

Three mutations repurpose a plant karrikin receptor to a strigolactone receptor

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29 Uncovering the basis of small molecule hormone receptors evolution is paramount to a 30 complete understanding of how protein structure drives function. In plants, hormone 31 receptors for strigolactones are well suited to evolutionary inquires because closely related 32 homologs have different ligand preferences. More importantly, because of facile plant 33 transgenic systems, receptors can be swapped and quickly assessed functionally in vivo. 34 Here, we show only three mutations are required to switch the non-strigolactone receptor, 35 KAI2, into a strigolactone receptor. This modified receptor still perceives KAI2 ligands and 36 does not require receptor hydrolysis for activity. Structural and molecular dynamic 37 modeling suggest receptor pocket flexibility is important for ligand specificity and 38 downstream signaling partner affinity. These findings indicate a few keystone mutations 39 link strigolactone signaling to germination, which explains how parasitic plants that 40 devastate African agriculture evolved SL receptors to sense the presence of a host plant.

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42 Introduction

43 Plants and animals use small molecule hormones to drive growth and development and for this reason much effort is placed on understanding how hormone receptors perceive their ligands^{1,2}. 44 45 Most inquiries are tuned to identify receptor amino acids that contribute to ligand specificity using "loss-of-function" approaches where an amino acid is replaced with a chemically inert 46 moiety, usually an alanine, and binding is assessed^{4,5}. The less explored "gain of function" 47 48 approach involves swapping amino acids between related receptors that respond to different 49 ligands^{6,7}. Gain-of-function approaches have the potential to not only answer questions about 50 receptor-ligand relationships but also can give insights into how receptors evolve. As selection 51 drives changes in the receptor sequence to recognize new ligands, important questions regarding 52 this molecular process can be posed: is this process gradual occurring through many amino acid 53 changes or are only a few keystone substitutions required? When receptors evolve the ability to 54 recognize new ligands, do they lose the ability to bind the ancestral ligands? Gleaning this type of information through gain-of-function approaches, however, has imposing challenges⁸. First, 55 56 amino acids are not discrete entities acting in isolation but are a sea of interdependent residue. As 57 a result, swapping amino acids into a new context often can result in a non-functional protein due 58 to negative epistasis. Second, depending on the number of potential swaps, the number of variants can quickly scale limiting analysis to simple *in vitro* assays rather than more complex *in vivo* analysis.

61 With this said, the perception of the plant hormone strigolactone (SL) may be a useful 62 system to understanding the evolution of small molecule receptors. The root parasite Striga hermonthica (Striga), for example, uses a group of α/β 63 hydrolases designated HYPOSENSITIVE TO LIGHT (ShHTL) to perceive host-derived SLs allowing the parasite to 64 coordinate its germination with the lifecycle of a host plant⁹. By contrast, the homologous 65 66 protein, HYPOSENSITIVE TO LIGHT/KARRIKIN INSENSITIVE 2 (KAI2), which is also involved in germination in the model plant Arabidopsis thaliana (Arabidopsis), is not considered 67 an SL receptor^{10,11}. Plants only produce SLs with a 2^{R} enantiomeric configuration in D-ring but 68 this enantiomer is not recognized by KAI2 (Supplementary Fig. 1)^{12,13}. In Arabidopsis, (2'R)-69 70 SLs are recognized by a more distantly related α/β hydrolase, DWARF14 (AtD14), a receptor that is only involved in vegetative growth^{14,15}. Instead KAI2 responds to a collection of smoke-71 derived butenolides collectively called karrikins (KARs)¹⁶, and strangely is activated by non-72 natural (2'S)-SL enantiomers (Supplementary Fig. 1)¹³. This has led to suggestions that KAI2, 73 74 like ShHTL receptors, positively regulates germination not by using naturally occurring $(2^{\circ}R)$ -SLs but by perceiving an unidentified butenolide-based ligand, designated KAI2-ligand (KL)¹⁷. 75 76 Because parasitic lifestyles are derived from nonparasites, this implies that ShHTL parasitic receptors evolved the ability to perceive (2^{R}) -SLs from ancestor KAI2 receptors that bind KL¹⁸. 77

78 Structural analysis of KL and SL receptors generally conclude that ShHTL receptors have 79 larger binding sites with a broader set of active site amino acids allowing for greater responsiveness to an array of $(2^{r}R)$ -SL molecules, a trait thought to increase Striga host range¹⁹. 80 81 There are also suggestions that receptor affinity to downstream signaling partners also contributes to ligand responsiveness²⁰. The separation of ligand specificity versus partner affinity 82 83 helps to resolve the conundrum of how Striga SL receptors show decreased specificity but 84 increased sensitivity to the same ligand. These conclusions, however, are based solely on *in vitro* experiments because Striga is an experimentally intractable genetic system²¹. Fortunately, 85 ShHTL receptors function in Arabidopsis mutants deficient in KAI2 function and under specific 86 conditions confers seed germination sensitivity to SLs^{11,22}. Furthermore, model plants like 87 *Arabidopsis*, unlike their animal counterparts, are easily transformed²³ making high throughput 88 analysis at the whole organism level feasible. This cross-species functionality means 89

homologous-substitution mutagenesis between KL-based KAI2 and SL-based ShHTL receptors
 can be performed to probe evolutionary questions of ligand sensitivity, specificity and
 promiscuity in an *in vivo* context.

