1 **Title:**

2 **Rice perception of symbiotic arbuscular mycorrhizal fungi requires the karrikin**
3 **receptor complex**

4

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32 **Abstract:**

33 **In terrestrial ecosystems plants take up phosphate predominantly via association**
34 **with arbuscular mycorrhizal fungi (AMF). Here we identified loss of responsiveness to**
35 **arbuscular mycorrhizal fungi in the rice mutant *hebiba*, reflected by the absence of**
36 **physical contact and of characteristic transcriptional responses to fungal signals.**
37 **Among the 26 genes deleted in *hebiba,* the one responsible for loss of symbiosis encoded**
38 **the alpha/beta fold hydrolase, *DWARF 14 LIKE*, a component of an intracellular**
39 **receptor complex involved in the detection of the smoke-compound karrikin. Our**
40 **finding reveals an unexpected plant recognition strategy for AMF and a novel signaling**
41 **link between symbiosis and plant development.**
42
43
44 **One sentence summary:**
45 Widely beneficial symbiosis between plant and fungi shares signaling components with
46 wildfire ephemerals.
47
48
49 Most land plants establish symbioses with arbuscular mycorrhizal fungi (AMF) of the
50 phylum *Glomeromycota* (*1*). These symbioses contribute to global carbon and mineral
51 nutrient cycles, because AMF provide mineral nutrients to the plant and receive
52 carbohydrates in return. Colonization of plant roots by AMF requires reciprocal
53 recognition initiated by diffusible molecules before fungal attachment to the root surface
54 and root penetration *via* hyphopodia (*2*). Diffusible pre-colonization signals include
55 strigolactones released from plant roots that activate the fungus before physical
56 interaction (*3*), and fungal (lipo)chito-oligosaccharides (LCOs) and chitotetraose (CO4)
57 secreted by AMF that trigger plant calcium signaling, gene expression and lateral root
58 formation (*4, 5*). Plant LysM receptor-like kinases (RLKs, *6*) are required for perception
59 of chitinaceous microbial molecules that trigger either symbiosis or defense signaling (*7),*
60 but are not indispensable for fungal colonization *(8, 9*). Plant signaling mutants impaired
61 in root colonization by both AMF and nitrogen-fixing bacteria still exhibit transcriptional
62 responses to fungal signaling molecules (*10-12*). Therefore, additional signaling modules

63 have been postulated *(12)*. Here we identify the rice receptor for karrikin, a plant growth
64 regulator first identified in smoke (*13-16*), as a necessary signaling component for
65 establishment of AM symbiosis.
66
67 We found that the jasmonate-deficient rice mutant *hebiba* (*17*) was unable to
68 establish symbiosis with either of two AMF *Rhizophagus irregularis* and *Gigaspora*
69 *rosea* as reflected by the absence of hyphopodia, intraradical colonization and induction
70 of colonization marker genes (Fig. 1A-C, *10*). The lack of fungal interaction persisted
71 upon increased inoculum strength imposed by growing *hebiba* alongside colonized wild
72 type plants (Fig. 1D). This suggested that the mutant is compromised at a very early stage
73 of the interaction, during pre-symbiotic signaling.
74 The *hebiba* mutant is due to a genomic deletion of 169 kb, which contains 26
75 annotated genes (*17, 18*). One of the genes encodes *Allene Oxide Cyclase* (AOC), part of
76 the jasmonate biosynthetic pathway, loss of which leads to jasmonate deficiency *(17)*.
77 However, transgenic complementation of *hebiba* with AOC (*hebiba*^{AOC}) did not restore
78 AM symbiosis (fig. S1, *17*, *19*). Therefore, another gene contained within the deleted
79 interval must be required for AM development.
80 We identified the gene responsible for AM symbiosis by transforming *hebiba*^{AOC}
81 with genomic clones of individual genes from the deleted interval (tab. S1, *17, 18*).
82 Reintroduction of the *LOC_Os03g32270* gene restored fungal colonization of *hebiba*^{AOC}
83 roots in independent rice transformants (Fig. 2A and B, tab. S1). Quantitative
84 measurements of colonization correlated (R^2=0.84) with the amount of transcript
85 accumulation from the *LOC_Os03g32270* transgene (Fig. 2C). Transgenic lines such as
86 C10 (Fig. 2B) with transgene mRNA levels below the detection limit retained the *hebiba*
87 mutant phenotype. *LOC_Os03g32270* encodes the alpha/beta-fold hydrolase
88 DWARF14LIKE/KARRIKIN INSENSITIVE2/HYPOSENSITIVE TO LIGHT
89 (D14L/KAI2/HTL). In *Arabidopsis thaliana,* this hydrolase acts together with the F-box
90 protein DWARF3/MORE AXILLIARY GROWTH2/ (D3/MAX2) in the perception of
91 karrikins, a group of butenolide compounds found in smoke that induce seed germination
92 in fire-chasing plants (*13-16*). The structurally related strigolactones are perceived by a
93 receptor complex involving D3 and the alpha/beta-fold hydrolase DWARF14 (D14), the

