

1 **A barley gene cluster for the biosynthesis of diterpenoid**  
2 **phytoalexins**

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24 **Abstract**

25 Phytoalexins are specialized metabolites that are induced upon pathogen infection and  
26 contribute to the defense arsenal of plants. Maize and rice produce multiple diterpenoid  
27 phytoalexins and there is evidence from genomic sequences that other monocots may also  
28 produce diterpenoid phytoalexins. Here we report on the identification and characterization of  
29 a gene cluster in barley (*Hordeum vulgare* cv. Golden Promise) that is involved in the  
30 production of a set of labdane-related diterpenoids upon infection of roots by the fungal  
31 pathogen *Bipolaris sorokiniana*. The cluster is localized on chromosome 2, covers over 600  
32 kb and comprises genes coding for a (+)-copalyl diphosphate synthase (HvCPS2), a kaurene  
33 synthase like (HvKSL4) and several cytochrome P450 oxygenases (CYPs). Expression of  
34 HvCPS2 and HvKSL4 in yeast and *Nicotiana benthamiana* resulted in the production of a  
35 single major product, whose structure was determined to be of the cleistanthane type and  
36 was named hordediene. Co-expression of HvCPS2, HvKSL4 and one of the CYPs from the  
37 cluster (CYP89E31) afforded two additional products, hordetriene and 11-hydroxy-  
38 hordetriene. Both of these compounds could be detected in extracts of barley roots infected  
39 by *B. sorokiniana*, validating the function of these genes *in planta*. Furthermore, diterpenoids  
40 with multiple oxidations and with molecular masses of 316, 318 and 332 were induced in  
41 infected barley roots and secreted in the medium, indicating that additional oxidases,  
42 possibly from the same genomic cluster are involved in the production of these phytoalexins.  
43 Our results provide the basis for further investigation of the role of this gene cluster in the  
44 defense of barley against pathogens and more generally in the interaction with the  
45 microbiome.

## 46 **Introduction**

47 Monocotyledons contribute some of the most important staple crops worldwide, including the  
48 three major ones maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*) that  
49 cover a large part of calorie intake by humans worldwide (Awika, 2011). Behind these three  
50 species, barley (*Hordeum vulgare*) is the fourth most important grain crop with an annual  
51 production of over 140 million tons and with an harvest area of almost 48 million ha  
52 worldwide in 2018 (**Table S1**). Barley is grown in temperate climates and primarily for animal  
53 feed, but also to provide substrate for the fermentation of beverages (e.g. beer), and for a  
54 range of health promoting products. It is one of the earliest cultivated crops with supporting  
55 archaeological evidence from the fertile crescent region dating to as far back as over 10,000  
56 years before present (BP) (Badr et al., 2000). The initial use of barley was for food, but it was  
57 then later replaced by wheat (Riehl, 2019). Cultivated barley is one of 31 *Hordeum* species,  
58 with *H. vulgare* subsp. *spontaneum* believed to be the wild ancestor of cultivated barley  
59 (Badr et al., 2000). The close relatedness of barley to wheat and its diploid nature ( $2n = 14$ )  
60 make it a relevant model species for the study of temperate cereal crops. A first draft of its  
61 genome was published in 2016 and was recently updated (Beier et al., 2017; Mascher et al.,  
62 2017; Monat et al., 2019). Furthermore, genetic transformation and gene editing in barley are  
63 now well established, providing a complete toolbox for functional genetics in this species  
64 (Hensel, 2020).

65 Plants synthesize a complex array of secondary metabolites that contribute to the response  
66 and adaptation to a range of biotic or abiotic stresses. These metabolites can be produced  
67 constitutively, in a tissue specific manner or upon challenge by specific stresses, be they  
68 biotic or abiotic. Whereas some metabolites are common to a wide range of species, others  
69 are restricted to a species or to a taxon, thereby determining a species or taxon metabolite  
70 signature, a feature that led to the denomination “specialized metabolites” (Pichersky et al.,  
71 2006; Pichersky and Lewinsohn, 2011).

72 Plant pathogenic fungi impose a major burden on crop yield, and this impact is expected to  
73 increase with climate change (Miedaner and Juroszek, 2021). As the use of agrochemicals is  
74 increasingly under scrutiny by environmental agencies and while single gene-for-gene  
75 resistance may be rapidly overcome in a changing climate, there is a strong need for more  
76 durable resistance traits. The fungus *Bipolaris sorokiniana* (syn. *Cochliobolus sativus*) is the  
77 pathogenic agent of spot blotch and root rot in wheat and barley and is particularly prevalent  
78 in regions with a warmer climate (Rosyara et al., 2010) and therefore represents a typical  
79 future important threat in regions with a temperate climate in the context of global warming.  
80 *B. sorokiniana* can infect both aerial and underground parts of the plant but knowledge on  
81 how it interacts with roots is still rather limited (Sarkar et al., 2019).

### *Barley diterpenoid gene cluster*

82 There is widespread evidence that infection of plants by microbial pathogens triggers the  
83 production of a secondary metabolites (also called specialized metabolites) that exhibit  
84 antimicrobial or antioxidant activities (Ahuja et al., 2012). These compounds, called  
85 phytoalexins, are not restricted to a particular chemical class, with examples among  
86 phenylpropanoids, alkaloids or terpenoids (Ahuja et al., 2012). This is also the case in  
87 Monocotyledons, a plant clade that includes some of the most important food crops  
88 worldwide. In barley, there is a number of reports of various phytoalexins produced in  
89 response to diverse pathogens. These include phenylamides, such as the dimeric hordatine  
90 A and B, the indole-derived gramine, benzoxazinones such as 2,4-dihydroxy-1,4-benzoxazin-  
91 3-one (DIBOA), methoxychalcones as well as tyramine and related amines (Ishihara et al.,  
92 2017; Ube et al., 2017; Ube et al., 2021). However, in contrast to other monocotyledon crops  
93 such as maize and rice, no sesqui- or diterpenoid phytoalexins have been identified in barley  
94 yet. There is now extensive data available on the nature and biosynthesis of a range of  
95 terpenoid phytoalexins in these important crop species. Rice produces several classes of  
96 labdane-related diterpenoids, including momilactones (A and B) (Kato et al., 1973; Cartwright  
97 et al., 1977), phytocassanes, oryzalexins (Akatsuka et al., 1983; Kono et al., 1984; Sekido et  
98 al., 1986; Kato et al., 1993, 1994) and oryzalides (Watanabe et al., 1990; Kono et al., 1991)  
99 as well as the macrocyclic *ent*-oxodepressin (Inoue et al., 2013). Maize produces  
100 dolabralalexins and kauralexins, both labdane-related diterpenoid phytoalexins (Schmelz et al.,  
101 2011), as well as zealexins, which are sesquiterpenoids (Huffaker et al., 2011).

102 Biosynthesis of terpenoids starts by the conversion of linear isoprenyl diphosphate chains by  
103 terpene synthases to either linear or cyclic terpenes or terpene alcohols. In the case of  
104 diterpenoids, the precursor is typically all-*trans*-geranylgeranyl diphosphate (here  
105 abbreviated as GGPP) (Bohlmann et al., 1998), although in some isolated cases it is the all-  
106 *cis*-isomer, nerylneryl diphosphate (Zi et al., 2014). Diterpene synthases (diTPS) are classified  
107 according to the mechanism underlying the initiation of the cyclization reaction. Thus class I  
108 diTPS initiate the reaction by dephosphorylation whereas class II do it by protonation of the  
109 terminal isoprenic double bond (Peters, 2010). The main products of class II diTPS are *ent*-  
110 copalyl diphosphate (*ent*-CPP), the precursor of the gibberellins, and the other stereoisomers  
111 *syn*-copalyl diphosphate (*syn*-CPP) and CPP of normal configuration (Peters, 2010). In  
112 addition, there are other products of class II diTPS with a slightly different core structure,  
113 such as clerodienyl or halimadienyl diphosphates, as well as products with an alcohol  
114 function (Nakano et al., 2005; Sallaud et al., 2012; Pelot et al., 2017). Because the products  
115 of class II diTPS still contain a diphosphate group, class I diTPS can convert them to olefinic  
116 diterpenes or diterpene alcohols. The sequential reactions catalysed by class II and class I  
117 diTPS lead to the broad group of labdane-related diterpenes, which have in common a core  
118 bicyclic decalin ring structure (Peters, 2010). Apart from *ent*-oxodepressin, which has a

119 macrocyclic structure, all diterpenoid phytoalexins from monocots identified so far belong to  
120 the labdane-related group. Following cyclization by diTPS, diterpene backbones are then  
121 oxidized at different positions and in a stereospecific way. Cytochrome P450 oxygenases  
122 (CYPs) are the most frequently involved in these oxidations (Bathe and Tissier, 2019), but  
123 other classes of enzymes such as 2-oxoglutarate dependent dioxygenases in gibberellin  
124 biosynthesis (Hedden and Kamiya, 1997) or short-chain dehydrogenases/reductases as in  
125 momilactone biosynthesis (Kitaoka et al., 2016) can also play a role in functionalizing  
126 diterpenes. These oxidations can sometimes lead to backbone rearrangements and,  
127 importantly, provide anchoring points, such as hydroxyl or carboxyl groups, for further  
128 modifications by conjugating enzymes (Long et al., 2008; Rontein et al., 2008). Thus, sugar,  
129 acyl, or benzoyl groups can decorate the oxidized diterpene core and provide added  
130 functionalities.

131 In recent years the elucidation of the biosynthesis of rice and maize diterpenoid phytoalexins  
132 has progressed significantly (Murphy and Zerbe, 2020). In rice, the labdane-related  
133 diterpenoids are derived from either *ent*- or *syn*-copalyl diphosphate, whereas the maize  
134 diterpenoid phytoalexins derive from *ent*-CPP. In addition to the diTPS, which produce the  
135 diterpene backbones of these phytoalexins, a number of CYPs are involved in the  
136 functionalization of these backbones. Notably, both in maize and rice, some of the genes for  
137 the biosynthesis of terpenoid phytoalexins occur are physically associated in chromosomal  
138 clusters (Shimura et al., 2007; Wang et al., 2011; Ding et al., 2020; Liang et al., 2021).

139 Here we present the identification and characterization of a diterpenoid phytoalexin  
140 biosynthesis cluster in barley. We show that genes in this cluster are strongly induced in  
141 roots by a barley fungal pathogen, *B. sorokiniana* and we characterize the first biosynthesis  
142 steps, consisting of a copalyl diphosphate synthase, a kaurene synthase like and a CYP.  
143 Notably, the diterpene backbone produced by the CPS/KSL enzymes belong to the  
144 cleistanthane group of labdane-related diterpenoids. To the best of our knowledge, this  
145 diterpene backbone has not been detected yet in grasses. We also detected diterpenoid  
146 phytoalexins in barley roots and in high amounts in the root exudate as well as the  
147 intermediates produced by the first three enzymes of the pathway.