93 With this in mind, here we systematically substituted key amino acids from the highly 94 responsive ShHTL7 SL receptor at homologous positions in KAI2 and assayed their ability to 95 sense SLs in vivo. We show using both yeast- and plant-based assays, that only three amino acid 96 changes were required to repurpose KAI2 into an SL receptor that recognizes (2'R)-SL 97 enantiomers. The emergent properties of this chimeric KAI2 receptor does not appear to require 98 loss of KL perception. Furthermore, ligand perception does not require receptor hydrolysis. 99 Structural and molecular dynamic modeling indicates substitution of three active site amino acids 100 from ShHTL7 into KAI2 produces a larger more flexible binding pocket that better 101 accommodates SLs including $(2^{2}R)$ -SL enantiomers and most likely allows better interactions 102 with downstream signaling partners. The relatively simple mutational path to convert KAI2 into 103 a SL receptor together with the central role of KAI2 in non-parasitic plant germination has 104 implications with respect to the molecular mechanism of how Striga co-opted this regulator to be 105 a germination sensor of host-derived SLs.

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107 Results

108 **Identifying amino acids involved in SL binding.** KAI2 and ShHTL receptors share β-sheet cores flanked by α -helices with their ligand binding pockets covered by a V-shaped cap²⁴⁻²⁷. 109 110 Within the binding pocket is a catalytic triad of amino acids that is involved in ligand hydrolysis^{9,27-29}. To identify important amino acid differences between a KL and an SL receptor 111 112 we compared amino acid sequences within the ligand binding pocket of KAI2 to the Striga SL receptor, ShHTL7, which both have experimental 3D structures available^{11,22}. Because the larger 113 ShHTL7 binding pocket can accommodate $(2^{\circ}R)$ -SLs^{19,20}, we focused on ShHTL7 residues that 114 115 were less bulky and/or more polar than their homologous counterparts in KAI2 (Supplementary 116 Fig. 2). This identified eight ShHTL7 amino acids (Leu26, Tyr124, Thr142, Leu153, Thr157, 117 Tyr174, Thr190, Cys194) that we substituted into the equivalent homologous position in KAI2 in 118 all single, double and triple combinations to generate 92 chimeric KAI2 gene variants. 119

120 Three substitutions change KAI2 responsiveness to SL. To systematically test the 92 chimeric 121 KAI2 receptors, we developed two in vivo high throughput SL receptor responsive assays. KAI2 interacts with its signaling partner MORE AXILLARY MERISTEMS 2 (MAX2)³⁰ making this 122 interaction a good candidate for a yeast two-hybrid assay (Y2H). KAI2, however, autoactivates 123 in Y2H assays in some conditions³¹ and MAX2 is also unstable in yeast¹⁹. Therefore, we 124 125 developed Y2H growth conditions where KAI2 did not autoactivate (see Supplementary 126 Materials & Methods) and fragmented MAX2 into nine constructs (N1-N9) to identify a 127 fragment that produced a positive Y2H signal in the presence of a SL (Supplementary Fig. 3). 128 Although KAI2 (Var1) and the chimeric receptor variants (Var2-Var93) showed no or poor 129 interactions with most MAX2 fragments, the N3 fragment interacted with some chimeric 130 receptors to varying degrees, in the presence of a racemic mixture $(2^{\circ}R, 2^{\circ}S)$ of the artificial SL, 131 rac-GR24 (Fig 1a). With a cut off of four standard deviations above the mean interaction 132 intensity this experiment identified seven variants (Var21, 37, 62, 64, 65, 66, 67) that showed 133 rac-GR24-dependent N3 interactions (Fig. 1b). Six of these variants contained a ShHTL7 amino 134 acid substitution at position 190 and four variants contained substitutions in positions 124 or 157 135 suggesting that these amino acids influence the rac-GR24-dependent interactions in yeast 136 (Supplementary Fig. 3). The identification of amino acids positioned at 124, 157 and 190 is consistent with *in vitro* studies that show these amino acids are important for SL binding^{19,20}. 137

138 Parallel to our Y2H assays, we evaluated all 92 of the chimeric KAI2 receptors by transforming them into Arabidopsis deficient for KAI2 function $(htl-3)^{30}$. Using an SL-dependent 139 germination assay involving GA-depleted seeds¹¹, we found the one line, Var64 (Trp153Leu, 140 141 Phe157Thr, Gly190Thr), germinated well upon rac-GR24 addition (Fig. 1a). Subsequent analysis of three independent transgenic lines (64A, 64B, 64C) showed 50 percent germination 142 (EC₅₀) at high nanomolar concentrations of GR24_{rac} (Fig. 1c). Interestingly, Var19 lines 143 144 (Trp153Leu, Gly190Thr), which contains a subset of Var64 amino acids, showed the next 145 highest level of rac-GR24-dependent germination in our in planta assay (Fig. 1a, 146 Supplementary Fig. 4). Notably, the Var64 receptor variant was also identified in our Y2H 147 assay, indicating that two independent assays showed that substitution of Leu153, Thr157 and 148 Thr190 into KAI2 greatly improved its responsiveness to rac-GR24.

The identification of Leu153, Thr157 and Thr190 as having roles in SL perception encouraged us to determine the prevalence of these amino acids in KAI2-related proteins across 151 a large collection of land plants. Focusing on clades of receptors that are not expected to bind SLs (conserved) versus clades of receptors that are expected to bind SLs (divergent)¹⁸ we found 152 153 conserved clades were mostly devoid of ShHTL7-related amino acids whilst the divergent clades 154 were highly populated with ShHTL7-related residues (Fig. 2). Divergent KAI2 proteins were 155 particularly enriched for 153Leu or hydrophobic amino acids at this position, and proteins more 156 closely related to ShHTL7 possessed similar amino acids at positions 157Thr and 190Thr (Fig. 157 2). Interestingly, within the divergent group, the one clade of proteins depleted of ShHTL7 158 amino acid identities were most closely related to receptors ShHTL10 and ShHTL11 (e.g. 159 SaKA12d2, 10, 11, 16, ShKA12d10, 11) which do not have a role in germination as assayed by Arabidopsis²². Together, the distribution of ShHTL7 amino acids across land plants suggests that 160 161 our in vivo screening identifies important amino acids involved in the evolutionary switch of 162 KAI2 to an SL receptor. Finally, the observation that both our assays led to the Var64 amino acid 163 combination suggests that the simpler and less time consuming yeast-based protein interaction 164 assays may be a good first screen before plant-based assays.

