature. These phenotypes are also characteristic of the strigolactone-insensitive mutant *more axillary branches2* (*max2*) (Stirnberg *et al*. 2002). This insight led Nelson *et al*. (2011) to make an educated guess, and upon sequencing they found that both *kai1* alleles contained frameshift mutations in *MAX2*. This was a spectacular discovery because not only did it demonstrate that *MAX2* had a new role in seed germination, but also it showed that the mechanism for the perception of strigolactones and karrikins shared a common component (Waters *et al*. 2011).

MAX2 is an F-box protein, a family of leucine-rich-repeat proteins that form one part of the SCF class of E3 ubiquitin ligase complexes (Xu *et al*. 2009). The F-box component confers substrate specificity to polyubiquitination, a process that labels target proteins for proteolytic destruction via the 26S proteasome. An SCF E3 ubiquitin ligase that contains MAX2 can be denoted $SCF^{MAX2}$. The perception mechanisms for several plant hormones, including auxins, gibberellins and jasmonates make use of F-box proteins to selectively target downstream repressor proteins (McGinnis *et al*. 2003; Dharmasiri *et al*. 2005; Kepinski and Leyser 2005; Katsir *et al*. 2008). Thus, following the identification of MAX2, additional karrikin signalling and response components were anticipated.

### *2012:* KAI2/HTL *and* D14

MAX2 (and its homologue in rice, DWARF3) was known to be essential for strigolactone perception. As the auxin receptor, TIR1, was also an F-box protein, it was possible that MAX2 also served as a receptor strigolactones and karrikins. However, there was no molecular evidence for this hypothesis, and no obvious mechanism for how MAX2 might mediate such different physiological responses to the two different classes of compounds. In 2009, the rice mutant *dwarf14* (*d14*; also known as *d88* or *htd2*) was described as having a shoot architecture phenotype largely indistinguishable from *d3* (Arite *et al*. 2009; Liu *et al*. 2009). Crucially, like *d3*, *d14* was also strigolactone-insensitive, and double mutant analysis placed it within the strigolactone response pathway (Arite *et al*. 2009). *DWARF14* encodes an α/β-fold hydrolase, and its function was not described until 2012, but it was known to be part of a larger family of proteins common to all land plants. Phylogenetic analysis indicated that the *Arabidopsis* and rice genomes each contained a single *D14* orthologue, as well as a more distant paralogue dubbed *D14-LIKE*. At the time, this gene also had no known function, with no described mutant phenotype in any species. As the gibberellin receptor GID1 is an α/β-fold hydrolase (Ueguchi-Tanaka *et al*. 2005), the possibility that D14 might serve as the strigolactone receptor was an appealing hypothesis. D14 and D14-LIKE thus represented potentially valuable signalling components that remained to be investigated.

On the supposition that D14 and D14-LIKE proteins might be receptor proteins, and in turn might provide MAX2 with a means to discriminate between karrikins and strigolactones, Waters *et al*. (2012*a*) set out to analyse the function of D14 family members in *Arabidopsis*. As expected, the *d14* mutant of *Arabidopsis* exhibited an increased shoot branching phenotype and strigolactone insensitivity (Waters *et al*. 2012*a*; Chevalier *et al*. 2014). By a remarkable stroke of serendipity, we converged upon the function of D14-LIKE by two parallel routes. First, as part of a systematic search for karrikin insensitive mutants among candidate genes known to be involved in seed germination and dormancy, we discovered a mutant allele of the gene *SPATULA* (*At4g36930*). Although this allele, *spt-1*, was almost completely insensitive to karrikins in seed germination and seedling hypocotyl elongation assays, several other *spt* alleles (including predicted null alleles) responded normally to karrikins. Eventually we were forced to conclude that a second, unknown mutation elsewhere in the *spt-1* genome was responsible for the karrikin insensitive phenotype, and we renamed this mutant *karrikin insensitive2–1* (*kai2–1*) after backcrossing to segregate away the *spt-1* mutation. Recombinant $F_2$ individuals were much less frequent than expected assuming independent assortment, suggesting that the two mutations were closely linked.