148

## 149 **Results**

150

### 151 **Identification of a diterpenoid biosynthesis gene cluster in barley chromosome 2**

152 In previous work, we generated transcriptome data of barley roots infected with either a  
153 fungal pathogen, *Bipolaris sorokiniana*, or a beneficial root endophyte, *Serendipita vermifera*,  
154 or both in local or systemic context (Sarkar et al., 2019). We observed that a number of  
155 genes from the MEP pathway as well as a number of genes from a region in chromosome 2

156 encoding terpene synthases and cytochrome P450 oxygenases are strongly induced by the  
157 fungal pathogen *B. sorokiniana* and moderately by *S. vermifera* (**Fig. 1**). In the MorexV2  
158 version of the barley genome (Monat et al., 2019), this cluster spans over 600 kb and  
159 contains one gene encoding a copalyl diphosphate synthase (CPS), one kaurene-synthase  
160 like (KSL), 8 CYP and one asparaginase (**Fig. 1**). The cluster also harbors a number of  
161 pseudogenes, including five CYPs and four CPS. As is frequently the case in regions of the  
162 genome with multiple duplications, the early versions of the genome sequence (including  
163 MorexV1 and MorexV2) still contain a number of gaps, and it is likely that the number of  
164 genes and pseudogenes in this cluster evolves as more accurate sequences become  
165 available.

166

### 167 **Phylogenetic analysis of the CPS and KSL genes in the chromosome 2 cluster**

168 To better characterize the genes of the cluster, we performed a phylogenetic analysis. Earlier  
169 publications reported on the identification and biochemical characterization of HvCPS1 as an  
170 *ent*-CDP synthase (Spielmeyer et al., 2004; Wu et al., 2012). The strong similarity of  
171 HvCPS1 to TaCPS3 and TaCPS4, both of which are *ent*-CDP synthases, and the exclusive  
172 presence of *ent*-CDP synthases in the same branch underscores the distinct evolutionary  
173 conservation of *ent*-CDP synthases in monocots (**Fig. 2**). The CPS in the chromosome 2  
174 cluster has not been characterized yet and we propose to name it HvCPS2. HvCPS2 shares  
175 high similarity to TaCPS2, a (+)-CDP synthase, and belongs to the same branch as OsCPS4,  
176 a *syn*-CDP synthase, and TaCPS1, an *ent*-CDP synthase. Thus, although these data support  
177 a role of HvCPS2 in specialized diterpenoid metabolism, they do not allow us to predict its  
178 actual biochemical activity with high confidence.

179 The kaurene synthase-like of the chromosome 2 cluster was already identified previously  
180 and named HvKSL4 (Li et al., 2016). In the phylogenetic tree comprising other monocot KSL  
181 enzymes (**Fig. 2**), it is in the same branch as TaKSL1, TaKSL4 and OsKSL4. The substrate  
182 of TaKSL1 and TaKSL4 is (+)-CDP (Zhou et al., 2012), while that of OsKSL4 is *syn*-CDP  
183 (Otomo et al., 2004). In the neighboring branch are TaKSL2 and TaKSL3. The function of  
184 TaKSL3 is unknown but TaKSL2 also uses (+)-CDP as substrate (Zhou et al., 2012). The  
185 clear separation from KSL enzymes that use *ent*-CDP as substrate, including the barley *ent*-  
186 kaurene synthase (HvKS), indicates that HvKSL4 most likely uses either (+)-CDP or *syn*-  
187 CDP as substrate.

188

### 189 **HvCPS2 is a (+)-CDP synthase and HvKSL4 produces a cleistanthane-type backbone**

190 To determine the biochemical activity of HvCPS2, we expressed it in yeast using our Golden  
191 Gate yeast cloning system (Scheler et al., 2016) together with RoMiS, the miltiradiene  
192 synthase from rosemary (*Rosmarinus officinalis*) (Brückner et al., 2014), with CcKS, an *ent*-

193 kaurene synthase from coffee (*Coffea canephora*), or with HvKSL4. Co-expression with  
194 RoMiS yielded the expected diterpene product (miltiradiene), whereas no *ent*-kaurene could  
195 be detected with CcKS, demonstrating that HvCPS2 produces (+)-CDP (**Fig. 3**). Co-  
196 expression of HvCPS2 and HvKSL4 in yeast yielded a novel product with a molecular ion of  
197 272 (**Fig. 3**), indicating that it is a diterpene olefin. Besides this major product, several much  
198 more minor products could also be detected. When expressing truncated versions of  
199 HvCPS2 and HvKSL4 together with a cytosolic geranylgeranyl diphosphate synthase  
200 (GGPPS) and a truncated version of hydroxymethylglutaryl CoA-reductase (tHMGR) in  
201 *Nicotiana benthamiana*, the same product could be detected (**Fig. S1**). Since there was no  
202 significant match in the NIST database (Mass Spectrometry Data Center,  
203 <http://chemdata.nist.gov>), we purified the main product and determined its structure by  
204 nuclear magnetic resonance (NMR) spectroscopy (see **Table S4**). The product was  
205 determined to have a cleistanthane backbone, with two double bonds in the C-ring at  
206 positions C8-C9 and C12-C13, a methyl group attached to C13 and an ethyl group attached  
207 to C14 in the  $\alpha$ -configuration (**Fig. 3**). Cleistanthanes constitute a relatively small group of  
208 diterpenes that occur in some plants and fungi [see for example: (Kaufman et al., 1987; Riehl  
209 and Pinto, 2000; Shiono et al., 2010; Zheng et al., 2018)]. We could not find published  
210 reports of the exact same structure and we therefore named it hordediene (alternatively:  
211 cleistantha-8(9),12(13)-diene).

212

### 213 **Phylogenetic analysis of the CYPs in the chromosome 2 cluster**

214 Of the eight CYP-encoding genes in the chromosome 2 cluster, only five are expressed at  
215 significant levels and display an expression pattern similar to that of *HvCPS2* and *HvKSL4*.  
216 Furthermore in the latest annotation of the barley genome, two of these genes merged into a  
217 single one so that only four CYP-encoding genes can be considered in the current version of  
218 the barley genome (**Table 1**). A phylogenetic analysis of these sequences shows that three  
219 of them are most similar to OsCYP99A2 and to OsCYP99A3 from rice (see **Fig. S2 and**  
220 **Table S5**). Although no biochemical function could be determined for OsCYP99A2 yet,  
221 OsCYP99A3 plays a role in the biosynthesis of momilactones by oxidizing *syn*-labdane  
222 related diterpenes - *syn*-pimaradiene and *syn*-stemodene - at the C19 position to generate a  
223 carboxylic acid function (Wang et al., 2011). Interestingly, OsCYP99A2 and OsCYP99A3 are  
224 located in the same tandem cluster next to OsCPS4 and OsKSL4, a situation highly  
225 reminiscent of the barley cluster presented here (Shimura et al., 2007).

226 The fourth CYP shares strongest similarity to enzymes of the CYP89 clan, with the closest  
227 homolog in *Arabidopsis thaliana* being CYP89A2 (AT1G64900), whose biochemical function  
228 is unknown yet. The most closely related protein sequences in the databases are homologs

229 from other crops, e.g. *Triticum aestivum* or *Setaria viridis*, but these are of unknown function  
230 as well. The assigned identification for this CYP is CYP89E31.

231

### 232 **Characterization of HvCYP89E31**

233 We first focused on the characterization of HvCYP89E31 by expressing it in yeast. We  
234 cloned a yeast optimized sequence in a vector together with HvCPS2 and HvKSL4 and in  
235 addition with the cytochrome P450 reductase from Arabidopsis. Analysis of the extracts by  
236 gas chromatography couple to electron ionization mass spectrometry (GC/EI-MS) showed  
237 the presence of two additional peaks, with a molecular  $m/z$  of 270 (product **2**) and 286  
238 (product **3**), respectively (**Fig. 4**). These masses indicate one and two additional degrees of  
239 oxidation. Next, we also isolated microsomes from a yeast strain expressing CYP89Ax and  
240 performed in vitro assays with hordediene as a substrate. GC-MS analysis of the extracts  
241 from this reaction showed the same products that are produced by yeast expressing all three  
242 genes (**Fig. 4**). This conclusively demonstrates that the new products detected are directly  
243 derived from hordediene. We also expressed those three genes in *N. benthamiana* as  
244 described above for HvCPS2 and HvKSL4 and found the exact same products present in  
245 hexane extracts (**Fig. S1**). The products were purified from a large scale yeast culture and  
246 their structure determined by NMR (**Tables S6 and S7**). Compound **2** has an aromatized C-  
247 ring whereas compound **3** has a hydroxyl group on position 11 (**Fig. 4**). We propose to name  
248 these compounds hordetriene (**2**) and 11-hydroxy-hordetriene (**3**).

249

### 250 **Hordetriene and 11-hydroxy-hordetriene are present in barley roots infected with *B.*** 251 ***sorokiniana***

252 To determine if the diterpene products identified in yeast and *N. benthamiana* by metabolic  
253 engineering are also produced in barley plants, we infected barley roots with the pathogen *B.*  
254 *sorokiniana*, or with the beneficial fungus *Serendipita vermifera* (syn. *Sebacina vermifera*) or  
255 with both as described in Sarkar et al. (2019). Hexane extracts from the roots were then  
256 analyzed by GC-MS and compared to an extract from a yeast strain expressing HvCPS2,  
257 HvKSL4 and CYP89E31 (**Fig. 5**). Whereas hordediene (**1**) could not be detected in any of  
258 the root extracts, **2** and **3** were detected in roots infected with *B. sorokiniana* or with *B.*  
259 *sorokiniana* and *S. vermifera*. In mock-infected roots or in roots infected with *S. vermifera*  
260 alone, smaller amounts of **3** could be detected compared to roots infected with *B.*  
261 *sorokiniana*, but **2** could not be detected. These results demonstrate that the diterpenoids  
262 produced by enzymes of the chromosome 2 cluster are indeed induced during fungal  
263 pathogen infection but not significantly by beneficial endophytic colonization, an observation  
264 consistent with the transcriptome data generated previously (Sarkar et al., 2019).

265



266 **Detection/identification of diterpenoid phytoalexins induced by *B. sorokiniana***  
267 **infection**

268 Given the presence of additional CYP enzymes in the barley chromosome 2 cluster, we  
269 wondered whether further oxidized diterpenoids could be detected in barley roots infected  
270 with *B. sorokiniana*. We thus performed untargeted LC-QToF-MS (negative mode) analysis  
271 of extracts from the root and from the medium. Searching for masses corresponding to  
272 additional oxidations of **3 (Fig. 4)**, i.e. 301.2, 317.2, 315.2 and 331.2, a number of peaks  
273 could be detected with a strong increase in samples from roots and medium of plants  
274 infected with *B. sorokiniana* (**Fig. 6**). By comparison, under mock control conditions this  
275 accumulation was not observed. LC-TOF-MS with negative electrospray ionization detected  
276 [M-H] adducts of six major compounds of which two products with the monoisotopic neutral  
277 mass or 316.21 Da dominated. High enrichments were also observed for three compounds  
278 with the monoisotopic formula mass 332.20 Da and for one with 318.22 Da, indicating that  
279 these main products carry 3 or 4 oxygen atoms. MS/MS spectra of these compounds imply a  
280 diterpene character based on the fragment ions 269.19, 271.21, 287.20 and 301.1 (**Fig. S3**).  
281 Neutral losses of 43.99 and 46.01 suggest the presence of lactones or carboxyl groups in all  
282 six diterpenes. As well, compound 6 has an additional carbonyl group as indicated by a  
283 neutral loss of 30.01 Da between the molecular ion and m/z 287.203.