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166 KAI2 variant 64 recognizes natural occurring (2'R)-SL. The SL responsiveness of Var64 seed may be due to increased sensitivity to either or both enantiomers of $GR24_{rac}$. To 167 168 differentiate between these possibilities, we measured the germination response of three 169 independent Var64 lines (64A, 64B, 64C) to either (2'S)- or (2'R)-GR24 in our GA-depletion assay¹¹. Compared to misexpressed KAI2, the Var64 lines showed more sensitivity to 2'S-GR24 170 171 but importantly showed responsiveness to $(2^{2}R)$ -GR24, a property not present in the wild type 172 KAI2 receptor (Fig. 3a). We also noticed that compared to KAI2 seed, Var64 lines germinated at low levels, even in the absence of rac-GR24_{rac}, suggesting a weakly constitutive germination 173 174 phenotype in the absence of SL addition (Fig. 3a). Suppressor analysis in rice identify GID1 α/β 175 hydrolase GA receptor mutants with weak GA constitutive phenotypes that are enhanced by GA addition³². This is thought to be due to alterations in the conformation of the receptor's lid 176 subdomain that increases its affinity for interactions with its downstream signaling partners³². By 177 178 analogy, ShHTL7 amino acids may alter the active site of KAI2 to a conformation closer to the 179 "on state", which may improve downstream protein interactions. Consistent with this, the positions 153 and 157 are important in MAX2 and SMAX1 interactions with ShHTL7 in vitro²⁰. 180

181 To biochemically characterize the molecular mechanism of Var64 signalling, we first measured hydrolysis activity of Var64 variant receptors using the YLG assav⁹. Var64 receptors 182 183 showed weak YLG hydrolysis activity compared to WT KAI2 enzyme, but this activity was 184 inhibited by addition of rac-GR24_{rac}, indicating that SL indeed bind the variant receptor (Fig. 185 **3b**). To more directly measure SL hydrolysis we monitored D-ring production by HPLC from 186 hydrolysis of either (-)-(2'S)- or (+)-(2'R)-GR24 and again found neither GR24 enantiomer was 187 a substrate compared to KAI2, which has a preference for (2'S)-SLs, or ShHTL7, which has a 188 preference for (2'R)-SLs (Fig. 3c). In summary, although Var64 seed showed improved 189 germination on $(2^{\circ}S)$ -GR24 and an emergent ability to germinate on the $(2^{\circ}R)$ -GR24 the Var64 190 protein at best, has poor hydrolysis activity for these two enantiomers. This suggests this receptor 191 variant functions in response to SL in vivo but with minimal hydrolase activity.

192

193 KAI2 variant 64 responds to karrikins. Because the identity of KL is unknown, we cannot 194 directly assess its binding to our variant receptors. However, KL also has a role in Arabidopsis leaf development³³, which means rescue of the htl-3 leaf mutant phenotype by variant receptors 195 196 in transgenic plants can be used as a readout of endogenous KL binding to its receptor. Under 197 our growth conditions, *htl-3* leaf length to width ratios are smaller (≤ 1.85) versus wild type 198 leaves (≥ 1.92) and this difference can be used to quantify the degree to which chimeric variants 199 can complement *htl-3* leaf shape defects (Supplementary Fig. 5). Generally, higher order 200 mutant lines showed less rescue of *htl-3* leaf mutant phenotype than lower order mutants, which 201 could reflect a loss of endogenous KL recognition (Fig. 1a). This pattern, however, may also be 202 due to increasing negative epistasis as newly introduced amino acids pile up resulting in nonfunctional proteins⁸. We noticed, however, that triple substitution variant lines that carried a 203 204 ShHTL7 amino acid at position 190 complemented *htl-3* leaf defects more often than other triple 205 mutant or even double mutants lacking this substitution (Fig. 1a). Deep sequencing analysis 206 suggest that amino acid substitutions can be neither positive nor negative in terms of protein 207 function, but simply stabilize proteins and allow for a larger number of substitutions to be tolerated during evolution³⁴. Perhaps, Threonine at position 190 is a permissive substitution that 208 209 opens alternative mutational paths for ligand recognition. With this said, Var64 lines, which 210 contain a Thr190 substitution, complemented *htl-3* leaf defects suggesting that this chimeric 211 receptor retained an ability to recognize KL (Fig. 1a). Although this cannot be directly tested,

Var64 seed germinated on KAR_2 to a similar level seen for KAI2 seed, indicating that this chimeric variant retains the capacity to recognize a native KAI2 ligand.