Meanwhile, in the second route, we used reverse genetics to isolate a *Ds* transposon insertion mutant in the *Arabidopsis*

orthologue of rice *D14-LIKE*, *At4g37470*. We soon noticed that *SPATULA* (*At4g36930*) and *At4g37470* were closely located on the same chromosome, prompting us to sequence *At4g37470* in the *kai2–1* mutant background. In doing so, we discovered a G-to-A transition in the coding sequence, modifying a conserved glycine residue to glutamic acid (Waters *et al*. 2012*a*). Coupled with allelism tests, this finding indicated that *At4g37470* was in fact *KAI2*, and we renamed the *Ds* insertion allele *kai2–2*. In the course of our work, another group identified a further *kai2* allele in a screen for photomorphogenesis mutants (Sun and Ni 2011). They named the mutant *hyposensitive to light* (*htl*) on the basis of its elongated hypocotyl phenotype. We felt that *KAI2* was a more accurate description of the function of the gene, but both names are now widely adopted in the literature (e.g. Toh *et al*. 2015), as is *D14-LIKE* in studies on rice (e.g. Kameoka and Kyozuka 2015).

A key observation from isolating the *Arabidopsis d14* and *kai2* mutants was that each one exhibited different components of the *max2* phenotype. Although *d14* seedlings were phenotypically normal, *kai2* seedlings were indistinguishable from *max2* seedlings. Meanwhile, *d14* mutants exhibited the same increased shoot branching phenotype as *max2*, but shoot branching was normal in *kai2*. Thus, the *max2* phenotype could be considered to be an amalgamation of *kai2* and *d14* phenotypes; accordingly, a *kai2 d14* double mutant fully recapitulates the *max2* phenotype (Fig. 2). Importantly, *kai2* mutants retain the ability to respond to exogenous strigolactones, and *d14* mutants to karrikins (Waters *et al*. 2012*a*; Scaffidi *et al*. 2014). By extension, some *max2* phenotypes are thus strigolactone related (as mediated by D14), and others are strigolactone-independent (as mediated by KAI2).

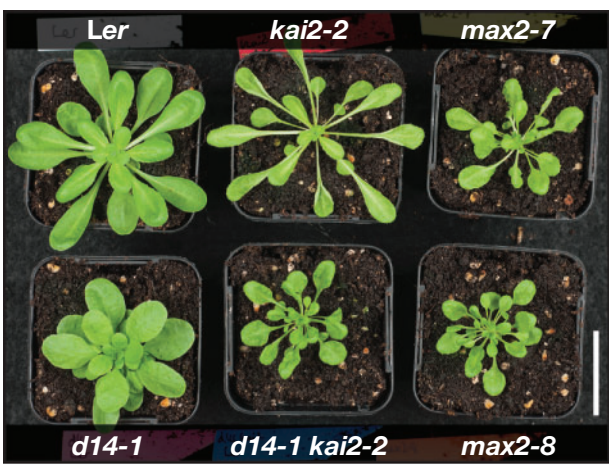


**Fig. 2.** D14 and KAI2 encompass the developmental functions of MAX2. Rosette phenotypes of *d14*, *kai2* and *max2*. Under these growth conditions and in the Landsberg *erecta* background, the *kai2* mutation results in long, slender leaf blades and elongated petioles, and a corresponding 'open' rosette structure. The *d14* mutation has the opposite effect. Combining both mutations fully recapitulates the *max2* phenotype, which exhibits aspects of each single mutant. Plants were grown under short day conditions (8 h light/16 h dark photoperiod) and a 22°C light/16°C dark temperature cycle. Plants were photographed six weeks after germination. The *d14-1* allele was introgressed into the L*er* background by six consecutive backcrosses; all other mutants were generated in the L*er* background. Scale bar = 4 cm.