284

285 **Discussion**

286

287 **Evolution and diversity of diterpenoid phytoalexins in monocots**

288 Since the discovery of diterpenoid phytoalexins in rice and the elucidation of their  
289 biosynthesis, there has been an increasing number of reports on investigations of specialized  
290 diterpenoid metabolism in monocots. This includes maize (*Zea mays*) (Schmelz et al., 2011;  
291 Ding et al., 2020), switchgrass (*Panicum virgatum*) (Pelot et al., 2018; Muchlinski et al.,  
292 2021), and wheat (*Triticum aestivum*) (Wu et al., 2012; Zhou et al., 2012). In the Triticeae,  
293 which comprise wheat, barley and rye (*Secale cereale*), despite the publication of a report  
294 describing the characterization of wheat diterpene synthases, (Wu et al., 2012; Zhou et al.,  
295 2012), to the best of our knowledge no diterpenoid phytoalexins had been identified yet. Our  
296 work shows that like maize and rice, barley is also able to produce diterpenoid phytoalexins  
297 when challenged with a pathogen. Thus, production of diterpenoid phytoalexins appears to  
298 be a common feature of monocots. It is also noteworthy that the genes involved in the  
299 biosynthesis of these compounds are frequently localized in clusters encoding diterpene  
300 synthases (CPS and KSL) as well as CYPs or in the case of rice a short chain  
301 dehydrogenase reductase (OsMAS) (Shimura et al., 2007; Ding et al., 2020; Muchlinski et  
302 al., 2021). Clusters of genes involved in specialized metabolism are encountered in plants

### *Barley diterpenoid gene cluster*

303 and typically consist of tandem duplicated copies of one or more gene families (Nützmann et  
304 al., 2016). The molecular mechanisms driving the formation and evolution of these clusters  
305 are still enigmatic, but the physical association and duplication of genes offer a number of  
306 potential advantages. First, physical association means that possible toxic intermediates due  
307 to mutation or loss of one of the genes are less likely to accumulate. Second, the presence of  
308 multiple copies of highly similar genes provides opportunities for gene rearrangements and  
309 independent evolution, thereby opening the door to rapid chemical innovations. The capacity  
310 to evolve novel defenses rapidly constitutes a key evolutionary adaptation in the arms race  
311 against pests and pathogens.

312 Despite the relatedness of wheat and barley, the characterization of wheat diterpene  
313 synthases indicates that none of them can produce the cleistanthane backbone identified  
314 here (Awika, 2011). This is additional evidence that the gene clusters allow for rapid  
315 evolution of enzyme function and chemical diversification.

316 A search for regions in rice that are syntenic to the barley cluster on chromosome 2 using the  
317 SynFind tool (Tang et al., 2015) was unsuccessful (data not shown). This could be due to the  
318 fact that this chromosomal region is subject to frequent rearrangements. Nonetheless, in a  
319 publication investigating the synteny between rice and barley (Thiel et al., 2009), much of  
320 barley chromosome 2 was found to be syntenic with rice chromosome 4, which is where the  
321 main cluster for momilactone biosynthesis is located. Thus, it is possible that the evolution of  
322 this genomic cluster predates the divergence between Triticeae and rice.

323

#### 324 **Hordediene: an unprecedented diterpene backbone in grasses**

325 HvKSL4 catalyzes the cyclization of (+)-CPP to hordediene, a cyclization reaction which, to  
326 the best of our knowledge, has not been reported previously, at least not in monocots.  
327 Hordediene belongs to the cleistanthane group of labdane-related diterpenes, which is  
328 characterized by the presence of an ethyl group attached to C17, instead of C13 as in  
329 pimaradiene. In the cyclization pathway we propose, after the initial dephosphorylation, the  
330 first electron migrations lead to the formation of the pimaradienyl cation (**Fig. S4**). At this  
331 point the pathway diverges from the pimaradiene cyclization via capture of a proton from C9  
332 by the C14-C15 double bond of pimaradienyl. This results in formation of the C8-C9 double  
333 bond and the C14 carbocation. A hydride shift from C17 to C14 would then allow the  
334 migration of the ethyl group to C17 and consequently result in the presence of carbocation at  
335 C13. Resolution of this cation by proton loss from C12 would then lead to hordediene.

336 A survey of the literature on cleistanthane diterpenoids reveals a modest number of reports  
337 (65 in Web of Science as of May 2021), with occurrence either in plants or fungi. Many of the  
338 cleistanthanes reported are of the *ent*- configuration, such as Fimbricalyxoid A from  
339 *Strophoblachia fimbricalyx* (Cheng et al., 2016) but some of normal (+) configuration, like

340 hordediene, have also been described, such as zythiostromic acids (Ayer and Khan, 1996).  
341 There is little information on the biological function of these diterpenoids, although in some cases  
342 anticancer activity in the  $\mu\text{M}$  range could be determined (Cheng et al., 2016).

343

#### 344 **Production and potential role of diterpenoid phytoalexins in barley**

345 We characterized three genes from the cluster (HvCPS2, HvKSL4 and HvCYP89E31) and  
346 showed that co-expression of these three genes results in the production of two major  
347 products, hordetriene and 11-hydroxy-hordetriene. The fact that we could detect both of  
348 these products in barley roots infected with *B. sorokiniana*, strongly supports a role of this  
349 cluster in the production of diterpenoid phytoalexins and in the defense reaction of barley.  
350 LC-MS analysis indicated the presence of further oxidized diterpenoids, however at this  
351 stage we were not able to isolate and purify sufficient quantity of these compounds for  
352 structural elucidation. Thus it remains to be determined whether these are also derived from  
353 the same diterpene backbone as compounds **2** and **3**. The fact that no other diterpene  
354 synthase is induced by *B. sorokiniana* in our transcriptome data argues in favor of  
355 this hypothesis. At this stage, we can only speculate on the biological activity of these  
356 compounds. Their biosynthesis is only minimally induced by *S. vermifera*, but very strongly  
357 by the pathogenic fungus *B. sorokiniana* (Sarkar et al., 2019). Whether the induction is  
358 specific to *B. sorokiniana* or is a general response to infection by pathogens remains to be  
359 determined. Interestingly, in a recent report on the metabolic profiling of barley plants  
360 infected with *Fusarium graminearum*, which causes head blight, two diterpenoids with  
361 masses of 302.2 and 318.2 were the most strongly induced compounds (respectively 32 and  
362 22 fold change) (Karre et al., 2017). They were annotated as neoabietic and 7-  
363 hydroxykaurenoic acid respectively, although there was no formal identification of these  
364 compounds. We suspect that these compounds also belong to the diterpenoid group that we  
365 identified, which would suggest that they are not induced specifically by *B. sorokiniana*.  
366 Availability of larger amounts of pure compounds in the future will allow us to perform  
367 bioassays and determine their potential antimicrobial activity. Of relevance to their biological  
368 function is the fact that these compounds are for the most part secreted into the medium by  
369 the roots. Since they are present at a low basal level in non-infected plants, it is likely that  
370 their presence in the rhizosphere could influence the composition of the root microbiome.  
371 There is already evidence of specialized metabolites secreted by the roots that impact the  
372 composition of the microbiome (Massalha et al., 2017; Huang et al., 2019; Murphy et al.,  
373 2021). Conversely, specific members of the microbial community can induce secretion of  
374 specialized metabolites by the roots (Massalha et al., 2017), suggesting a complex network  
375 of signals and effectors between the microbial community and the plant in the rhizosphere.

376 Generating barley plants mutated in HvCPS2 or HvKSL4 by CRISPR-Cas gene editing will  
377 allow us to address these questions in this important crop species in the future.

378

## 379 **Materials and Methods**

380

### 381 **Plant growth and fungal inoculations**

382 Barley seeds (*Hordeum vulgare* L. cv Golden Promise) were sterilized in 70% ethanol for 1  
383 min, followed by washing with sterile distilled water and 1.5 h incubation in 12% sodium  
384 hypochloride under continuous shaking. After 3 times 30 min washing, the seeds were  
385 placed on wet filter paper in darkness and at room temperature for 4 days for germination.  
386 Four seedlings were transferred to 1/10 PNM (Plant Nutrition Medium, pH 5.7) (Wawra et al.,  
387 2016) in sterile glass jars and grown in a day/night cycle of 16/8 h at 22/18 °C, 60 % humidity  
388 under 108  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.

389 *Bipolaris sorokiniana* (ND90Pr) and *Serendipita vermifera* (MAFF305830) were used in this  
390 study. *Bs* was propagated on modified CM medium with 1.5% agar and *Sv* on MYP medium  
391 with 1.5% agar in the dark at 28 °C for 21 days and 14 days before inoculation respectively.  
392 *Bs* conidia and *Sv* mycelial were collected according to the procedures which were described  
393 in (Sarkar et al., 2019). Barley roots were inoculated with 3 ml of either *Sv* mycelium  
394 (2g/50ml), *Bs* conidia (5000 spores/ml) or a 1:1 mixture of the two fungi per jar for 6 days.  
395 Sterile water was used as a mock treatment. Roots washed thoroughly and the  
396 corresponding medium were collected and snap-frozen in liquid nitrogen for extraction of  
397 metabolites.

398

### 399 **qRT-PCR**

400 RNA isolation from roots was performed using the Spectrum Plant Total RNA kit (Sigma-  
401 Aldrich). The complementary DNA (cDNA) was synthesized using ProtoScript II First Strand  
402 cDNA Synthesis Kit (New England Biolabs) following the manufacturer's instructions with  
403 primer d(T)<sub>23</sub> VN. Quantitative real-time PCR was performed in triplicates using 10-20 ng  
404 cDNA as template and gene specific primer pairs shown in **Table S8** in CFX Connect Real-  
405 Time PCR System (Bio-Rad). The PCR conditions were 95 °C for 15 min; 40 cycles of 95  
406 °C for 15 s, 56 °C for 30 s; 95 °C for 10 s. The melting curve was measured from 65 °C to  
407 95 °C with a step of 0.1 °C per second. Relative expression of targeted genes was  
408 calculated using delta Ct method (Livak and Schmittgen, 2001) and barley ubiquitin genes as  
409 references (Deshmukh et al., 2006).

410

### 411 **Phylogenetic analysis**

412 Amino acid sequences (**Tables S2, S3 and S5**) were aligned and phylogenetic trees were  
413 generated using MEGA X (Kumar et al., 2018). ClustalW was used for the alignment and the  
414 maximum likelihood method with a bootstrap of 1,000 for the phylogenetic tree. For the other  
415 parameters, default settings were used.