94 paralogue of D14L (*20-22*). However, the strigolactone insensitive rice mutant *d14* is not
95 perturbed in AM symbiosis (*23,* Fig. 3A), thus the strigolactone receptor gene *D14* is not
96 required establishment of the itneraction. A rice *d3* mutant was also severely impaired in
97 AM colonization and marker gene induction (Fig. 3A and B, *23*) revealing the importance
98 of the karrikin receptor complex for the earliest stages of AM development. We further
99 confirmed the requirement of D14L in AM development using a set of RNAi lines
100 generated in the *Oryza sativa* cv. Nipponbare background. The RNAi lines displayed
101 diverse levels of AM suppression that correlated (R^2=0.69) with the degree of
102 downregulation of endogenous *LOC_Os03g32270* (fig. S2A-C). The *D14L* RNAi line
103 Ri43 supports AMF colonization (*23*), however we found a decrease (p = 0.047) in total
104 fungal colonization relative to wild type in this line. The phenotypic diversity among the
105 *D14L* RNAi lines suggests a low transcript threshold for AM symbiosis establishment.
106 In Arabidopsis *KAI2*/*HTL* controls hypocotyl elongation in response to light and
107 karrikin (*16, 24*). Over expression of rice *D14L* in an *Arabidopsis htl-2* mutant restored
108 wild type hypocotyl length in two independent F3 populations homozygous for *htl-2* (fig.
109 S3A). Mesocotyl elongation assays in rice demonstrated that *hebiba*^{AOC} is insensitive to
110 karrikin but responds to the synthetic strigolactone GR24 (fig. S3B). In contrast,
111 mutations of *D14* specifically compromised strigolactone but not karrikin responses in
112 rice whereas mutation of the F-box protein encoding *D3* resulted in insensitivity to both
113 (fig. S3B). Thus, in rice D14L and D14 mediate perception specificity to karrikin vs.
114 strigolactone in an overall similar manner to *Arabidopsis* (*16*). However, the partial
115 response of Arabidopsis *d14* to racemic GR24 (*16*) was not reproduced in rice *d14*
116 mutants (fig. S3B, *25*), suggesting D14L to have less redundant activity in rice.
117 Fluorescently tagged D14L in both Arabidopsis (*24*) and rice localized to both nucleus
118 and cytoplasm (Fig. 2E). D14L in rice (Fig. 2D) as in Arabidopsis (*24*) is expressed in all
119 rice organs and transcript accumulation in roots is not altered during AM colonization.
120 We asked whether D14L is required for suppression of defense responses against
121 AMF. We found no evidence for increased activation of selected defense marker genes (*26*)
122 during the early stages of mycorrhizal colonization (fig. S4A and B). Moreover, *hebiba*^{AOC}
123 was susceptible to colonization by the root endophyte *Piriformospora indica* and the

124 pathogen *Magnaporthe oryzae* (fig. S4C-D), implicating D14L in symbiotic
125 compatibility.