KAI2 and D14 are capable of mediating plant responses to very similar compounds. Although KAI2 is essential for karrikin responses, in *Arabidopsis* it can also detect the synthetic strigolactone GR24 (Waters *et al*. 2012*a*). At first glance, this result suggests that KAI2 can serve as a receptor for natural, endogenous strigolactones; however, in *Arabidopsis* at least, this is not the case. Careful mutant analysis indicated that the KAI2-dependent activity of GR24 is an artefact of the racemic composition of standard preparations of synthetic GR24 (Scaffidi *et al*. 2014). Although all natural strigolactones contain a 2′*R*-configured D-ring, racemic GR24 (*rac*-GR24) contains an equal proportion of 2′*R* and 2′*S* stereoisomers (Fig. 1). These mirror images, or enantiomers, have been given the simplified names of GR24$^{5DS}$ and GR24$^{ent–5DS}$, respectively, in reference to the natural strigolactone 5-deoxystrigol (5DS) that they most closely resemble (Scaffidi *et al*. 2014; Flematti *et al*. 2016). GR24$^{ent–5DS}$ can substitute for karrikins in a range of KAI2-dependent assays, albeit with reduced sensitivity (Scaffidi *et al*. 2014; Waters *et al*. 2015*a*, 2015*b*). Likewise, D14 responds preferentially to GR24$^{5DS}$ over GR24$^{ent–5DS}$ (Nakamura *et al*. 2013; Scaffidi *et al*. 2014; Waters *et al*. 2015*b*; Zhao *et al*. 2015). The biological significance of KAI2 recognising non-naturally-configured strigolactones is unclear, but it does demonstrate that KAI2 and D14 exhibit distinct substrate preferences, which in part explains their functional differences. In *Arabidopsis* at least, KAI2 does not appear to have any role in mediating responses to endogenous strigolactones.

Several angiosperms, including *Arabidopsis* and rice, contain a third member of the D14 family. This protein – D14-LIKE2 (DLK2) – is a close paralogue of D14, suggesting that it arose through a duplication of *D14* before the divergence of monocots and dicots. At the time of writing, the function of DLK2 remains an enigma, but a very curious one. *DLK2* transcripts are strongly upregulated in response to application of karrikins, making it a convenient transcriptional marker for KAI2-dependent activity (Waters *et al*. 2012*a*). *DLK2* transcripts are also repressed in *kai2* and *max2* seedlings, but not in *d14* seedlings; however, D14-mediated signalling of exogenous strigolactones can increase the abundance of *DLK2* transcripts (Waters *et al*. 2012*a*; Scaffidi *et al*. 2014). These observations could suggest that DLK2 has some form of negative feedback role, perhaps serving to repress KAI2- or D14-dependent signalling. However, *dlk2* mutants have no obvious seed germination or seedling development phenotype, and shoot branching is apparently normal (Waters *et al*. 2012*a*). A closer examination of *dlk2* phenotypes, perhaps in conjunction with higher order mutants, will doubtless reveal DLK2 function in time.

### *2013: The* SMXL *family*

The newly identified function for MAX2 in karrikin signalling presented a new angle to discover the putative repressor proteins that are targeted for proteolysis by SCF$^{MAX2}$. A suppressor screen for the karrikin-related *max2* phenotypes relating to seed germination and seedling growth identified SUPPRESSOR OF MAX2 1 (SMAX1) (Stanga *et al*. 2013). *Arabidopsis* seedlings deficient in SMAX1 and, to a lesser extent its close paralogue SMAX1-LIKE 2 (SMXL2), resemble wild type seedlings treated with karrikins – i.e. they exhibit constitutive karrikin responses

(Stanga *et al*. 2016). Shortly after the discovery of SMAX1, the rice protein DWARF53 (D53) was identified thanks to a dominant rice mutant that had similar phenotypes to *d3* and *d14*, such as high tillering and increased strigolactone production (Jiang *et al*. 2013; Zhou *et al*. 2013). D53 is another member of the SMXL family, all of which share similarity to the ClpB/HEAT SHOCK PROTEIN 100 (HSP100) class of heat shock proteins. In *Arabidopsis*, three D53 homologues (SMXL6, SMXL7 and SMXL8) act redundantly to mediate all tested strigolactone-related components of MAX2 function (Soundappan *et al*. 2015; Liang *et al*. 2016; Bennett *et al*. 2016). This redundancy might explain why suppressor screens for the increased shoot branching phenotype of *max2* had not uncovered this protein family. Instead, one such screen identified a role for the transcription factor FAR RED ELONGATED HYPOCOTYL3 in the regulation of auxin-dependent axillary bud outgrowth (Stirnberg *et al*. 2012). The functions of other SMXL members – SMXL3, SMXL4 and SMXL5 in *Arabidopsis* – are yet to be resolved (Khosla and Nelson 2016). However, it is clear that the diverse functions of MAX2 relating to karrikins and strigolactones are a combinatorial result of two different receptor proteins (KAI2 and D14), and two subclasses of downstream repressor proteins (SMAX1/SMAXL2, and SMXL6/7/8) (Fig. 3). A significant body of work has resulted in a molecular model for strigolactone and karrikin signalling mechanisms, and this has been reviewed recently (Waters *et al*. 2017).