416

#### 417 **Heterologous expression of diterpenes in yeast**

418 Plasmids containing *GGPPS* and *ATR1* were kindly provided by colleagues in the group and  
419 have been described previously (Scheler et al., 2016). Codon-optimized DNA sequences of  
420 *HvCPS2*, *HvKSL4* and *HvCYP89E31* were synthesized by Thermo Fisher Scientific Inc. for  
421 yeast expression. Each gene was further cloned into Golden Gate compatible yeast  
422 expression level 1 vector, together with a synthetic galactose-inducible promoter and a  
423 terminator. Different gene combinations were finally assembled into one yeast expression  
424 level M vector by a 50 cycle restriction–ligation reaction with Bpil and T4-Ligase.

425 Constructs were then transformed into *S. cerevisiae* strain INVSc1 (Thermo Fisher Scientific  
426 Inc.) and plated out onto uracil-free (Ura-) selection medium (1 g l<sup>-1</sup> Yeast Synthetic Drop-  
427 out Medium Supplements without uracil (Sigma-Aldrich), 6.7 g l<sup>-1</sup> Yeast Nitrogen Base with  
428 Amino Acids (Sigma-Aldrich) and 20 g l<sup>-1</sup> Micro Agar (Duchefa Biochemie)). Three positive  
429 colonies were picked and inoculated into 5 ml yeast extract-peptone-dextrose (YPD)  
430 medium (20 g l<sup>-1</sup> tryptone and 10 g l<sup>-1</sup> yeast extract) containing 2% of glucose and grown  
431 for 24 h with shaking at 30 °C. To induce protein expression, the cell pellet was  
432 resuspended in fresh YPD medium containing 2% galactose. After another 24 h of growth,  
433 the whole culture was extracted with 2 ml *n*-hexane.

434

#### 435 **Transient expression in *Nicotiana benthamiana***

436 Transit peptides of protein *HvCPS2* and *HvKSL4* were predicted by two online tools, ChloroP  
437 1.1 (<http://www.cbs.dtu.dk/services/ChloroP/>) and LOCALIZER (<http://localizer.csiro.au/>).  
438 Truncated sequences without the predicted transit peptides of these two genes were  
439 generated by PCR reactions using designed primers and subsequently sequenced. The  
440 cDNAs of *HMG reductase*, *GGPPS* in plasmids have been described previously (Scheler et  
441 al., 2016; Yadav et al., 2019). The *HMG reductase*, *GGPPS*, *trHvCPS2*, *trHvKSL4* and  
442 *HvCYP89E31* were cloned into T-DNA vectors (binary vector pL1F-1) driven by the 35S  
443 promoter and flanked by the Ocs terminator (Weber et al., 2011). The resulting T-DNA  
444 plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90 and  
445 plated out onto LB agar plates with appropriate antibiotics. Bacteria were harvested and  
446 resuspended in infiltration medium (10 mM MgCl<sub>2</sub>, 10 mM MES, 20 μM acetosyringone,  
447 pH=5.6) after 48 h inoculation at 28 °C. To co-infiltrate several genes, each bacteria  
448 suspension was diluted to a final OD<sub>600</sub> of 0.4, then all strains were mixed equally to an

449 appropriate volume for infiltration. The suspension was infiltrated into the abaxial side of  
450 several leaves in three individual 4-week-old *N. benthamiana* plants using a syringe without  
451 needle. After treatment, the plants were cultivated in a climate controlled phytochamber for 4  
452 days. Three leaf discs (9 mm diameter) per infiltrated spot were harvested and extracted by 2  
453 ml *n*-hexane, followed by drying down under nitrogen flow and GC-MS analysis.

454

#### 455 **Microsome isolation and *in vitro* enzyme assay**

456 A protocol from the literature with slight modification was used for microsome isolation  
457 (Urban et al., 1997; Scheler et al., 2016). The construct carrying *HvCYP89E31* and *ATR1*  
458 were transformed into yeast strain INVSc1. A single positive colony was picked to inoculate  
459 5 ml of Ura-medium with 2% glucose and grown for 24 h at 30 °C with shaking. The  
460 culture was then used to inoculate 100 ml of Ura-medium with 2 % glucose in a 500 ml  
461 flask at 30 °C for 24 h. The cells were then collected by centrifugation, resuspended in  
462 100 ml fresh YPD medium with 2 % galactose to induce protein expression and inoculated  
463 under shaking for another 24 h at 30 °C. All the following steps were carried out at 4 °C.  
464 The cells were harvested by centrifugation and resuspended in 30 ml of pre-chilled TEK  
465 buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM KCl), centrifuged again and  
466 resuspended in 2 ml TES buffer (50 mM Tris-HCl pH 7.5, 600 mM sorbitol, 10 g l<sup>-1</sup> BSA,  
467 1.5 mM β-mercaptoethanol) and transferred to a 50 ml tube. Acid-washed autoclaved 450–  
468 600 μm diameter glass beads were added into the tube until the surface of the cell  
469 suspension are reached. The suspension was shaken vigorously by hand for 1 min and  
470 returned back to ice for 1 min. This step was repeated four times. The glass beads were  
471 washed by 5 ml TES buffer three times, and the supernatant was collected and combined to  
472 a new tube, followed by centrifugation at 7,500 g for 10 min. The supernatant was  
473 transferred to ultracentrifugation tubes and centrifuged for 2 h at 100,000 g. The pellet  
474 was gently washed successively with 5 ml TES and 2.5 ml TEG buffer (50 mM Tris-HCl  
475 pH 7.5, 1 mM EDTA and 30% glycerol) after the supernatant was removed, then transferred  
476 to a Potter homogenizer with a spatula. 2 ml TEG buffer was added to the homogenizer and  
477 the pellet was carefully homogenized. 100 μl aliquots were transferred to 1.5 ml microtubes  
478 and stored at –80 °C until used.

479 *In vitro* CYP enzyme assays were performed in a 600 μl reaction volume, containing 40 μl  
480 of microsome preparation, 100 μM substrate, 1 mM NADPH, 50 mM sodium phosphate  
481 pH 7.4. The solution was incubated at 30 °C for 2 h with gentle shaking. Products were  
482 extracted with 1 ml *n*-hexane under strong agitation (vortex). After centrifugation, the  
483 organic phase was collected, then dried under a N<sub>2</sub> stream and resuspended in 100 μl *n*-  
484 hexane for GC-MS analysis.

485

486 **Purification of diterpenes by silica gel column chromatography or SPE**

487 For the purification of hordediene, 1 l of yeast culture was grown and extracted with 1 l *n*-  
488 hexane. The raw extracts were dried in a rotary evaporator and resuspended in 4 ml *n*-  
489 hexane, then loaded into two properly conditioned SiOH SPE cartridges (500 mg,  
490 MACHEREY-NAGEL). The cartridges were then washed with 2 ml *n*-hexane. The  
491 breakthrough and washing fraction were collected and combined. After drying down under  
492 nitrogen stream, an aliquot was measured by GC-MS to check the purity of the product and  
493 the rest, with an amount of around 2 mg was used for NMR structure elucidation.

494 Hordetriene and 11-hydroxy-hordetriene were first extracted from three liters of yeast culture.  
495 After concentration, the raw extracts were dissolved in 4 ml *n*-hexane and loaded into a pre-  
496 conditioned self-packed silica gel column (5 g, 15 mm x 100 mm). The column was then  
497 eluted by *n*-hexane and successively by 99:1, 98:2, 97:3, 96:4, 96:5 *n*-hexane: ethyl acetate  
498 solutions. The volume of each elution solution was 10 ml but the elution was separately  
499 collected in five 2 ml microtubes. An aliquot of each fraction was measured by GC-MS and  
500 the fractions with the same product were combined and then used for NMR structure  
501 elucidation. The yield of hordetriene and 11-hydroxyhordetriene was around 0.5 mg.

502

503 **Nuclear magnetic resonance (NMR) conditions**

504  $^1\text{H}$ ,  $^{13}\text{C}$ , 2D ( $^1\text{H}$ ,  $^1\text{H}$  gDQCOSY;  $^1\text{H}$ ,  $^1\text{H}$  zTOCSY;  $^1\text{H}$ ,  $^1\text{H}$  ROESYAD;  $^1\text{H}$ ,  $^{13}\text{C}$  gHSQCAD;  
505  $^1\text{H}$ ,  $^{13}\text{C}$  gHMBCAD), selective ( $^1\text{H}$ ,  $^1\text{H}$  zTOCSY1D;  $^1\text{H}$ ,  $^1\text{H}$  ROESY1D), and band  
506 selective ( $^1\text{H}$ ,  $^{13}\text{C}$  bsHMBC) NMR spectra were measured with an Agilent VNMR5  
507 600 instrument at 599.83 MHz ( $^1\text{H}$ ) and 150.84 MHz ( $^{13}\text{C}$ ) using standard  
508 CHEMPACK 8.1 pulse sequences implemented in the VNMRJ 4.2A spectrometer  
509 software. TOCSY mixing time: 80 ms; ROESY mixing time: 300 ms; HSQC optimized  
510 for  $^1J_{\text{CH}} = 146$  Hz; HMBC optimized for  $^nJ_{\text{CH}} = 8$  Hz. All spectra were obtained with  
511  $\text{C}_6\text{D}_6 + 0.03\%$  TMS as solvent at  $+25^\circ\text{C}$ . Chemical shifts were referenced to internal  
512 TMS ( $\delta$   $^1\text{H} = 0$  ppm) and internal  $\text{C}_6\text{D}_6$  ( $\delta$   $^{13}\text{C} = 128.0$  ppm).

513

514 **Metabolites extraction from barley roots and PNM medium**

515 100 mg (fresh weight) of frozen and cryo-ground root matter was extracted using 900  $\mu\text{L}$   
516 dichloromethane/ethanol (2:1, v/v) and 100  $\mu\text{l}$  hydrochloric acid solution (pH 1.4). Extraction  
517 and duplicate removal of hydrophilic metabolites was achieved by 1 min FastPrep bead  
518 milling (FastPrep24, MP Biomedicals) followed by phase separation during centrifugation.  
519 For extraction 1.6 mL wall-reinforced cryo-tubes (Biozyme) each containing steel and glass  
520 beads were used. The upper aqueous phase was discarded and replaced for a second round  
521 of bead mill extraction/ centrifugation. Thereafter, the aqueous phase was removed and the

522 lower organic phase was collected. Subsequently 600  $\mu$ L tetrahydrofuran (THF) was used for  
523 exhaustive extraction (FastPrep). After centrifugation the organic THF extract was combined  
524 with the first extract and dried under a stream of  $N_2$ .

525 Root exudates were extracted from 60 mL of gelrite media. For this, the gel was distributed  
526 into two 50 ml Falcon tubes. To each tube 4 g of NaCl and 3 mL ethyl acetate were added.  
527 The tubes were thoroughly shaken by hand and centrifuged. The upper phase (organic  
528 extract) was collected before fresh ethyl acetate was added for another two consecutive  
529 extractions. The combined extracts of three extraction rounds were combined and dried in a  
530 stream of  $N_2$ .