214

215 KAI2 variant 64 protein-ligand dynamics. Structural comparisons of the Var64 protein model 216 to a wild type KAI2 crystallographic structure (PDB 4IH1^{xx}) showed that replacing KAI2 217 aromatic residues Trp153 and Phe157 with Leu and Thr, disrupted several hydrogen bond 218 interactions that would occur between the αB , αC , and αD helices of the lid domain (Fig. 4a, b). This change would increase the pocket mouth area from 45.5 \AA^2 to 57 \AA^2 , which would allow for 219 larger substrates, such as GR24, to access the active site of the receptor more easily. These 220 221 disruptions would also result in the increase of the overall pocket solvent-accessible volume by approximately 140 Å³ (Fig. 4a, b). Larger binding pockets should increase ligand accessibility 222 223 but to explore this in more detail we modeled $(2^{2}R)$ -GR24 ligand-receptor interactions using 224 molecular dynamics (MD) simulations. Unlike traditional structural docking analysis, which is a 225 static snapshot of the protein structure, MD simulations take into account the temporal evolution 226 and velocities of all of the atoms of a protein averaged over a specific period of time to give a 227 more realistic atomic-level detail of the protein. MD simulations of Var64 and KAI2 receptors in 228 the presence of $(2^{\circ}R)$ -GR24, as well as their apo states, were conducted in triplicate 10 ns 229 simulations to evaluate the dynamics of these ligand-receptor complexes in contrast with the 230 unbound receptor, calculating the number and distance of hydrogen bonds that form between 231 receptor and ligand, and measuring the fluctuation of the ligand positions throughout the 232 simulation. To quantitatively distinguish between ligand binding states, we defined productive 233 modes to include states in which $(2^{\circ}R)$ -GR24 was close to the receptor (≤ 5.0 Å) and its surface 234 area was mostly covered by the active site of the protein. The unproductive mode incorporates instances where the ligand was further away from the pocket opening (5.1-7.0 Å) and not 235 236 interacting with the active site. Finally, a dissociated mode comprised states where $(2^{2}R)$ -GR24 237 was far from the protein (\geq 7.5 Å) thus any interactions are very unlikely. Using these criteria, 238 (2'R)-GR24 was in a productive binding mode with the Var64 receptor almost 50% of the 239 simulation time versus only 2.1% for KAI2 (Fig. 4c). Furthermore, when docked on KAI2, 240 (2'R)-GR24 spent approximately 65% of the simulation time in a dissociated mode versus 10% 241 for Var64. Finally, we calculated the magnitude of location change of the position of $(2^{2}R)$ -GR24 242 on the two receptors. For a productive ligand-receptor interaction, a minimal fluctuation is

expected, as the ligand is tightly bound until it is metabolized or released. The simulations showed that the position of $(2^{2}R)$ -GR24 in KAI2 fluctuates between 0.02 and 0.18 nm once initially bound to the protein, by contrast, this oscillation is greatly reduced in the Var64 protein, confirming the ability of the new variant to form a stable interaction with an SL ligand (**Fig. 4d**).

247 Visualization of the trajectory of the MD simulations revealed that the motility of the αE 248 loop of unbound Var64 is increased, consistent with our model of increased flexibility of the 249 receptor's active site and the expansion of the cavity depth and volume (Supplementary Movie 250 1). The trajectories also show three main changes between bound and unbound receptors. First, 251 the hydrophobicity of the binding domain of Var64 was slightly reduced throughout the 252 simulation after binding of $(2^{r}R)$ -GR24, which is qualified by the change in coloration from 253 orange to blue in the binding domain region. Second, $(2^{2}R)$ -GR24 appears to be more attracted 254 by the polar ShHTL7 Thr190 in Var64 than the KAI2 Gly190. Possibly, this substitution is the 255 reason for the higher ligand stability provided by Var64. Lastly, we observe a conformational 256 change of the αD helix of Var64 upon binding to $(2^{2}R)$ -GR24 (Supplementary Movie 2). This 257 conformational change is expected in the SL perception pathway, as SL bound receptors have 258 been shown to adapt the conformation of the αD helix to accommodate interactions with its downstream partners^{20,35,36}. 259

260

261 Discussion

262 Structurally guided mutational analysis is an effective approach to dissecting how a protein's 263 sequence contributes to biochemical activity, but how these biochemical properties evolve is more experimentally challenging⁸. From an evolutionary perspective, loss-of-function 264 265 approaches are not necessarily informative and often experiments on protein evolution are not 266 performed in an *in vivo* context which allows proteins to interact with other cellular constituents 267 and processes. In this study, we used a gain-of-function approach with active site amino acid 268 targeted substitutions informed by structural and evolutionary considerations to determine if a 269 non-parasitic plant orphan receptor, KAI2, that is incapable of recognizing natural SL ligands 270 harnessed by parasitic plants, could be converted into a receptor that recognizes such ligands. 271 Importantly, we assayed these receptor variants using yeast- and Arabidopsis-based systems to 272 evaluate receptor function under in vivo conditions.

273 Our approach unambiguously determined that only three amino acid substitutions were 274 necessary to convert KAI2 into a functional SL receptor. By introducing smaller, more polar 275 amino acids into KAI2, we produced a protein with a more flexible pocket and lid; this affects 276 receptor function in two ways. First, it increases the pocket volume to accommodate natural SL 277 conformations like that of (2R')-GR24. Second, it increases the overall elasticity of the protein, which may allow better interactions with signaling partners 20,35,36 . To this end, the identification 278 279 of positions 153, 157 and 190 in our in vivo experiments is consistent with the in vitro conclusions about these amino acids^{19,20}. Our in vivo findings contribute to discussions 280 281 concerning the role of gradual versus keystone mutations in small molecule receptor evolution. 282 Animal studies using ancestral gene reconstructions involving steroid receptors show as few as two mutations can switch ligand specificity³⁷. Our results now extend the importance of keystone 283 284 mutations to plant hormone receptor evolution.

285 General models of hormone evolution suggest promiscuous ancestral forms evolve specificity so as to prevent deleterious ligand crosstalk³⁸. Three lines of evidence in this study, 286 287 however, suggest a path to SL perception in KAI2 does not necessarily require loss of 288 responsiveness to previous ligands. First, our variant receptor complements the KAI2 loss-of-289 function phenotype suggesting it still recognizes the endogenous KAI2 ligand. Second, variant 290 receptor seeds are sensitive to (2'S)-GR24 enantiomers, a known substrate for KAI2. Finally, 291 variant lines respond to the KAI2-specific exogenous ligands, karrikins. The ability of variant 292 receptors to respond to both ancestral as well as derived ligands supports the notion that SL 293 perception can evolve through sufficient promiscuity rather than by a narrowing of specificity. 294 The sufficient promiscuity models receptor specificity as being adequate but not overly so to distinguish endogenous ligands³⁹. This "just enough" specificity explains why receptors often 295 296 show sensitivity to drug structures unrelated to their natural ligands. The discovery of an 297 artificial femtomolar responsive ShHTL7 agonist with little similarity to SLs further supports a sufficient promiscuity model for these receptors⁴⁰. 298