### *KAI2 and D14 are both receptor proteins and enzymes*

D14 and KAI2 are unusual among hormone receptor proteins because they are also functional enzymes with hydrolytic activity. In 2012, the structure of the D14 homologue in *Petunia hybrida*, DAD2, was solved (Hamiaux *et al*. 2012). Shortly thereafter, structures of KAI2 and D14 from *Arabidopsis* and rice were also published (Bythell-Douglas *et al*. 2013; Guo *et al*. 2013; Kagiyama *et al*. 2013; Zhao *et al*. 2013). Both KAI2 and D14 contain a catalytic triad of Ser, His and Asp residues at the end of a hydrophobic ligand-binding cavity. The overall structure is globular, consisting of a core domain of seven α-helices and seven β-sheets linked to a 'lid' domain of four α helices. The ligand binding cavity sits between these two domains.

As part of the strigolactone signalling pathway, D14 has received much attention to demonstrate its role as a receptor, a position supported by a substantial body of evidence. As described above, loss-of-function *d14* mutants are consistently SL-insensitive in several species (Arite *et al*. 2009; Waters *et al*. 2012*a*; Hamiaux *et al*. 2012; Chevalier *et al*. 2014; de Saint Germain *et al*. 2016). Extensive biochemistry experiments have enhanced this genetic evidence. D14 hydrolyses strigolactones, albeit very slowly, by nucleophilic attack upon the strigolactone D-ring, which is oriented towards the catalytic serine (Hamiaux *et al*. 2012; Yao *et al*. 2016; de Saint Germain *et al*. 2016). Mutation of the catalytic triad residues renders D14 non-functional as an enzyme, and also as a transducer of strigolactone signalling *in planta* (Hamiaux *et al*. 2012). A critical observation is that binding of strigolactone to D14 induces a marked change in protein melting temperature,

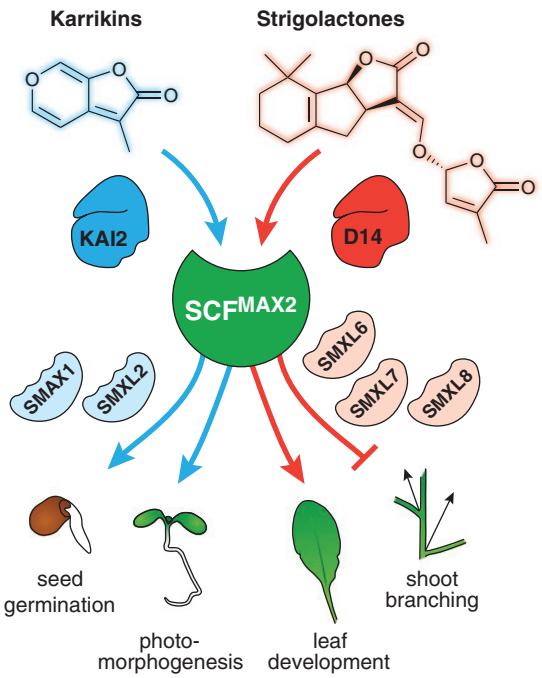


**Fig. 3.** Simplified model of karrikin and strigolactone perception and response. Two receptor proteins, KAI2 and D14, mediate the perception of karrikins and strigolactones respectively. Upon binding their ligand, each receptor protein associates with MAX2 and their cognate SMXL family members, here grouped by colour. The SMXL proteins are degraded by $SCF^{MAX2}$ upon receptor activation. Events downstream of SMXLs are unclear, but may involve transcriptional regulation. The various SMXL members regulate distinct aspects of plant development, some of which are shown here. The association between D14 and SMXL7/D53 has been demonstrated (Wang *et al*. 2015; Liang *et al*. 2016), but a physical association between KAI2 and SMAX1/SMXL2 is only inferred genetically. In this model, the specific ligand–substrate–repressor associations explain the observed physiological outcomes. For example, karrikins cannot regulate shoot branching because karrikins cannot activate D14 and thus stimulate the degradation of SMXL6, SMXL7 and SMXL8. However, there may be some promiscuity between receptor–repressor pairings under certain circumstances; for a more detailed model and discussion, see Waters *et al*. (2017).