531

### 532 **GC-MS**

533 Dried extracts were suspended in 200  $\mu$ l n-hexane. The analysis of yeast and plant extracts  
534 was carried out using a Trace GC Ultra gas chromatograph (Thermo Scientific) coupled to an  
535 ATAS Optic 3 injector and an ISQ single quadrupole mass spectrometer (Thermo Scientific)  
536 with electron impact ionization. Chromatographic separation was performed on a ZB-5ms  
537 capillary column (30  $\mu$ m  $\times$  0.32  $\mu$ mm, Phenomenex) using splitless injection and an injection  
538 volume of 1  $\mu$ l. The injection temperature rose from 60  $^{\circ}$ C to 250  $^{\circ}$ C with 10  $^{\circ}$ C  $s^{-1}$  and  
539 the flow rate of helium was 2  $ml \cdot min^{-1}$ . The GC oven temperature ramp was as follows:  
540 50  $^{\circ}$ C for 1  $min$ , 50 to 300  $^{\circ}$ C with 7  $^{\circ}$ C  $min^{-1}$ , 300–330  $^{\circ}$ C with 20  $^{\circ}$ C  $min^{-1}$  and  
541 330  $^{\circ}$ C for 5  $min$ . Mass spectrometry was performed at 70 eV, in a full scan mode with  
542  $m/z$  from 50 to 450. Data analysis was done with the device specific software Xcalibur  
543 (Thermo Scientific).

544

### 545 **RP-UPLC-ESI-MS/MS**

546 For UPLC-MS analysis dried extracts were suspended in 100  $\mu$ L 80% methanol/ 20% water.  
547 Separation of medium polar metabolites was performed on a Nucleoshell RP18 (2.1 x 150  
548 mm, particle size 2.1  $\mu$ m, Macherey & Nagel, GmbH, Düren, Germany) using a Waters  
549 ACQUITY UPLC System, equipped with a Binary Solvent Manager and Sample Manager (20  
550  $\mu$ l sample loop, partial loop injection mode, 5  $\mu$ l injection volume, Waters GmbH Eschborn,  
551 Germany). Eluents A and B were aqueous 0.3 mmol/L  $NH_4HCOO$  (adjusted to pH 3.5 with  
552 formic acid) and acetonitrile, respectively. Elution was performed isocratically for 2 min at 5%  
553 eluent B, from 2 to 19 min with a linear gradient to 95% B, from 19-21 min isocratically at  
554 95% B, and from 21.01 min to 24 min at 5% B. The flow rate was set to 400  $\mu$ l  $min^{-1}$  and the  
555 column temperature was maintained at 40  $^{\circ}$ C. Metabolites were detected by positive and  
556 negative electrospray ionization and mass spectrometry.

557 Mass spectrometric analysis of small molecules was performed by MS-TOF-SWATH-MS/MS  
558 (TripleToF 5600, AB Sciex GmbH, Darmstadt, Germany) operating in negative or positive ion



## *Barley diterpenoid gene cluster*

559 mode and controlled by Analyst 1.7.1 software (AB Sciex GmbH, Darmstadt, Germany). The  
560 source operation parameters were as follows: ion spray voltage, -4500 V / +5500 V;  
561 nebulizing gas, 60 psi; source temperature 600°C; drying gas, 70 psi; curtain gas, 35 psi.  
562 TripleToF instrument tuning and internal mass calibration were performed every 5 samples  
563 with the calibrant delivery system applying APCI negative or positive tuning solution,  
564 respectively (AB Sciex GmbH, Darmstadt, Germany).  
565 TripleToF data acquisition was performed in MS1-ToF mode and MS2-SWATH mode. For  
566 MS1 measurements, ToF masses were scanned between 65 and 1250 Dalton with an  
567 accumulation time of 50 ms and a collision energy of 10V (-10V). MS2-SWATH-experiments  
568 were divided into 26 Dalton segments of 20 ms accumulation time. Together the SWATH  
569 experiments covered the entire mass range from 65 to 1250 Dalton in 48 separate scan  
570 experiments, which allowed a cycle time of 1.1 s. Throughout all MS/MS scans a  
571 declustering potential of 35 (or -35 V) was applied. Collision energies for all SWATH-MS/MS  
572 were set to 35 V (-35) and a collision energy spread of  $\pm 25$ V, maximum sensitivity scanning,  
573 and otherwise default settings.

574

### 575 **Acknowledgments**

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578 of the DFG SPP2125 “Deconstruction and Reconstruction of the Plant Microbiota,  
579 DECRyPT.” (<https://ag-zuccaro.botanik.uni-koeln.de/decrypt>).

580

### 581 **Conflict of interest**

582 The authors have no conflict of interest to declare.

583

### 584 **Author Contributions**

585 YL performed the LC-MS, GC-MS, yeast and *N. benthamiana* expression and gene  
586 expression analysis. GB provided supervision for LC-MS and data analysis. AS-H provided  
587 assistance for LC-MS sample preparation. AP performed the NMR measurements and  
588 analyses. UB supervised the yeast expression experiments. LM and AZ provided infected  
589 barley samples and transcriptomic data. AT and GB designed the project. AT designed and  
590 supervised the project. AT wrote the abstract, introduction, results and discussion. YL wrote  
591 the materials and methods, except the NMR part (AP) and LC-MS (GB). AT, YL and GB  
592 prepared the figures. All authors read and approved the manuscript.

593

### 594 **Tables**

595 **Table 1 List of genes from the chromosome 2 diterpenoid phytoalexin cluster.**

*Barley diterpenoid gene cluster*

Genome version			Gene status	Transcript ID	Protein ID	Name
MorexV1 (2017)	MorexV2 (2019)	MorexV3 (2021)				
2Hr1G004480	2HG0081780	2HG0099280	FL	AK252527.1		CYP99A66
2Hr1G004510 2Hr1G004520	2HG0081840	2HG0099340	Pseudo			
2Hr1G004530	2HG0081840	2HG0099350	FL		KAE8771735	CYP99A67
2Hr1G004540	2HG0081850	2HG0099360	FL	AK370792		HvKSL4
2Hr1G004550	2HG0081860 UnG0627890 UnG0631950	2HG0099370 <sup>a</sup> 2HG0099420 <sup>a</sup> 2HG0099430 <sup>a</sup>	FL	AK369243		CYP89E31
NP	2HG0081880	2HG0099470	Pseudo			ψCPS1
2Hr1G004600	2HG0081930	2HG0099550	FL			CYP99A68 <sup>b</sup>
2Hr1G004610	2HG0081980	2HG0099550	FL			CYP99A68 <sup>b</sup>
2Hr1G004640 2Hr1G004650	2HG0081890 2HG0082010 2HG0081930 UnG0636400	2HG0099480	FL			CYP99A68 <sup>b</sup>
	2HG0081920	2HG0099500	Pseudo			ψCPS2
2Hr1G004620	2HG0082000	2HG0099570	FL	AK364238		HvCPS2

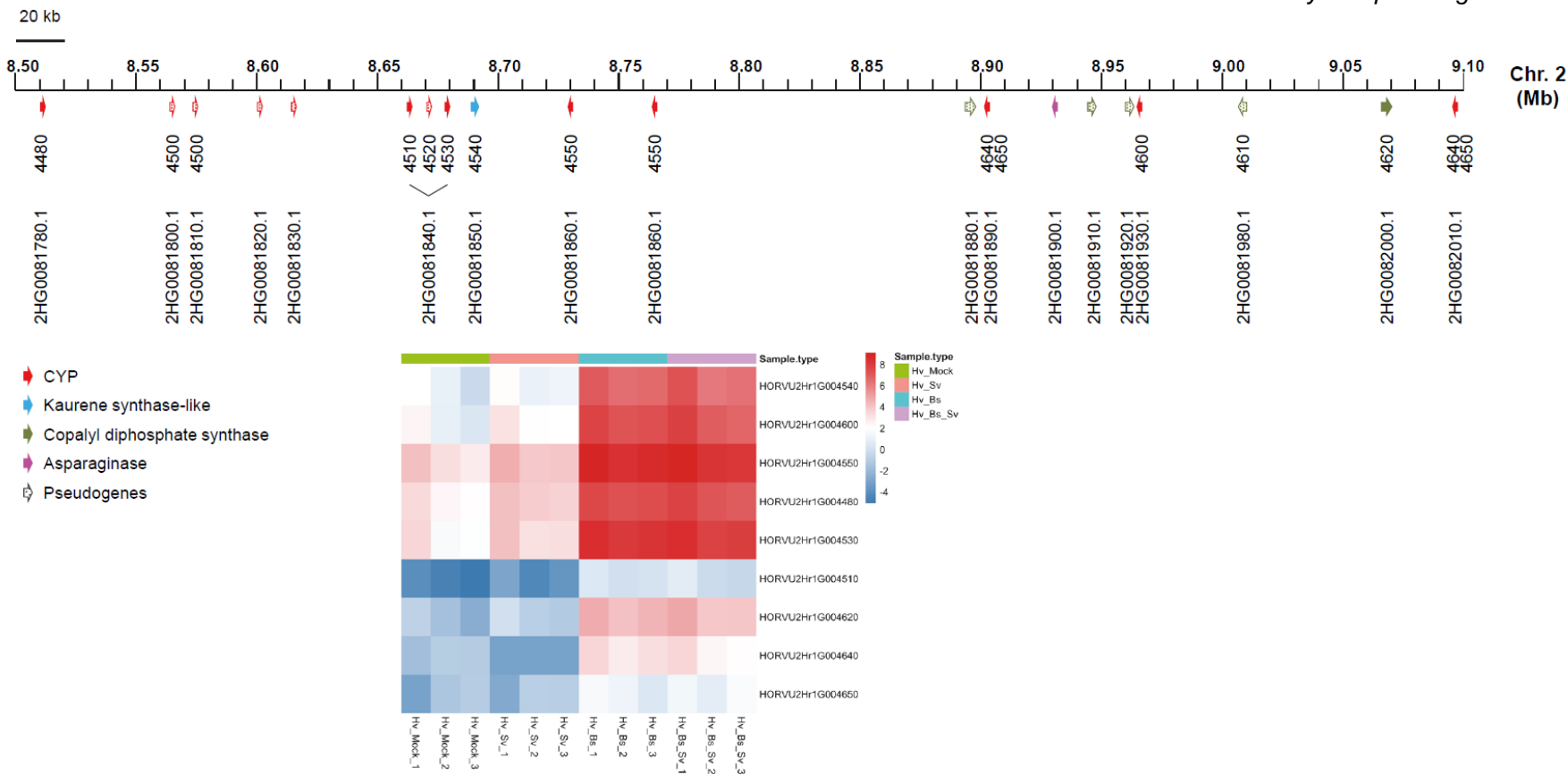
596

597 Notes: (a) the amino acid sequences of these two genes are identical. The sequences only  
598 differ in the 5'-UTR. (b) the amino acid sequences are identical except for one amino acid  
599 change in 2Hr1G004600