It also appears that the tight connection between SL hydrolysis and SL perception is not necessary as Var64 variant seed responded to GR24 enantiomers as the Var64 protein showed little hydrolase activity. The role of ligand hydrolysis in SL signaling is somewhat contentious with structural studies producing conflicting models^{41,42}. Chemical biology suggests hydrolysis is important to signaling sensitivity but again may not be essential^{30,40}. Finally, mutational analysis

of the D14 receptor in *Arabidopsis* indicates hydrolysis is both necessary²⁹ and not necessary⁴² 304 305 for SL signaling. Although our results support a less direct connection between of hydrolysis and 306 signaling, our variant receptor most likely does not reflect the natural progression by which 307 KAI2 genes evolved to become SL receptors in Striga. Our KAI2 variants were not under the 308 constant constraints seen in natural environments that are important for fitness. This may explain 309 why in contrast to the yeast-based interaction assay, we only found one variant with high SL 310 responsiveness in planta. We understand that monitoring KAI2 function using the output of 311 Arabidopsis germination is superior to in vitro based experiments but even this system almost 312 certainly overestimates the frequency of beneficial mutations relative to natural evolution. With 313 this said, the correlation of ShHTL7 153, 157 and 190 amino acids in divergent versus conserved 314 KAI2 is consistent with these residues playing key roles in SL perception. Finally, directed 315 protein engineering studies tell us that enzymes usually possess minor non-selective activities that are often enhanced by laboratory evolution^{44,45}. If true, KAI2 may possess a to-date 316 317 undetected low level of responsiveness to 2'R enantiomers. In vitro, KAI2 only appears to bind (2'S)-GR24²⁹ but *in vivo*, KAI2 can perceive (2'R)-GR24 but only when the SL receptor, D14, is 318 defective⁴⁶, suggesting that the absence of D14 uncovers the latent binding ability of KAI2 for 319 320 2^{R} enantiomers as it would be free from competition for (2^{R}) -SL substrates with endogenous D14. 321

322 The role of KAI2 in germination together with its ability to perceive small molecules 323 makes it easy to understand why this gene was co-opted during the evolution of Striga 324 parasitism. Striga plants not only requires a host for growth but also requires a cue to signal the 325 proximity to a host, and a long-term survival strategy in the absence of a host. Since SLs are needed for beneficial plant root-AM fungi interactions⁴⁷, secretion of these small molecules 326 327 makes it an excellent plant root positional cue. Seeds are an attractive solution to establishing 328 long-term viability in the absence of a host as dormant seeds can survive long periods under a 329 variety of environmental conditions. An unintended agricultural consequence of using dormant 330 seeds as the protective state in the absence of a host is that they easily contaminate soil and are 331 difficult to purge from farmers fields. For these reasons, Striga has contaminated over 20 African nations and is the largest biological impediment to food security on the continent^{48,49}. 332 333 Understanding the steps by which KAI2 evolved to become a SL receptor will give insights not 334 only into the role of this key regulator of the Striga lifecycle but will also help define the

- 335 chemical space of this receptor, which should open leads to new chemical solutions to this
- African scourge.

337	Ref	Verence
338	1.	Evans, R. M. & Mangelsdorf, D. J. Nuclear Receptors, RXR, and the Big Bang. Cell,
339		157 , 255–266 (2014).
340	2.	Lumba, S., Cutler, S. & McCourt, P. Plant nuclear hormone receptors: a role for small
341		molecules in protein-protein interactions. Annu Rev Cell Dev Biol. 26, 445-469 (2010).
342	3.	Morrison ,K. L. & Weiss, G. A. Combinatorial alanine-scanning. Curr Opin Chem Biol.
343		5, 302-307 (2001).
344	4.	Howlader, M.T. et al. Alanine scanning analyses of the three major loops in domain II of
345		Bacillus thuringiensis mosquitocidal toxin Cry4Aa. Appl Environ Microbiol. 76, 860-
346		865 (2010).
347	5.	Zhao, L., et al. Destabilization of strigolactone receptor DWARF14 by binding of ligand
348		and E3-ligase signaling effector DWARF3. Cell Res 25, 1219–1236 (2015).
349	6.	Kobilka, B.K., et al. Chimeric alpha 2-, beta 2-adrenergic receptors: delineation of
350		domains involved in effector coupling and ligand binding specificity. Science 240,
351		1310–16 (1988).
352	7.	Cunningham, B. C., Jhurani, P., Ng, P. & Wells, J. A. Receptor and antibody epitopes in
353		human growth hormone identified by homolog-scanning mutagenesis. Science 243,
354		1330–36 (1989).
355	8.	Hochberg, G. K. A. & Thornton, J. W. Reconstructing Ancient Proteins to Understand
356		the Causes of Structure and Function. Annu Rev Biophys. 46, 247-269 (2017).
357	9.	Tsuchiya, Y., et al. PARASITIC PLANTS. Probing strigolactone receptors in Striga
358		hermonthica with fluorescence. Science. 349, 864-868 (2015).
359	10	Scaffidi, A., et al. Strigolactone Hormones and Their Stereoisomers Signal through Two
360		Related Receptor Proteins to Induce Different Physiological Responses in Arabidopsis.
361		Plant Physiol. 165, 1221-1232 (2014).
362	11.	Bunsick M, et al. SMAX1-dependent seed germination bypasses GA signalling in
363		Arabidopsis and Striga. Nat Plants. 6, 646-652 (2020).
364	12	Yoneyama, K., Xie, X., Yoneyama, K., et al. Which are the major players, canonical or
365		non-canonical strigolactones? J Exp Bot. 69, 2231-2239 (2018).
366	13	Flematti, G. R., Scaffidi, A., Waters, M. T. & Smith, S. M. Stereospecificity in
367		strigolactone biosynthesis and perception. Planta. 243,1361-1373 (2016).