126 On the basis of the early and pronounced *hebiba* mutant phenotype, we
127 hypothesized that functional D14L is required for the perception of AM fungi prior
128 contact. Germinated spore exudates of AMF activate pre-contact plant responses (*27*).
129 Therefore, we used RNAseq to monitor the transcriptional changes of *hebiba*^{AOC} and wild
130 type roots in response to germinated spore exudates over the first 24 hours post treatment
131 (hpt, Supplementary Materials, tab. S2 and S3). Overall 140 genes showed statistically
132 significant differences in average expression upon germinated spore exudates treatment in
133 wild type plants (Fig. 4A, tab. S4 and S5). In *hebiba*^{AOC} plants only six genes responded
134 significantly to GSE, of which only two genes (predicted to encode an expressed and a
135 hypothetical protein) overlapped with the genes responding in wild type (Fig. 4A, tab.
136 S4) suggesting that the transcriptional response observed in the wild-type relied on
137 functional D14L. Time resolved gene ontology (GO) analyses of genes differerentially
138 regulated in response to germinated spore exudates in wild type but not in *hebiba*^{AOC}
139 demonstrated an overrepresentation of terms associated with responses to extra cellular
140 and biotic stimuli. Genes were induced or repressed at the earliest time points, 3 and 6
141 hpt, and in a *D14L* dependent fashion, consistent with D14L playing a role in early
142 signaling activation (Fig. 4B, tab. S6A and B). The expression pattern of representative
143 genes was validated by quantitative RT-PCR on the same RNA used for the RNAseq
144 experiment (fig. S5A) and on RNA from two additional biological replicates which
145 included the complemented line C11 (fig. S5B). Thus, D14L is required to support initial
146 colonization events by AMF. Despite its effect on mesocotyl elongation, treatment with
147 karrikin did not induce significant gene expression changes in roots of wild-type rice (tab.
148 S4). Also the exogenous application of karrikin did not stimulate colonization of wild
149 type roots by *R. irregularis* (fig. S6).

150 We found that a total of 104 transcripts differed significantly between untreated
151 *hebiba*^{AOC} and wild type roots (tab. S4) derived from genes with borderline GO-term
152 enrichment for metabolic processes (tab. S6C). Whereas mRNA levels of known genes
153 essential for AM symbiosis accumulated independently of functional D14L, transcript
154 levels of the rice homolog of *DLK2* (*16*), *LOC_Os05g51240,* depended on D14L as

155 earlier observed in Arabidopsis (*13*, tab. S4). In contrast to Arabidopsis however, karrikin
156 treatment of rice roots did not induce this gene. Because D14L is found in genomes of
157 plants that germinate without fire stimulation, and because Arabidopsis mutants lacking
158 D14L show developmental phenotypes, we hypothesize that an endogenous ligand exists
159 and is required for wild type seedling development (*28*). In rice, the differences in
160 transcriptomes between germinated spore exudates and mock or karrikin treated wild-
161 type plants indicates either that this ligand is not karrikin, or that D14L acts upstream of
162 the germinated spore exudates response thereby possibly creating a condition permissive
163 for AM symbiosis.

164 We show that the karrikin receptor complex is central to the everyday interaction
165 of plants with AMF, involving more than 80% of all plant species as opposed to 1200
166 smoke-responsive plant species (*29*). Conservation of D14L in early land plants such as
167 liverworts (*30*) suggests that it has served this purpose since AMF started supporting
168 terrestrial plant life. On poor natural soils, plants rely on AMF for mineral nutrient supply
169 and need to coordinate AMF development with their physiological and developmental
170 needs. The karrikin receptor complex may represent a node in the crosstalk between plant
171 development and AM signaling.