600

601 **Figures**

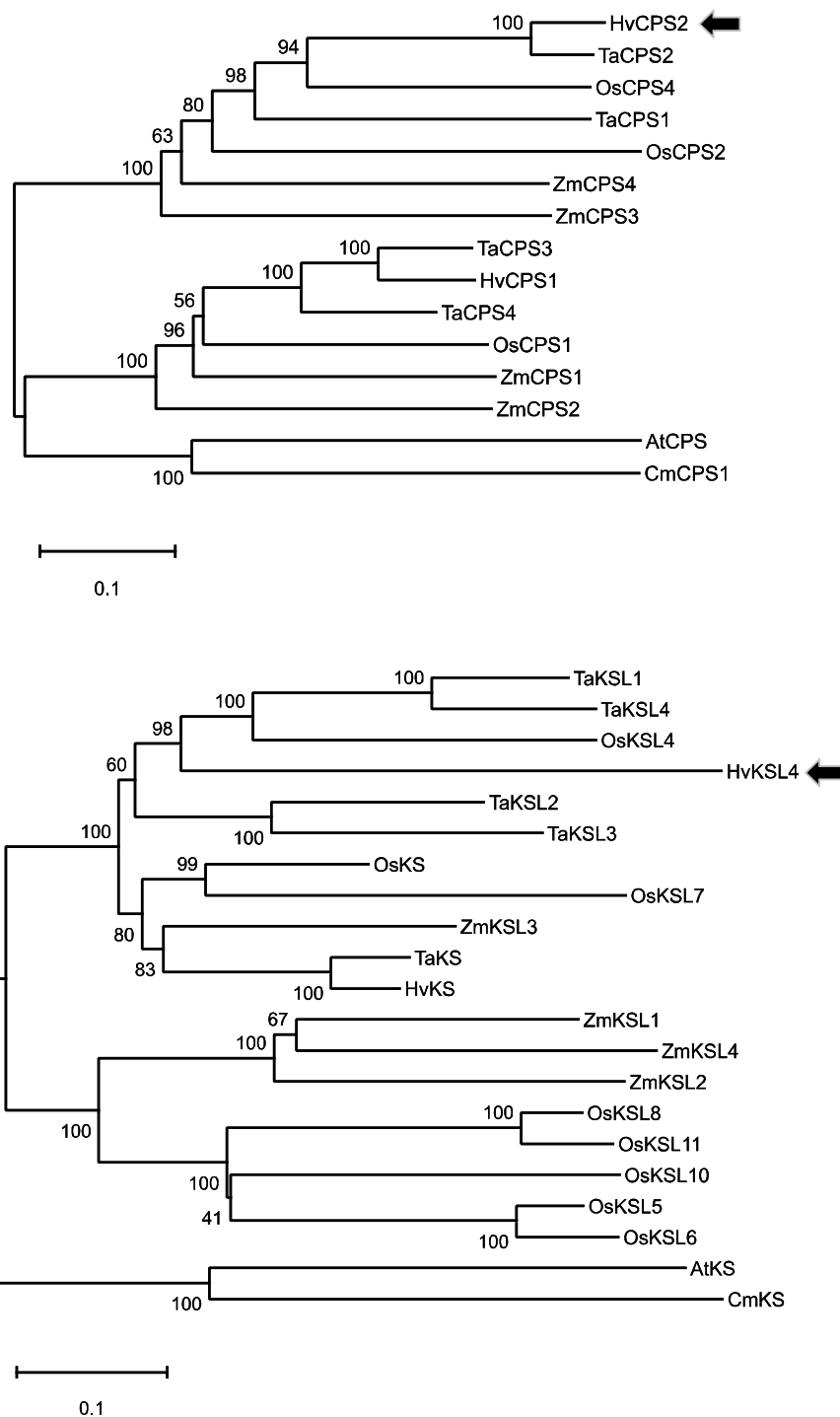
Barley diterpenoid gene cluster



602

603 **Figure 1. Overview of the barley chromosome 2 cluster for diterpenoid biosynthesis.** The scale on top indicates the position on chromosome  
 604 2 based on the latest annotation of the barley genome (Monat et al., 2019). The genes are represented by arrows underneath the scale. The color  
 605 code indicates to which gene family they belong and the dotted pattern the presence of a pseudogene. The first row of numbers below the scale  
 606 indicates the last 4 digits of the gene ID according to MorexV1 gene models and the second row gives the gene identification in the MorexV2  
 607 version of the barley genome. The colored bars in the lower part represent gene expression values as log2-transformed FKPM values for genes  
 608 that show differential gene expression ( |fold change| > 2, data from Sarkar et al., 2019). Each square for a sample type represents data from a  
 609 biological replicate. The samples types are indicated by the following color code: purple: barley root co-inoculated with *B. sorokiniana* and *S.*  
 610 *vermifera*; turquoise blue: barley roots inoculated with *B. sorokiniana*; pink: barley roots inoculated with *S. vermifera*; green: barley root mock  
 611 inoculated.

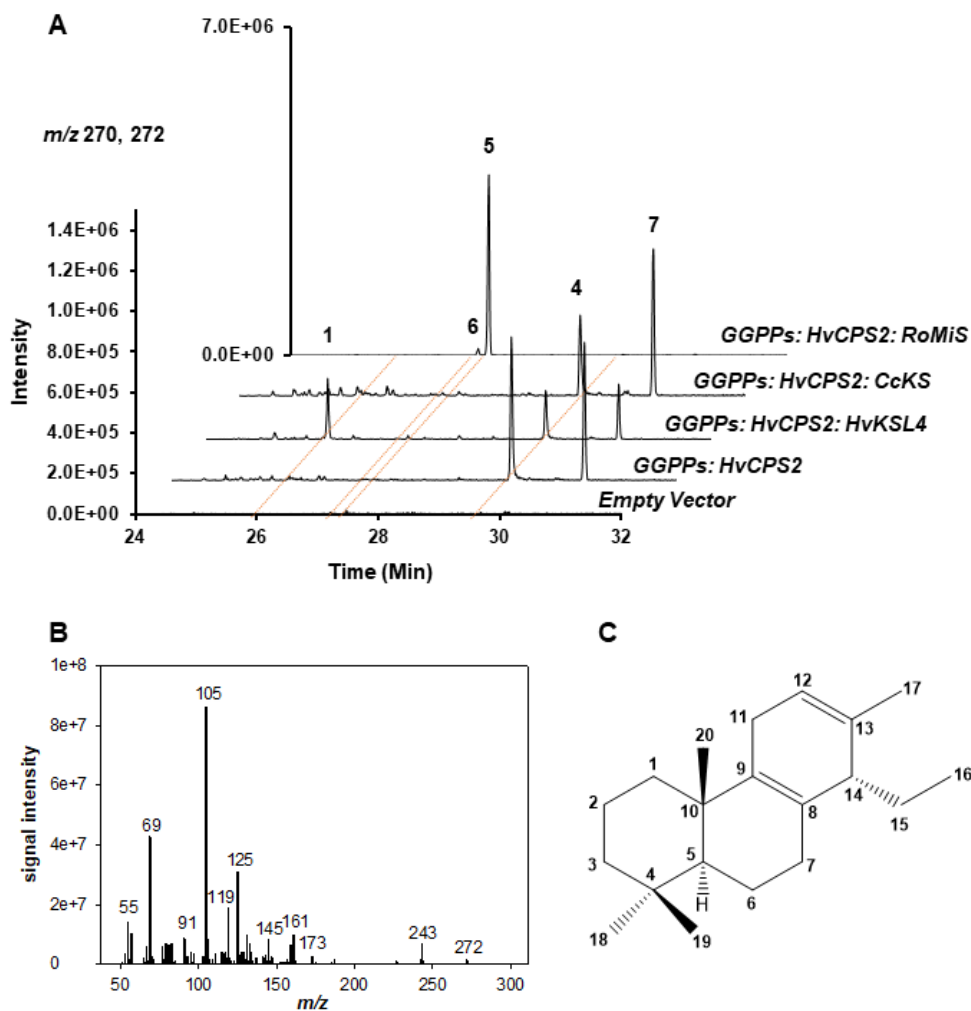
*Barley diterpenoid gene cluster*



612

0.1

613 **Figure 2. Phylogenetic analysis of HvCPS2 and HvKSL4.** The sequences indicated were  
614 aligned and processed with the MEGA X software (Kumar et al., 2018) with the maximum  
615 likelihood method and 1000 bootstrap replications. The consensus trees are shown with the  
616 bootstrap values for the individual branches shown. The HvCPS2 and HvKSL4 sequences  
617 are indicated by grey arrows. The list of sequences used is provided in **Tables S2 and S3.**