suggesting that the ligand-protein interaction imparts a change in D14 conformation (Hamiaux *et al*. 2012; Zhao *et al*. 2013; Abe *et al*. 2014; Waters *et al*. 2015*b*). Consistent with this interpretation, crystallography demonstrated the ligand-dependent interaction of D14 and D3/MAX2, together with the *Arabidopsis* SCF. component ASK1 (Yao *et al*. 2016). This crucial evidence revealed that binding of strigolactone to GR24 causes the lid domain of D14 to flatten and partially collapse the ligand-binding cavity, trapping a strigolactone hydrolysis product within. At the same time, another study determined that this hydrolysis product was covalently linked to the D14 catalytic His residue (de Saint Germain *et al*. 2016). Thus, the significance of the enzymatic activity of D14 has become clearer: substrate hydrolysis is an integral step to form a catalytic intermediate that enhances the formation of a protein complex, which in turn transduces the signal. Hydrolysis might also serve as a mechanism for destroying the strigolactone ligand and deactivating the signal. At least *in vitro*, the hydrolysis product is slowly released, suggesting

that D14 might relax after activation. However, in plants D14 undergoes proteolysis in response to strigolactone application (Chevalier *et al*. 2014), so this may be the dominant mechanism for removal of D14 in its activated and closed state. Finally, strigolactone hydrolysis by D14 is insufficient to confer a signalling function, because the non-functional d14–5 protein exhibits enhanced rates of hydrolysis, but strongly impaired interaction with D3/MAX2 (Yao *et al*. 2016). Collectively, these data demonstrate conclusively that D14 is not merely an enzyme, but also serves as a strigolactone receptor that binds and subsequently hydrolyses its ligand to transduce a signal.

Given this abundant knowledge about D14, the case for KAI2 being the karrikin receptor is very strong. KAI2 is an active hydrolase, and mutagenesis of the catalytic triad renders it inactive both *in vitro* as an enzyme and *in planta* as a signal transducer (Waters *et al*. 2015*a*, 2015*b*). Several studies have detected direct interaction between $KAR_1$ and KAI2 homologues using isothermal calorimetry and/or fluorescence-based binding assays (Guo *et al*. 2013; Kagiyama *et al*. 2013; Xu *et al*. 2016). Two of these studies have also used crystallography to determine the position of $KAR_1$ in the binding pocket (Guo *et al*. 2013; Xu *et al*. 2016). However, each study revealed $KAR_1$ to sit in quite different orientations, perhaps because two different KAI2 proteins were investigated – one from *Arabidopsis* and one from the parasitic plant *Striga hermonthica*. Neither structure showed a dramatic change in conformation between the $KAR_1$-bound and $KAR_1$-free states. Curiously, karrikins do not induce any thermal destabilisation upon purified KAI2 in the same way that strigolactones do for D14 (Waters *et al*. 2015*b*). Thermal destabilisation of KAI2 can, however, come about through addition of the non-natural enantiomer $GR24^{ent-5DS}$, which is also a hydrolytic substrate for KAI2, suggesting that mechanistically KAI2 and D14 work in a similar fashion as enzyme–receptors. At this time there is no direct evidence that karrikins are hydrolysed by KAI2, perhaps because karrikins are not expected to be destroyed by nucleophillic attack upon the butenolide ring (Scaffidi *et al*. 2012; Waters *et al*. 2012*b*). Thus some data do not fully support a simple interpretation that KAI2 binds and hydrolyses $KAR_1$ directly, with the same outcomes as when D14 binds strigolactones. It is possible that, without additional protein components (i.e. MAX2 and ASK1) to stabilise any conformational change in KAI2, the natural orientation of $KAR_1$ in the KAI2 cavity will not be detected. Alternatively, karrikins might undergo metabolism *in vivo* to generate a bioactive ligand that is capable of fully activating KAI2.

There are several additional aspects of karrikin signalling that remain unclear. For example, although a physical interaction between D14 and D53/SMXL7 has been demonstrated (Jiang *et al*. 2013; Zhou *et al*. 2013; Wang *et al*. 2015), the same has not yet been established for KAI2 and SMAX1 or SMXL2, despite very strong genetic evidence that they operate in the same pathway. In addition, KAI2 undergoes degradation via an unknown mechanism as a consequence of karrikin signalling, but this process is independent of MAX2 (Waters *et al*. 2015*a*). This is in stark contrast to D14, which is degraded via the proteasome in a process that requires MAX2 (Chevalier *et al*. 2014). These apparent discrepancies may represent functionally significant points of distinction from the proposed mechanism for strigolactone signalling.