172

173 **References and notes:**

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233
234 **Acknowledgements**

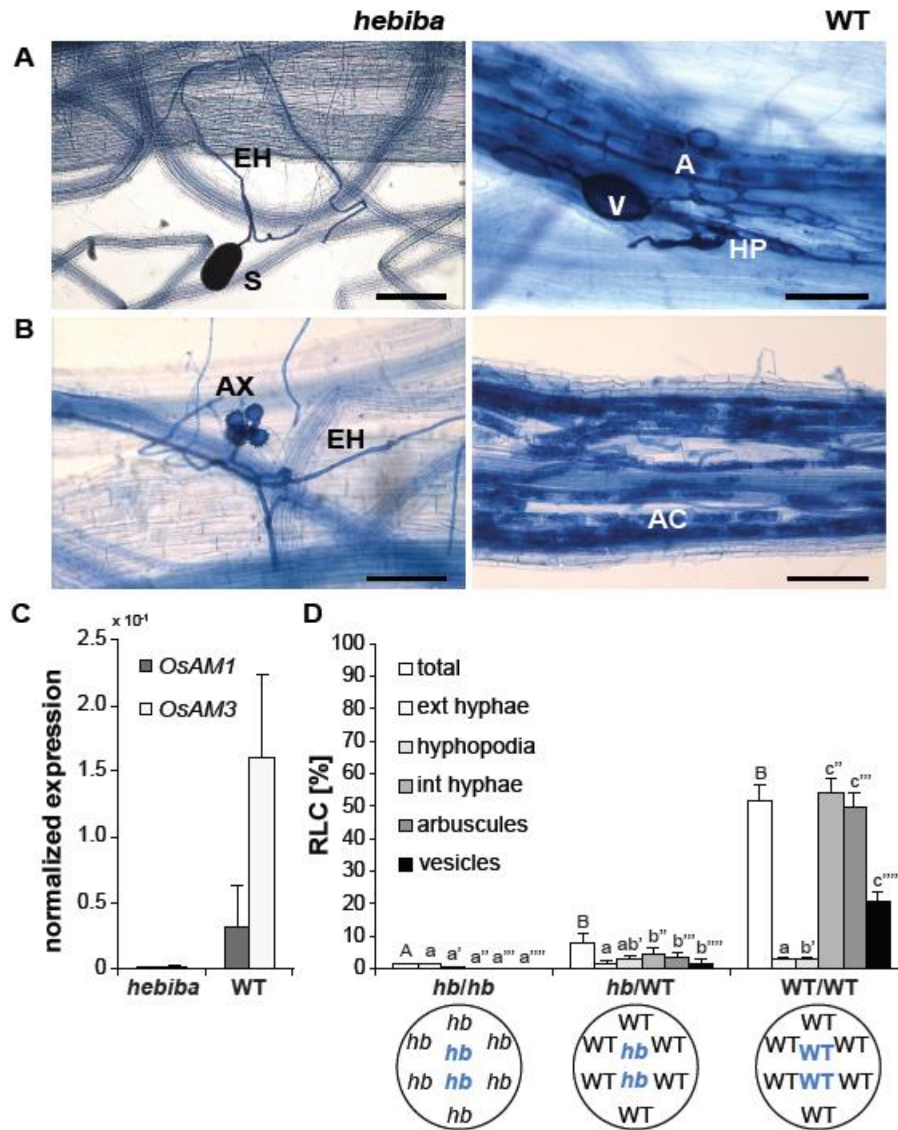
235 We thank Jacqueline Gheyselinck for technical assistance. We are grateful to Peter Nick
236 (Karlsruhe Institute of Technology, Germany) for providing *hebiba* and Nihonmasari wild
237 type seeds, Mikio Nakazono (University of Nagoya, Japan) for providing *d3* and Shiokari

wild type seeds, Junko Kyozuka (Graduate School of Agriculture and Life Sciences, University of Tokyo) for *d14* seeds and the *D14L* RNAi line Ri43 and Min Ni (University of Minnesota, USA) for providing Arabidopsis *htl-2* seeds. Tom Hardcastle helped by enhancing his R/Bioconduct or package baySeq to enable the RNAseq time course analysis. Moritz Meyer instructed the carbon-dioxide treatment of *R. irregularis*. C.G. received PhD fellowships from the German National Academic Foundation (*Studienstiftung des Deutschen Volkes*), the Roche Research Foundation and the Academic Society of Vaude (Société Académique Vaudoise). E.G. was supported by the Isaac Newton Trust grant RG74108. S.C. and research in the C.G. laboratory were funded by the Collaborative Research Center SFB924 "Molecular mechanisms regulating yield and yield stability in plants" of the German Research Council (DFG). Research in the U.P. laboratories was supported by the Swiss National Science Foundation 'professeur boursier' grants PP00A-110874, PP00P3-130704 and by the Gatsby Charitable Foundation grant RG60824.