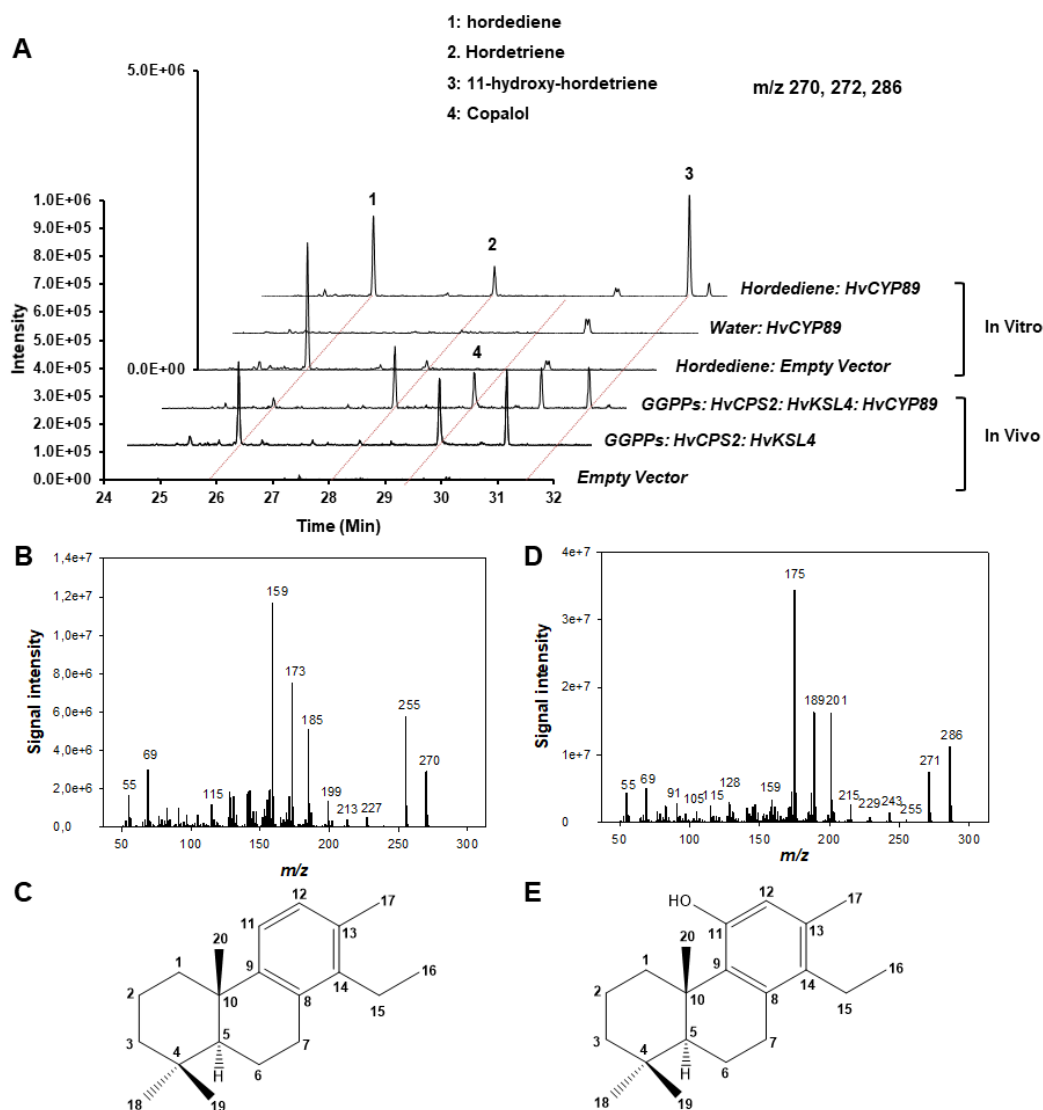
618

619

620 **Figure 3. Expression of HvCPS2 and HvKSL4 in yeast.** A. Selected ion ( $m/z$  270 and 272)  
621 GC-MS chromatograms of *n*-hexane extracts of yeast strains expressing the gene  
622 combinations indicated on the right. CcKS: *ent*-kaurene synthase from coffee (*Coffea*  
623 *canephora*); RoMiS, miltiradiene synthase from rosemary (*Rosmarinus officinalis*). **1**:  
624 hordediene; **4**: (+)-copalol; **5**: miltiradiene; **6**: abietatriene; **7**: unknown copalol derivative. B.  
625 EI mass spectrum of hordediene (**1**). Structure of hordediene as determined by NMR.

626

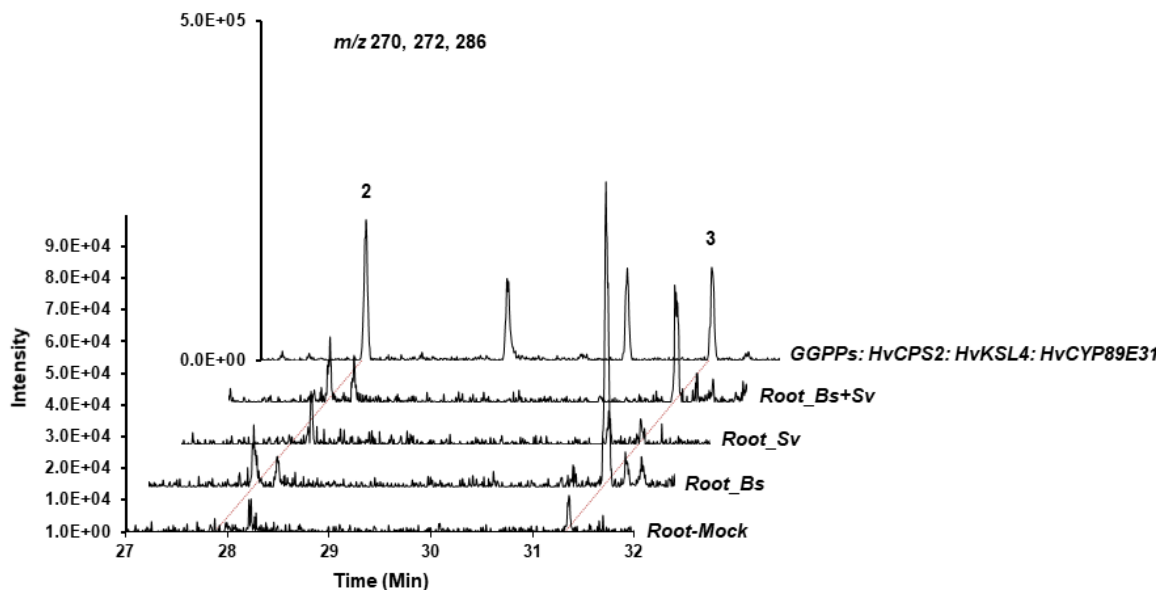
Barley diterpenoid gene cluster



627

628 **Figure 4. Characterization of HvCYP89E31.** A. GC-MS chromatograms (single ion  
 629 monitoring for  $m/z$  270, 272 and 286). The in vivo chromatograms show analysis of extracts  
 630 from transient assays in *N. benthamiana*. The in vitro chromatograms show analysis of in  
 631 vitro assays with microsomal fractions from yeast expressing HvCYP89E31. The identity of  
 632 peaks is indicated by numbers. B and C. Respectively EI mass spectrum and structure of  
 633 compound **2**, hordetriene. D and E. Respectively EI mass spectrum and structure of  
 634 compound **3**, 11-hydroxy-hordetriene.

635

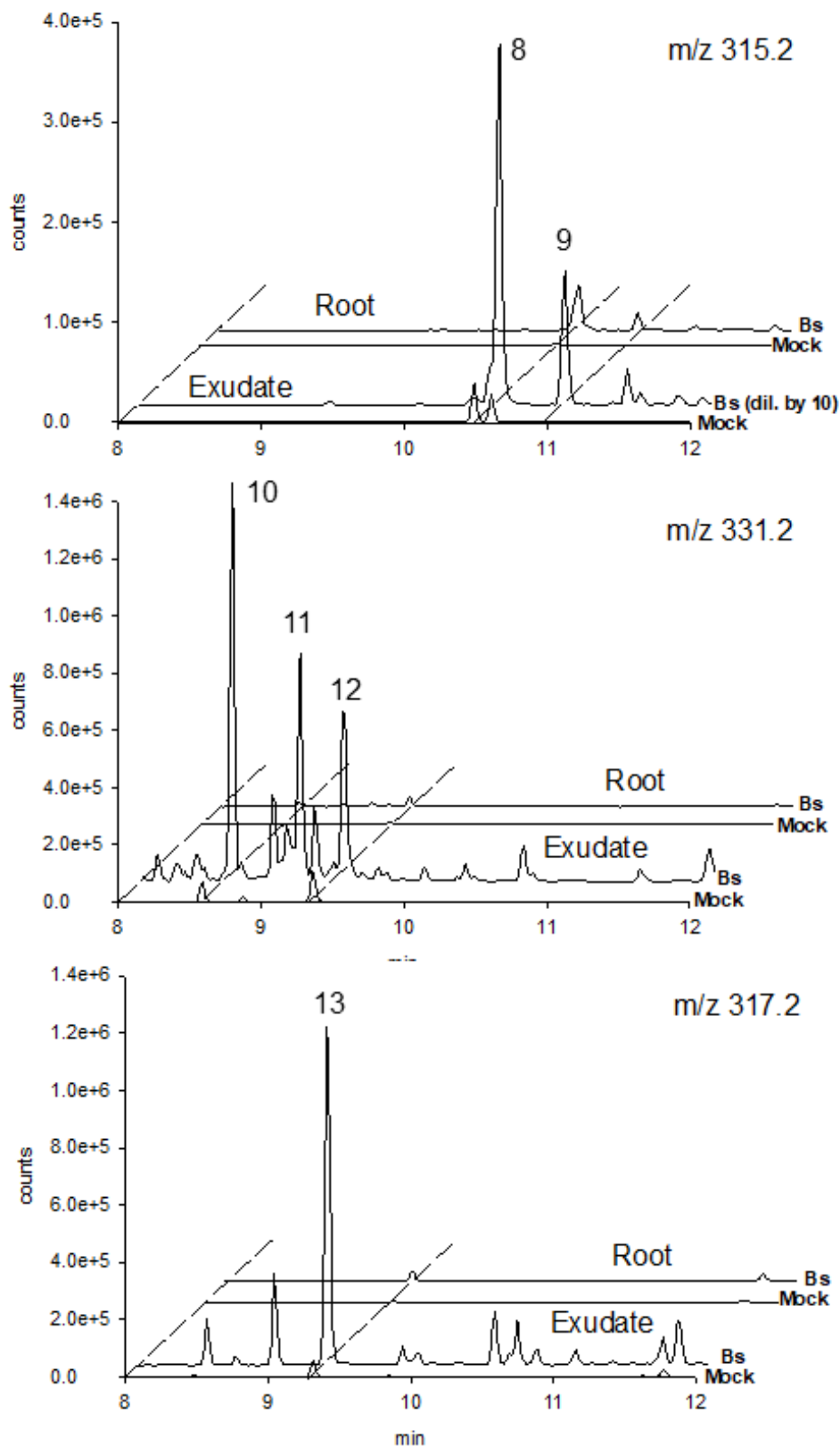


636

637

638 **Figure 5. GC-MS chromatograms of extracts from barley roots infected with *B.***  
639 ***sorokiniana* and/or *S. vermifera*.** Single ion monitoring ( $m/z$  270, 272 and 286) of extracts  
640 from roots infected with *B. sorokiniana* (Root\_Bs), *S. vermifera* (Root\_Sv) or both  
641 (Root\_Bs+Sv) or mock inoculated (Root-Mock). An extract of yeast strain expressing  
642 HvCPS2, HvKSL4 and HvCYP89E31 is shown as a reference. **2:** hordetriene; **3:** 11-hydroxy-  
643 hordetriene.

Barley diterpenoid gene cluster



644

645 **Figure 6. LC(-)ESI-QToF-MS of major diterpene metabolites in roots and root exudates**  
646 **of barley 6 dpi with *B. sorokiniana* (Bs) and in respective mock infected controls. HR-**  
647 **MS/MS spectra of compounds 8-13 are presented in Fig. S3.**