## *Beyond karrikins: what else does KAI2 do?*

The broad evolutionary conservation of KAI2 proteins – from algal relatives of land plants to angiosperms – clearly demonstrates that the primary function of KAI2 is unlikely to be the perception of compounds from fire. Although karrikins were instrumental in the discovery of KAI2 – not least because they are a potent activator of KAI2-dependent signalling, even in non-fire-prone plants – we now appreciate that KAI2 regulates some of the wide number of physiological processes that are dependent on MAX2. These include effects on seed germination and seedling development, as are well established, but effects on leaf development are also evident, at least in *Arabidopsis* (Waters *et al*. 2012*a*, 2015*b*; Bennett *et al*. 2016) (Fig. 2). In theory, any MAX2-dependent process that cannot be attributed to D14-dependent signalling (i.e. those processes regulated by strigolactones) might be under the influence of KAI2. A notable example of this was recently uncovered by studying the function of KAI2 in rice.

As noted above, strigolactones enhance the formation of AM symbioses by promoting the germination and hyphal development of fungal spores. Although strigolactone production is thus important for successful AM colonisation, it is not necessary, because strigolactone biosynthesis mutants nevertheless develop functional symbioses (Gomez-Roldan *et al*. 2008; Kohlen *et al*. 2012; Kretzschmar *et al*. 2012). Somewhat confusingly, the F-box protein D3/RMS4/MAX2 was shown to be essential for AM symbioses in both rice (Yoshida *et al*. 2012) and pea (Foo *et al*. 2013). However, it became clear that D14 was not responsible for regulating this aspect of MAX2 function, because rice *d14* mutants did not show the same AM-deficient phenotype as *d3* (Yoshida *et al*. 2012). These authors also excluded a role for *KAI2*/*D14-LIKE* in AM symbiosis, based on the analysis of RNAi knockdown lines that showed no phenotypic defect in this regard. This result was puzzling, as it implied that D3/MAX2 had a function in AM colonisation that was independent of both D14 and KAI2. However, in a classical genetic screen for rice mutants unable to form AM associations, Gutjahr *et al*. (2015) isolated the *hebiba* mutant, which showed several defects in the symbiotic process. Genetic mapping indicated that the *hebiba* phenotype resulted from a genomic deletion of 169 kb on chromosome 3. Complementation with genomic clones from this interval eventually identified the causative gene: *KAI2*, or *D14-LIKE* (Gutjahr *et al*. 2015). With this, order was restored: the AM-deficient phenotype of *d3* was shared with *d14-like*, reinforcing the prevailing notion that the functions of MAX2 are divided between D14 and KAI2. Thus, Gutjahr *et al*. (2015) demonstrated elegantly a new and critical role for KAI2 in the evolutionarily ancient symbiosis between plant roots and fungi. To date, the requirement for KAI2 in this process has not been demonstrated in other species, but it would be surprising if it were a function unique to rice. It remains to be elucidated exactly how KAI2 mediates AM symbiosis, but based upon the lack of AM development and induction of colonisation marker genes, KAI2 appears to act very early stage of the interaction, perhaps during presymbiotic signalling (Gutjahr *et al*. 2015). It is tempting to speculate that KAI2 perceives a compound present in the fungus. However, karrikins and extracts from germinating fungal spores do not

induce the same transcriptomic changes in rice (Gutjahr *et al*. 2015). Perhaps the level of the KAI2 ligand, or sensitivity to it, increases upon initial interaction with AM, and these changes are necessary to trigger the developmental changes in the plant. In this way, the signalling pathway defined by KAI2, MAX2 and SMAX1 might serve to create a permissive condition for symbiosis. One prediction of this hypothesis would be that loss-of-function *smax1* mutants are highly permissive, or at least would suppress the symbiosis-deficient phenotypes of *kai2* and *max2*. Although no *smax1* mutant outside *Arabidopsis* has yet been described, with modern genome editing tools such experiments in rice or other model systems are becoming increasingly feasible.