- 368 14. Brewer, P. B., Koltai, H. & Beveridge C. A. Diverse roles of strigolactones in plant
 369 development. *Mol Plant.* 6, 18-28 (2013).
- 370 15. Waldie, T., McCulloch, H. & Leyser, O. Strigolactones and the control of plant
 371 development: lessons from shoot branching. *Plant J.* **79**, 607-622 (2014).
- 372 16. Flematti, G. R., Ghisalberti, E. L., Dixon, K. W. & Trengove, R. D. A compound from
 373 smoke that promotes seed germination. *Science* 305, 977 (2004).
- 374 17. Conn, C. E. & Nelson, D. C. Evidence that KARRIKIN-INSENSITIVE2 (KAI2)
 375 Receptors may Perceive an Unknown Signal that is not Karrikin or Strigolactone. *Front* 376 *Plant Sci.* 6, 1219 (2016).
- 377 18. Conn, C.E., et al. PLANT EVOLUTION. Convergent evolution of strigolactone
 378 perception enabled host detection in parasitic plants. *Science*. 349, 540-543 (2015).
- 379 19. Xu, Y., et al. (2018). Structural analysis of HTL and D14 proteins reveals the basis for
 380 ligand selectivity in *Striga*. *Nat Commun.* 9, 3947 (2018).
- 381 20. Wang et al., *Plant Physiol*. 2020 in press
- 382 21. Spallek, T., Mutuku, M. & Shirasu K. The genus *Striga*: a witch profile. *Mol Plant*383 *Pathol.* 14, 861-869 (2013).
- 22. Toh, S., et al. Structure-function analysis identifies highly sensitive strigolactone
 receptors in *Striga. Science.* 350, 203-207 (2015).
- 23. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for Agrobacterium-mediated
 transformation of *Arabidopsis thaliana*. *Plant J*. 16, 735-743 (1998).
- 388 24. Kagiyama, M., et al. Structures of D14 and D14L in the strigolactone and karrikin
 389 signaling pathways. *Genes Cells.* 18, 147-160 (2013).
- 390 25. Guo Y., et al Smoke-derived karrikin perception by the α/β-hydrolase KAI2 from
 391 *Arabidopsis. Proc Natl Acad Sci U S A.* 110, 8284-8289. (2013).
- 392 26. Shahul Hameed, U., et al. Structural basis for specific inhibition of the highly sensitive
 393 ShHTL7 receptor. *EMBO Rep.* 19, e45619 (2018).
- 394 27. Hamiaux, C., et al. DAD2 is an α/β hydrolase likely to be involved in the perception of 395 the plant branching hormone, strigolactone. *Curr Biol.* **22**, 2032–2036. (2012).
- 396 28. de Saint Germain, A., et al. An histidine covalent receptor and butenolide complex
 397 mediates strigolactone perception. *Nat Chem Biol.* 12, 787–794 (2016).

398 29. Waters, M. T., Scaffidi, A., Flematti, G., & Smith, S. M. Substrate- induced degradation 399 of the α/β -fold hydrolase KARRIKIN INSENSITIVE2 requires a functional catalytic 400 triad but is independent of MAX2. Mol Plant. 8, 814-817 (2015). 401 30. Toh S, et al. (2014). Detection of Parasitic Plant Suicide Germination Compounds Using a High-Throughput Arabidopsis HTL/KAI2 Strigolactone Perception System. Chem 402 403 Biol. 2, 988-998 (2014). 404 31. Yao, J., et al. An allelic series at the KARRIKIN INSENSITIVE 2 locus of Arabidopsis 405 thaliana decouples ligand hydrolysis and receptor degradation from downstream 406 signalling. Plant J. 96, 75-89 (2018). 407 32. Yamamoto, Y. et al. A rice *gid1* suppressor mutant reveals that gibberellin is not always 408 required for interaction between its receptor, GID1, and DELLA proteins. *Plant Cell.* 22, 409 3589-3602 (2010). 410 33. Waters, M. T., et al. Specialisation within the DWARF14 protein family confers distinct 411 responses to karrikins and strigolactones in Arabidopsis. Development. 139, 1285-1295 412 (2012).413 34. Starr, T. N. & Thornton, J. W. Epistasis in protein evolution. Protein Sci. 25, 1204-1218 414 (2016). 415 35. Bürger, M., et al. Structural Basis of Karrikin and Non-natural Strigolactone Perception 416 in Physcomitrella patens. Cell Rep. 26, 855-865 (2019). 417 36. Lee, H. W., et al. Flexibility of the petunia strigolactone receptor DAD2 promotes its 418 interaction with signaling partners. J Biol Chem. 295, 4181-4193 (2020). 419 37. Harms, M. J., et al. Biophysical mechanisms for large-effect mutations in the evolution 420 of steroid hormone receptors. Proc Natl Acad Sci USA. 110, 11475-11480 (2013). 421 38. Tawfik, D. S. Messy biology and the origins of evolutionary innovations. *Nat Chem Biol* 422 6, 692–696 (2010). 423 39. Eick, G. N., et al. Evolution of minimal specificity and promiscuity in steroid hormone 424 receptors. PLoS Genet. 8, e1003072 (2012). 425 40. Uraguchi, D., et al. (2018). A femtomolar-range suicide germination stimulant for the 426 parasitic plant Striga hermonthica. Science. 362, 1301-1305 (2018). 427 41. Yao, R., et al. (2016). DWARF14 is a non-canonical hormone receptor for strigolactone. 428 *Nature* **536**, 469–473 (2016).