C.G., E.G., J.C., K.A.K. and U.P. designed the experiments. C.G., E.G., J.C., M.G.J., W.S., S.C., C.M., S.-Y.Y. M.N., I.A. performed the experiments. K.A.K., W.-B.J. and K.S. performed bioinformatics and statistical analysis of the RNAseq data. M.R. and M.T. provided access to unpublished mapping information. C.G., E.G., K.A.K. and U.P. wrote the manuscript.

The RNA-seq data have been deposited in ArrayExpress with Accession Numbers (to be supplied).

The authors declare no conflict of interest.



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262 **Fig. 1. Arbuscular mycorrhiza phenotype of *hebiba*.**

263 (**A** and **B**) Roots of *hebiba* and wild type stained with trypan blue to visualize AM fungal

264 structures six weeks post inoculation (wpi) with *Rhizophagus irregularis* (**A**) and *Gigaspora*

265 *rosea* (**B**). Labels refer to A, arbuscule; AC, arbuscular coil; AX, auxiliary cell; EH,

266 extraradical hypha; HP, hyphopodium; V, vesicle, size bar = 100 µm. (**C**) Expression of two

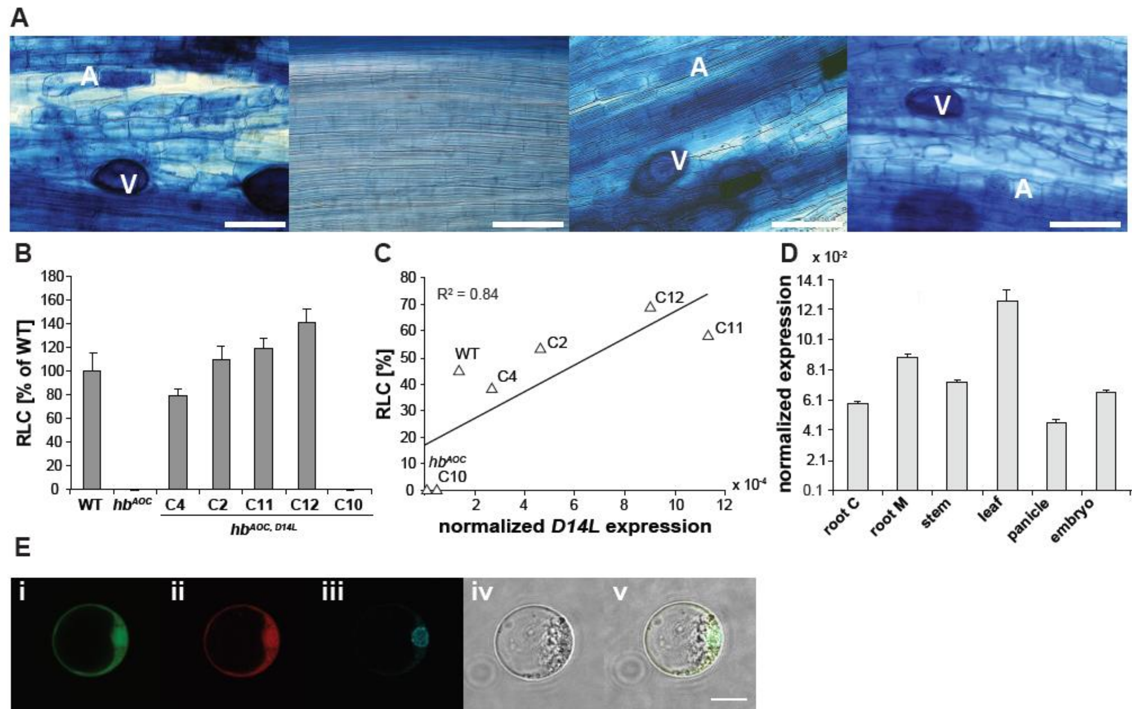
267 early AM marker genes in *hebiba* and wild type six wpi with *R. irregularis* as assessed by