KAI2 has another, more specialised role in mediating interactions between plant roots and another organism: the detection of host-derived strigolactones by parasitic weeds. Obligate parasitic weeds such as *Striga hermonthica* and *Phelipanche aegyptiaca* are widespread agricultural pests, especially in sub-Saharan Africa, where they infest staples such as sorghum, maize and millet, and cause substantial crop losses (Parker 2009). In many parasitic weed genomes, *KAI2* is present in multiple copies, having undergone extensive gene duplication. In contrast, although D14 is also present in these genomes – presumably to mediate endogenous strigolactone signalling within the parasite itself – this gene is almost always present in single copy, and only rarely duplicated (Conn *et al*. 2015). Some of the *KAI2* copies are unique to parasites and are particularly divergent from other angiosperm *KAI2* genes, suggesting that they underwent increased rates of evolution via positive selection (Conn *et al*. 2015). These '*KAI2d*' genes encode proteins with substantially enlarged ligand binding pockets relative to *Arabidopsis* KAI2, and structural modelling suggested that these pockets could accommodate a strigolactone molecule. Several *KAI2d* transgenes from *S. hermonthica* and *P. aegyptiaca* were able to confer strigolactone-specific germination responses to *Arabidopsis kai2* mutants, which normally are insensitive to these compounds (Conn *et al*. 2015; Toh *et al*. 2015). In addition, purified KAI2d proteins from *S. hermonthica* were able to hydrolyse strigolactones, with varying affinities for different strigolactones (Tsuchiya *et al*. 2015). Thus, these specialised versions of KAI2 are likely responsible for detection of strigolactones by parasitic weeds. It is noteworthy that the evolutionary target for selection in this case was KAI2, which regulates seed germination, rather than D14, which is the canonical strigolactone receptor. This scenario may reflect the relative ease of changing the ligand specificity of a receptor, rather than modifying tissue expression domains and affinities for partner proteins.

It is evident that KAI2 is sensitive to several compounds that regulate its activity and bring about downstream molecular and physiological changes. If the ancestral role for KAI2 is not to perceive karrikins, which are unusual substances for most plants to encounter, then what might this role be? One hypothesis is that KAI2 is a receptor for an unknown endogenous compound with structural similarity to karrikins, but which is distinct from strigolactones derived from carlactone (Waters *et al*. 2014; Conn and Nelson 2015). The arguments in favour of this notion are multiple, and mainly stem from evolutionary-genetic analysis and from analogy to strigolactones. First, phylogenetic analysis strongly suggests that a KAI2-like protein was the evolutionary ancestor of D14, implying that the signalling function of KAI2 is fundamental and has been retained throughout the diversification of land plants (Delaux *et al*. 2012; Waters *et al*. 2012*a*, 2015*b*). Given that D14 was an innovation of the seed plants but strigolactones are found in Charophyte algae, it is likely that KAI2 proteins in early land plants were receptors for strigolactones, and perhaps for other signalling compounds as well (Delaux *et al*. 2012; Waters *et al*. 2013; Lopez-Obando *et al*. 2016). Second, KAI2-dependent signalling requires an intact catalytic triad, and this requirement is evolutionarily conserved between lycophytes and angiosperms (Waters *et al*. 2015*a*, 2015*b*). It seems unlikely that a functional catalytic triad of KAI2 would persist across such long timescales were it not required for the perception of a signalling molecule with a similar mechanism to D14 and strigolactones. Fourth, the appearance of *KAI2d* homologues in parasitic weeds to detect strigolactones implies that the ancestral KAI2 also had the capacity to respond to a similar ligand. Crucially, these ancestral KAI2 homologues – which show relatively reduced rates of evolution and therefore higher degrees of conservation – have retained a strigolactone-independent function similar to that of KAI2 in *Arabidopsis* (Conn and Nelson 2015; Conn *et al*. 2015). Fifth, the fact that KAI2- and D14-dependent signalling both operate via the same family of SMXL repressor proteins also suggests that KAI2 likely modulates MAX2 activity through a similar ligand-dependent mechanism. Finally, *kai2* and *max2* mutants of *Arabidopsis* both share seed germination and seedling morphogenesis phenotypes that are opposite to the effects of karrikin treatment. These phenotypes imply that *kai2* and *max2* are unable to perceive some internal signal that karrikins enhance or mimic (Nelson *et al*. 2011; Waters *et al*. 2012*a*). Accordingly, there is good reason to believe that a hormone-like KAI2 ligand (KL) exists.