- 429 42. Shabek, N., et al. Structural plasticity of D3-D14 ubiquitin ligase in strigolactone
 430 signalling. *Nature*. 563, 652-656 (2018).
 431 43. Seto, Y., et al. Strigolactone perception and deactivation by a hydrolase receptor
 432 DWARF14. *Nat Commun.* 10, 191 (2019).
- 433 44. Bloom JD, Arnold FH (2009) In the light of directed evolution: pathways of adaptive
 434 protein evolution. *Proc Natl Acad Sci U S A* **106** Suppl 1, 9995–10000.
- 435 45. Khersonsky O, Tawfik DS (2010) Enzyme promiscuity: a mechanistic and evolutionary
 436 perspective. *Annu Rev Biochem* **79**, 471–505.
- 437 46. Villaecija-Aguilar, J. A., et al. (2019) SMAX1/SMXL2 regulate root and root hair
 438 development downstream of KAI2-mediated signalling in *Arabidopsis*. PLoS Genetics,
 439 15 (8) (2019).
- 440 47. Akiyama, K., Matsuzaki, K. & Hayashi, H. Plant sesquiterpenes induce hyphal
 441 branching in arbuscular mycorrhizal fungi. *Nature*. 435, 824-827 (2005).
- 442 48. Ejeta, G. (2007) The *Striga* scourge in Africa: A growing pandemic. Integrating New
 443 Technologies for *Striga* Control, pp. 3-16
- 444 49. Parker, C. Observations on the current status of *Orobanche* and *Striga* problems
 445 worldwide. *Pest Manag. Sci.* 65, 453–459 (2009).

446 Figure 1. Identification of a variant KAI2 that perceives SL. a. Heatmap representation of 447 SL-dependence of the 93 KAI2 chimeric variants. Var1 is the KAI2 wild type control. Lanes 1-2; 448 Yeast two hybrid assays where increased purple shading represent an increased interaction 449 intensity on rac-GR24 relative to the DMSO control for KAI2 chimeric variants queried against 450 the N3 MAX2 fragments (Supplementary Fig. 3). Interaction strengths were quantified using 451 colony color intensity after 72 hours. Representative colonies for Var27 and Var64 are shown on 452 the left inserts. Lanes 3-4; the mean germination percentage of three independent KAI2 chimeric 453 variants lines on PAC (20 µM), DMSO (0.1%) or rac-GR24 (1 µM). Lane 5-12; Graphic 454 representation of 93 insertion lines. Each blue box represents the amino acid position in KAI2 455 where a ShHTL7 amino acid is substituted. Lane 13; degree of rescue of the htl-3 leaf shape 456 phenotype by each KAI2 chimeric variant (Supplementary Fig 5). Colors are from 0% rescued 457 (yellow) to 100% rescued (dark green). Grey indicates that the variant was not available. 458 Representative pictures of htl-3, 35S::KAI2, Var19 and Var64 plants. Are shown on the right 459 inserts. **b**. Scatterplot showing the log-fold change scores calculated for each KAI2 chimeric 460 variant with full-length protein, empty vector control and MAX2 fragments using a Y2H protein 461 interaction assay (see Supplementary Fig. 3). 22 scores were calculated for each variant. Red 462 dotted line represents four standard deviations from the mean. c. SL dose response germination 463 curves. Percent germination of mis-expressed ShHTL7, KAI2 and three independent Var64 seed 464 (64A, 64B, 64C) on PAC (20 µM) and increasing rac-GR24 concentrations. Four-parameter 465 logistic curves were fitted to the data, and EC₅₀ values, \pm standard error, of rac-GR24 466 concentrations have been included for those lines showing response to SL. Bases on these curves 467 the effective concentration that germinates 50% of seed EC_{50} was calculated and is shown in the 468 box.

469

Fig. 2. Conservation of Var64 amino acids in land plants. a. Phylogenetic tree built using *KAI2/DLK/DDK* sequences from across the land plant phylogeny, rooted using *KAI2* sequences from hornworts. Sequences within each of the three coloured clades were investigated in more detail for their similarity with variant 64 at positions 153, 157 and 190. b. *KAI2* conserved sequences from angiosperms. c. *KAI2* divergent sequences from parasitic Orobanchaceae. Coloured boxes beside the clades indicate amino acid similarity with variant 64 at positions 153 476 (blue), 157 (green) or 190 (purple). James, we need a list of what the similar amino acids are
477 for each catagory!

478

479 Figure 3. A. Var64 responds to both GR24 enantiomers a. Percent germination of htl-3, mis-480 expressed KAI2 and three independent Var64 seed lines (64A, 64B, 64C) on PAC (20µM) with 481 DMSO, 1 μ M (-)-(2'S)-GR24 or (+)-(2'R)-GR24. **b**. SL inhibits YLG hydrolysis. Competitive 482 binding assay using YLG and rac-GR24 to evaluate the changes in YLG hydrolysis by KAI2 483 (black) and Var64 (blue) after incubation with 0.5 µM rac-GR24. Error bars represent SD values 484 from three independent experiments. c. Hydrolysis activity towards GR24 enantiomers. UPLC-485 UV (260 nm) analysis was used to detect the remaining amount of GR24 isomers. Box plots of 486 n=3 replicates represent the hydrolysis rate calculated from the remaining GR24 isomers and 487 analogs taking into account the hydrolysis in the buffer alone (non protein sample), quantified 488 using indanol as internal standard. Letters indicate different statistical groups (ANOVA, post-hoc 489 Tukey test). d. KAR₂ dose response germination curves. Mis-expressed ShHTL7, KAI2 and three 490 independent Var64 seed (64A, 64B, 64C) on PAC (20 µM) and increasing KAR₂ concentrations. 491 Four-parameter logistic curves were fitted to the data, and EC_{50} values, \pm standard error, of 492 KAR₂ concentrations have been included for those lines showing response to karrikin. Based on 493 these curves the effective concentration that germinates 50% of seed (EC_{50}) was calculated and is 494 shown in the box. Alex I need your data to be converted to box plots and I only need the 495 data for GR24. Also I need the stats included in the figure.