268 qPCR. Means and Standard Errors of six biological replicates from three independent

269 experiments are shown. (**D**) Percentage of root length colonization (RLC) by *R. irregularis* of

270 two central 'tester plants' surrounded by six 'donor plants' at seven wpi. Means and standard

errors (SEs) of five biological replicates are shown. Abbreviations refer to ext hyphae: extraradical hyphae, int hyphae: intraradical hyphae. For each of the six fungal structures in the figure, separate Kruskal-Wallis tests were performed, using the Benjamini-Hochberg adjustment for multiple testing for the post-hoc tests. The p-values were: p (total) ≤ 0.01, p (ext. hyphae) $= 0.43$, p (hyphopodia) ≤ 0.05, p (int. hyphae, arbuscules, vesicles) ≤ 0.001.



291 Figure 2

292

293 **Fig. 2. D14L is required for AM development.**

294 **(A-C)** AM phenotype of transgenic *hebiba*^{AOC,D14L} complementation lines. **(A)** Trypan blue
295 stained roots at six wpi with *Rhizophagus irregularis*: micrographs refer to (from left to right)
296 wild type Nihonmasari, *hebiba*^{AOC} mutant and two independent transgenically complemented
297 *hebiba*^{AOC,D14L} lines (C4, C11). A, arbuscule; V, vesicle. Size bar, 50 µm. **(B)** Root length
298 colonization (RLC) expressed as % of WT colonization at six wpi for independent
299 *hebiba*^{AOC,D14L} complementation lines. Values represent Means and Standard Errors from 2-5
300 replicate plants. **(C)** *D14L* transcript levels were assessed by real time RT-PCR in the
301 independent transgenic complementation lines. The averages for the wild type, *hebiba*^{AOC} and
302 the complementation *hebiba*^{AOC,D14L} lines were plotted against the corresponding averages for
303 total root length colonization (RLC). The Spearman rank correlation was calculated and
304 squared to give the proportion of the variation accounted for by the correlation. **(D)** Real
305 time RT-PCR-based expression of *D14L* in control root (C), mycorrhizal roots (M), stem,
306 leaf, panicle and embryo of Nipponbare rice. Expression values were normalized to the
307 expression values of the constitutively expressed gene *Cyclophilin2 (LOC_Os02g02890)*.
308 Means and standard deviations of three technical replicates are shown. **(E)** Subcellular

309 localization of D14L. A plasmid containing a *D14L* overexpression construct **(i)** maize
310 ubiquitin promoter:*D14L* cDNA:GFP was co-transfected with the plasmid containing a
311 genomic clone of *D14L* driven by its native promoter **(ii)** p*D14L*:g*D14L*:*RFP* in rice root
312 protoplasts. **(iii)** DAPI staining. **(v)** shows the overlay of all channels, including bright field
313 **(iv)**. Scale bar, 10μm.