## Chasing a new hormone

Historical discoveries of classical plant hormones have come about through the identification of an activity, followed by the identification of a source of this activity, and then isolation of the active compound. For example, gibberellins were discovered on the basis of the *bakanae* ('foolish seedling') disease of rice, caused by the fungus *Gibberella fujikuroi* (reviewed by Mander 2003). Overproduction of gibberellic acid by the fungus (the source) causes stem elongation and chlorosis (the activity). Similarly, ethylene was identified as the causative agent from gas streetlights that caused twisting and thickening of stems in nearby trees (Abeles *et al*. 1992). The discovery of strigolactones as a plant hormone was somewhat different; the existence of a mobile inhibitor of shoot branching was first predicted on the basis of genetics and physiological experiments. Likewise, the prediction of a KAI2 ligand (KL) comes primarily from inference and analogy, rather than from an observed bioactivity of a compound from a biological source.

Direct evidence for a compound that operates through KAI2 and is derived from plant material requires the development of a bioassay that provides a specific response. Recently, Sun *et al*. (2016) described the creation of a transgenic *Arabidopsis* line carrying a luciferase (*LUC*) coding sequence driven by the *DLK2*

promoter, which was chosen on the basis that *DLK2* transcripts are strongly induced by KAI2-dependent signalling. We showed that this reporter gene was sensitive to karrikins and GR24$^{ent-5DS}$ (the non-natural strigolactone enantiomer) but not to other plant hormones. These responses were abolished in a *kai2* mutant background, demonstrating the specificity of the reporter for KAI2-dependent activity. Importantly, we showed that extracts from *Arabidopsis* leaf material could activate expression of the reporter, suggesting that compounds present in leaf tissue were potential activators of KAI2 signalling (Sun *et al*. 2016). However, this activity was primarily found in the aqueous fraction of the plant extract, which would not be the case for hydrophobic compounds like karrikins and strigolactones. It is thus possible that the activity detected is not a direct ligand of KAI2, but may be a precursor of KL, or a stimulator of KL biosynthesis. Nevertheless, this work provides strong evidence for the existence of compounds that might act via KAI2, and lays out the groundwork for discovering a potential new hormone.

The challenges associated with identifying KL are formidable. The main difficulty will be the presumably very low abundance of KL in plant tissues. As a point of reference, strigolactones are present on the scale of 10 pg g$^{-1}$ FW in rice roots (Umehara *et al*. 2008). With the availability of a reporter assay, it should be possible to screen various sources for richer KL content, but without doubt considerable scaling-up of material will be necessary to obtain quantities sufficient for spectroscopic analysis. In addition, the compound(s) in question might be unstable, especially in aqueous conditions, which will impose difficulties during separation and purification steps. Ongoing development and improvement in bioassays that make full use of our knowledge of the molecular mechanisms of KAI2-dependent signalling, combined with modern synthetic biology approaches, will improve the chance of success.

## Concluding remarks

So, what has studying karrikins taught us? Their discovery came about through curiosity-driven investigations into a relatively obscure environmental sensing mechanism: the ability of seeds to detect a recent fire event. We now recognise that karrikins act by hijacking a taxonomically ubiquitous signalling system that regulates diverse aspects of plant development. This system was identified as a direct result of the study of karrikins. Armed with this knowledge, the focus of research in this area will doubtless move towards the wider biological significance and function of 'the karrikin pathway'. Several key questions remain to be addressed. Most prominently, what is the natural ligand(s) for KAI2, how is it synthesised, and how can we exploit it for beneficial use? The broad-ranging effects of the karrikin pathway provide several possible applications of this knowledge, such as enhancing seed germination rates in recalcitrant species, or boosting seedling establishment under stressful conditions. These applications will require a closer examination of physiological roles of KAI2-dependent signalling, under different growth conditions and in diverse species, so that we can understand precisely what aspects of growth might be manipulated. There are also numerous mysteries pertaining to the molecular mechanisms of the karrikin and strigolactone pathways. What are the specific functions of various SMXL proteins, especially SMAX1 and SMXL2? What are the downstream targets of each SMXL, and how are they distinct from each other? Finally, how does the karrikin pathway facilitate the formation of AM symbiosis? Is it also important in other plant–microbe interactions, such as nodulation or pathogenicity? Understanding these processes in particular will be of major agricultural significance. Answering these enigmas will take time and effort across collaborative and multidisciplinary teams. But without doubt, karrikins have made a permanent imprint upon plant chemical biology.

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