496

497 Figure 4. Var64 derived amino acids create a SL binding domain. a. The substitution of 498 KAI2 aromatic residues for ShHTL7 equivalents results in the disruption of several interactions 499 (purple dashed lines) along the X-axis of the protein. This disruption translates into increased 500 motility of the lid domain (Supplemental Movie 1), consistent with the receptor's new ability to 501 accommodate SL molecules. b. Pocket alignment of KAI2 and Var64 receptors reveals an 11.5 502 Å larger pocket mouth (orange shading) and a 29.7% increased pocket volume in the Var64 receptor. The pocket mouths are shown in yellow shading with width shown in $Å^2$. c. A 503 504 summary of the three most common binding states between Var64, KAI2, and 2'R-GR24. 505 Molecular dynamic simulations of Var64 and KAI2 receptors to determine binding modes of 506 ligand-protein complexes over a 10 ns simulation. Var64-(2'R)-GR24 complex was found in a 507 productive binding mode 47.8% of the time whist the same state was only found 2.1% of the 508 time for the KAI2 complex. **d.** Calculation of the magnitude change in the pose of the ligand 509 throughout the MD simulation. Both proteins stabilized the ligand after 1 ns of simulation, but its 510 position on the KAI2 pocket is compromised from the 4 ns mark as the interaction is not strong 511 nor stable.



b

htl-3

Figure 1





Figure 2



Variant 64

190 157 153

- PjKAl2d3 TvKAI2d2

PjKAI2d2

TvKAI2d3

PjKAl2d1

PaKAI2d2

PaKAI2d4

PaKAI2d1 PaKAI2d3

OceKAI2d2 OcKAI2d2

OcKAI2d4

-OmKAI2d1

OcKAl2d1

- OcKAl2d6

OceKAI2d3

OceKAI2d1

OcKAI2d3

OmKAI2d4

OmKAI2d2

TvKAl2d1

PjKAl2d4

PjKAl2d5

SaKAI2d17

ShKAI2d7

ShKAI2d8

ShHTL11

SaKAI2d12

SaKAI2d10

SaKAI2d11

SaKAI2d16

ShKAI2d10

ShHTL10

ShKAI2d2

ShHTL6 - SaKAI2d5

SaKAI2d9

ShHTL5

ShHTL4

SaKAI2d8 SaKAI2d2

ShHTL9

SaKAI2d1

- SaKAI2d14

SaKAI2d15 ShHTL8

SaKAI2d13

ShHTL7

SaKAI2d3

SaKAI2d4 - SaKAI2d7

ShKAI2d11

SaKAI2d6

OmKAI2d3

OmKAI2d5

OfKAI2d OcKAI2d5

Figure 3









d









Supplementary Fig. 1. Structures of 2'*R*- and 2'*S*-enantiomeric forms of strigolactones (SLs) and karrikin (KAR₂). For SLs the synthetic GR24 is shown and the 2' chirality is at the enol-ether bridge between the C and D-ring. For karrikin KAR₂ is shown.



Catalytic triad Polar side chain Non Polar side chain Electrically charged side chain **Supplementary Fig. 2 Selection of ShHTL7 residues for substitution in KAI2. a.** The choice of the eight substitutions (pink boxes) were made based on increased polarity and decreased size (blue amino acids) in ShHTL7 versus the KAI2 equivalent. Red boxes represent the three catalytic residues. **b.** Chemical structures of the amino acids at the positions substituted in KAI2 to generate JGI variants. Structures in black represent the amino acids present at these positions in KAI2, structures in red represent their counterparts in ShHTL7. Letter codes and numbers below the arrows denote the substitutions that were made to produce the JGI variants **c.** Crystal models representing the eight residues highlighted in pink, The three catalytic residues highlighted in red, Structures are visualized using PYMOL version 2.3.2.



С

Supplementary Fig. 3 Interactions of KAI2 chimeric variants with MAX2 fragments using yeast-two-hybrid (Y2H) (a) Map of MAX2 fragments (N) used as Y2H preys. (b) Heatmap of log-fold change scores based on Y2H interactions relative to DMSO conditions for 1-, 2-, and 3-substitution variants queried against all MAX2 fragments. EV, empty vector, F, full-length protein. Redder shading indicates increased interaction. Grey lines represent construct that could not be cloned into Y2H vectors (c) Statistically significant hits in Y2H screen.





Supplementary Fig. 4 Var19 germination on SL. Mis-expressed *ShHTL7*, *KAI2* and three independent Var19 lines (19A, 19B, 19C) on PAC (20μ M) plus or minus Gr24_{rac} concentrations. Each line was tested three times Bar = S.D



Supplementary Fig. 5 Standard distribution of length/width (major/minor) ratio of *htl-3* and wild type Columbia (WT) leaves. Each distribution was a normal distribution based on the population mean (μ) and standard deviation (σ) approximated by that of the 26 control samples. WT: $y=(1/(0.53332 \cdot \sqrt{(2\pi)})) \cdot e^{-(x-2.7996)^2/(2 \cdot (0.53332)^2)}; htl-3$: $y=(1/(0.2261 \cdot \sqrt{(2\pi)})) \cdot e^{-(x-1.48)^2/(2 \cdot (0.2261)^2)}.$ Three categories given by 5% cutoff: *htl-3*: x<1.85; WT: x>1.92; ambiguous: 1.85 < x<1.92. Based on the two distributions, in order to meet the 0.05 significance level, any sample data smaller than 1.85 would be considered as *htl-3*, while sample data larger than 1.92 would be considered as wild type. Note that there is an ambiguous region in between (1.82-1.92) where the phenotype can not be statistically determined in this case.