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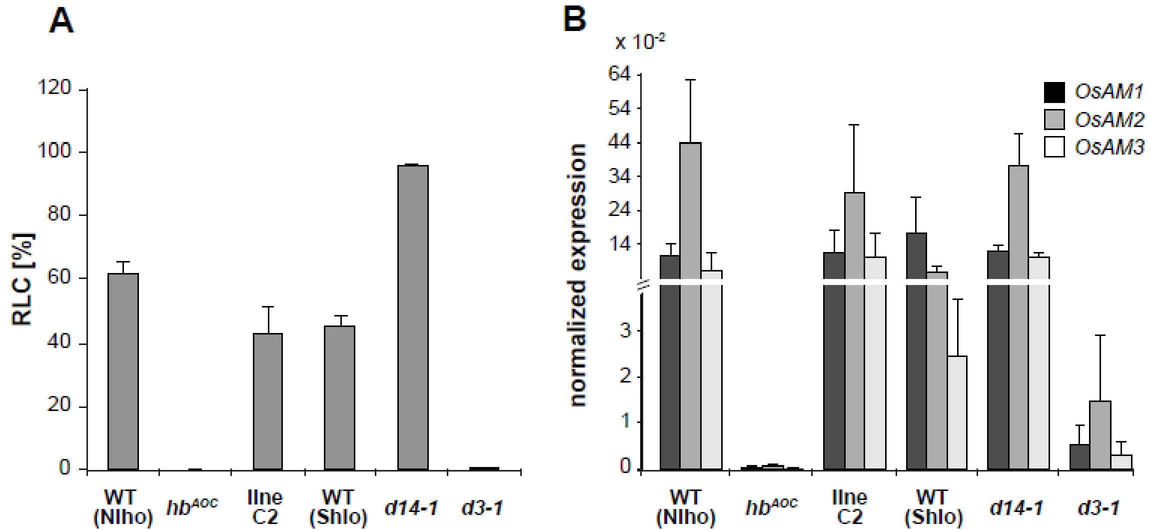

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318 **Fig. 3. AM phenotype of *d3* relative to *hebiba*^{AOC}, *d14* and corresponding wild type**
319 **cultivars.**

320

321 (**A**) Percentage of root length colonization and (**B**) induction of AM early marker genes at
322 seven wpi with *Rhizophagus irregularis* of *d3*, *d14, hebiba*^{AOC}, *hebiba*^{AOC,D14L}
323 complementation line C2 and corresponding wild type background Nihonmasari (Niho) and
324 Shiokari (Shio), respectively. Expression values were normalized to the expression values of
325 the constitutively expressed gene *Cyclophilin2 (LOC_Os02g02890)*. Values represent means
326 and standard errors from 3 biological replicates.

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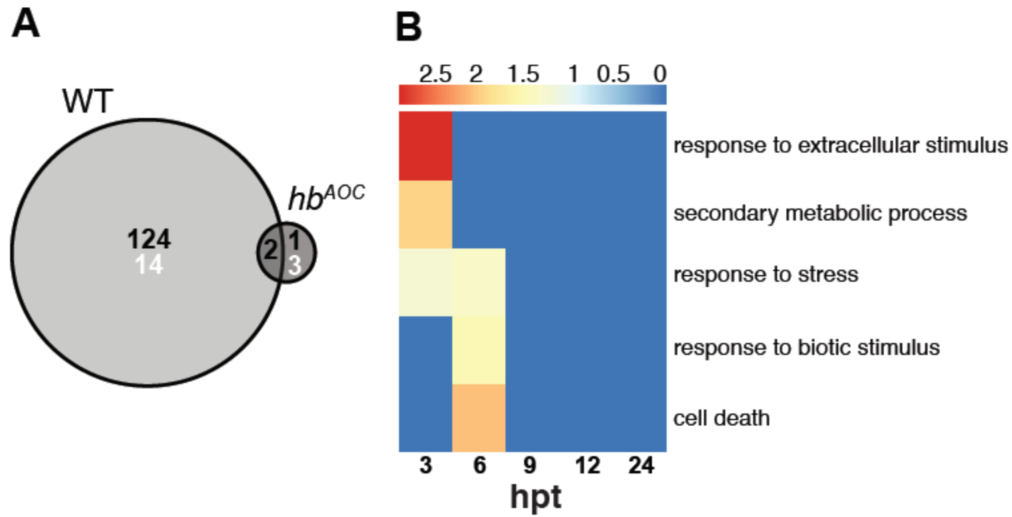

Figure 4

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332 **Fig. 4. GSE induced transcriptional responses of wild type and *hebiba*^{AOC}.**

333 (**A**) Venn diagram depicting the number of transcripts induced (black) and repressed (white)

334 in wild type and *hebiba*^{AOC} plants treated with GSE in comparison to plants receiving a mock

335 treatment. (**B**) Time resolved GO-term enrichment analysis ($p \leq 0.001$) for genes

336 differentially regulated in response to GSE in wild type but not in *hebiba*^{AOC}. The color code

337 represents odds ratios.

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