648 **References**

- 649 **Ahuja, I., Kissen, R., and Bones, A.M.** (2012). Phytoalexins in defense against pathogens.  
650 Trends in Plant Science **17**, 73-90.
- 651 **Akatsuka, T., Kodama, O., Kato, H., Kono, Y., and Takeuchi, S.** (1983). Short  
652 Communication 3-Hydroxy-7-oxo-sandaracopimaradiene (Oryzalexin A), a New  
653 Phytoalexin Isolated from Rice Blast Leaves. Agricultural and Biological Chemistry  
654 **47**, 445-447.
- 655 **Awika, J.M.** (2011). Major Cereal Grains Production and Use around the World. In Advances  
656 in Cereal Science: Implications to Food Processing and Health Promotion (American  
657 Chemical Society), pp. 1-13.
- 658 **Ayer, W.A., and Khan, A.Q.** (1996). Zythiostromic acids, diterpenoids from an antifungal  
659 Zythiostroma species associated with aspen. Phytochemistry **42**, 1647-1652.
- 660 **Badr, A., M, K., Sch, R., Rabey, H.E., Effgen, S., Ibrahim, H.H., Pozzi, C., Rohde, W., and**  
661 **Salamini, F.** (2000). On the Origin and Domestication History of Barley (*Hordeum*  
662 *vulgare*). Molecular Biology and Evolution **17**, 499-510.
- 663 **Bathe, U., and Tissier, A.** (2019). Cytochrome P450 enzymes: A driving force of plant  
664 diterpene diversity. Phytochemistry **161**, 149-162.
- 665 **Beier, S., Himmelbach, A., Colmsee, C., Zhang, X.-Q., Barrero, R.A., Zhang, Q., Li, L.,**  
666 **Bayer, M., Bolser, D., Taudien, S., Groth, M., Felder, M., Hastie, A., Šimková, H.,**  
667 **Staňková, H., Vrána, J., Chan, S., Muñoz-Amatriaín, M., Ounit, R., Wanamaker,**  
668 **S., Schmutzer, T., Aliyeva-Schnorr, L., Grasso, S., Tanskanen, J., Sampath, D.,**  
669 **Heavens, D., Cao, S., Chapman, B., Dai, F., Han, Y., Li, H., Li, X., Lin, C.,**  
670 **McCooke, J.K., Tan, C., Wang, S., Yin, S., Zhou, G., Poland, J.A., Bellgard, M.I.,**  
671 **Houben, A., Doležel, J., Ayling, S., Lonardi, S., Langridge, P., Muehlbauer, G.J.,**  
672 **Kersey, P., Clark, M.D., Caccamo, M., Schulman, A.H., Platzer, M., Close, T.J.,**  
673 **Hansson, M., Zhang, G., Braumann, I., Li, C., Waugh, R., Scholz, U., Stein, N.,**  
674 **and Mascher, M.** (2017). Construction of a map-based reference genome sequence  
675 for barley, *Hordeum vulgare* L. Scientific Data **4**, 170044.
- 676 **Bohlmann, J., Meyer-Gauen, G., and Croteau, R.** (1998). Plant terpenoid synthases:  
677 Molecular biology and phylogenetic analysis. Proc. Natl. Acad. Sci. U. S. A. **95**, 4126-  
678 4133.
- 679 **Brückner, K., Božić, D., Manzano, D., Papaefthimiou, D., Pateraki, I., Scheler, U., Ferrer,**  
680 **A., de Vos, R.C., Kanellis, A.K., and Tissier, A.** (2014). Characterization of two  
681 genes for the biosynthesis of abietane-type diterpenes in rosemary (*Rosmarinus*  
682 *officinalis*) glandular trichomes. Phytochemistry **101**, 52-64.
- 683 **Cartwright, D., Langcake, P., Pryce, R.J., Leworthy, D.P., and Ride, J.P.** (1977).  
684 Chemical activation of host defence mechanisms as a basis for crop protection.  
685 Nature **267**, 511-513.
- 686 **Cheng, L., Ji, K.-l., Liao, S.-g., Gan, L.-s., Yang, L., Cao, D.-h., Liu, Y.-q., Guo, J., Zhang,**  
687 **P., Lu, C.-l., Hu, H.-b., and Xu, Y.-k.** (2016). Diterpenoids and phenanthrenones  
688 from the leaves and stems of *Strophoblachia fimbriicalyx*. Tetrahedron Letters **57**,  
689 2262-2265.
- 690 **Deshmukh, S., Hueckelhoven, R., Schaefer, P., Imani, J., Sharma, M., Weiss, M., Waller,**  
691 **F., and Kogel, K.H.** (2006). The root endophytic fungus *Piriformospora indica*  
692 requires host cell death for proliferation during mutualistic symbiosis with barley. P  
693 Natl Acad Sci USA **103**, 18450-18457.
- 694 **Ding, Y., Weckwerth, P.R., Poretsky, E., Murphy, K.M., Sims, J., Saldivar, E.,**  
695 **Christensen, S.A., Char, S.N., Yang, B., Tong, A.-d., Shen, Z., Kremling, K.A.,**  
696 **Buckler, E.S., Kono, T., Nelson, D.R., Bohlmann, J., Bakker, M.G., Vaughan,**  
697 **M.M., Khalil, A.S., Betsiashvili, M., Dressano, K., Köllner, T.G., Briggs, S.P.,**  
698 **Zerbe, P., Schmelz, E.A., and Huffaker, A.** (2020). Genetic elucidation of  
699 interconnected antibiotic pathways mediating maize innate immunity. Nature Plants **6**,  
700 1375-1388.
- 701 **Hedden, P., and Kamiya, Y.** (1997). GIBBERELLIN BIOSYNTHESIS: Enzymes, Genes and  
702 Their Regulation. Annu Rev Plant Physiol Plant Mol Biol **48**, 431-460.

- 703 **Hensel, G.** (2020). Genetic transformation of Triticeae cereals – Summary of almost three-  
704 decade's development. *Biotechnology Advances* **40**, 107484.
- 705 **Huang, A.C.C., Jiang, T., Liu, Y.X., Bai, Y.C., Reed, J., Qu, B.Y., Goossens, A.,**  
706 **Nutzmann, H.W., Bai, Y., and Osbourn, A.** (2019). A specialized metabolic network  
707 selectively modulates Arabidopsis root microbiota. *Science* **364**, 546-+.
- 708 **Huffaker, A., Kaplan, F., Vaughan, M.M., Dafoe, N.J., Ni, X., Rocca, J.R., Alborn, H.T.,**  
709 **Teal, P.E.A., and Schmelz, E.A.** (2011). Novel Acidic Sesquiterpenoids Constitute a  
710 Dominant Class of Pathogen-Induced Phytoalexins in Maize *Plant Physiology* **156**,  
711 2082-2097.
- 712 **Inoue, Y., Sakai, M., Yao, Q., Tanimoto, Y., Toshima, H., and Hasegawa, M.** (2013).  
713 Identification of a Novel Casbane-Type Diterpene Phytoalexin, *ent-10-Oxodepressin*,  
714 from Rice Leaves. *Bioscience, Biotechnology, and Biochemistry* **77**, 760-765.
- 715 **Ishihara, A., Kumeda, R., Hayashi, N., Yagi, Y., Sakaguchi, N., Kokubo, Y., Ube, N.,**  
716 **Tebayashi, S.-i., and Ueno, K.** (2017). Induced accumulation of tyramine, serotonin,  
717 and related amines in response to *Bipolaris sorokiniana* infection in barley.  
718 *Bioscience, Biotechnology, and Biochemistry* **81**, 1090-1098.
- 719 **Karre, S., Kumar, A., Dhokane, D., and Kushalappa, A.C.** (2017). Metabolo-transcriptome  
720 profiling of barley reveals induction of chitin elicitor receptor kinase gene (*HvCERK1*)  
721 conferring resistance against *Fusarium graminearum*. *Plant Mol.Biol.* **93**, 247-267.
- 722 **Kato, H., Kodama, O., and Akatsuka, T.** (1993). Oryzalexin E, A diterpene phytoalexin from  
723 UV-irradiated rice leaves. *Phytochemistry* **33**, 79-81.
- 724 **Kato, H., Kodama, O., and Akatsuka, T.** (1994). Oryzalexin F, a diterpene phytoalexin from  
725 UV-irradiated rice leaves. *Phytochemistry* **36**, 299-301.
- 726 **Kato, T., Kabuto, C., Sasaki, N., Tsunagawa, M., Aizawa, H., Fujita, K., Kato, Y.,**  
727 **Kitahara, Y., and Takahashi, N.** (1973). Momilactones, growth inhibitors from rice,  
728 *oryza sativa* L. *Tetrahedron Letters* **14**, 3861-3864.
- 729 **Kaufman, T.S., Mischne, M.P., Gonzalez-Sierra, M., and Rueda, E.A.** (1987). Synthesis  
730 and <sup>13</sup>C nuclear magnetic resonance spectral analysis of some diterpenoids related  
731 to the cleistanthane type hydrocarbon isolated from *Amphibolis antarctica*. *Canadian*  
732 *Journal of Chemistry* **65**, 2024-2026.
- 733 **Kitaoka, N., Wu, Y., Zi, J., and Peters, R.J.** (2016). Investigating inducible short-chain  
734 alcohol dehydrogenases/reductases clarifies rice oryzalexin biosynthesis. *Plant J* **88**,  
735 271-279.
- 736 **Kono, Y., Takeuchi, S., Kodama, O., and Akatsuka, T.** (1984). Absolute Configuration of  
737 Oryzalexin A and Structures of Its Related Phytoalexins Isolated from Rice Blast  
738 Leaves Infected with *Pyricularia oryzae*. *Agricultural and Biological Chemistry* **48**,  
739 253-255.
- 740 **Kono, Y., Uzawa, J., Kobayashi, K., Suzuki, Y., Uramoto, M., Sakurai, A., Watanabe, M.,**  
741 **Teraoka, T., Hosokawa, D., Watanabe, M., and Kondo, H.** (1991). Structures of  
742 Oryzalides A and B, and Oryzalic Acid A, a Group of Novel Antimicrobial Diterpenes,  
743 Isolated from Healthy Leaves of a Bacterial Leaf Blight-resistant Cultivar of Rice  
744 Plant. *Agricultural and Biological Chemistry* **55**, 803-811.
- 745 **Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K.** (2018). MEGA X: Molecular  
746 Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol* **35**, 1547-  
747 1549.
- 748 **Li, L., Chen, X., Ma, C., Wu, H., and Qi, S.** (2016). *Piriformospora indica* requires kaurene  
749 synthase activity for successful plant colonization. *Plant Physiology and Biochemistry*  
750 **102**, 151-160.
- 751 **Liang, J., Shen, Q., Wang, L., Liu, J., Fu, J., Le, Z., Xu, M., Peters, R.J., and Wang, Q.**  
752 (2021). Rice contains a biosynthetic gene cluster associated with production of the  
753 casbane-type diterpenoid phytoalexin *ent-10-oxodepressin*. *New Phytol.*
- 754 **Livak, K.J., and Schmittgen, T.D.** (2001). Analysis of relative gene expression data using  
755 real-time quantitative PCR and the 2(- $\Delta\Delta C_T$ ) method. *Methods* **25**, 402-  
756 408.

- 757 **Long, R.M., Lagisetti, C., Coates, R.M., and Croteau, R.B.** (2008). Specificity of the N-  
758 benzoyl transferase responsible for the last step of Taxol biosynthesis. *Arch Biochem*  
759 *Biophys* **477**, 384-389.
- 760 **Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S.O., Wicker, T.,**  
761 **Radchuk, V., Dockter, C., Hedley, P.E., Russell, J., Bayer, M., Ramsay, L., Liu,**  
762 **H., Haberer, G., Zhang, X.-Q., Zhang, Q., Barrero, R.A., Li, L., Taudien, S., Groth,**  
763 **M., Felder, M., Hastie, A., Šimková, H., Staňková, H., Vrána, J., Chan, S., Muñoz-**  
764 **Amatriain, M., Ounit, R., Wanamaker, S., Bolser, D., Colmsee, C., Schmutzer, T.,**  
765 **Aliyeva-Schnorr, L., Grasso, S., Tanskanen, J., Chailyan, A., Sampath, D.,**  
766 **Heavens, D., Clissold, L., Cao, S., Chapman, B., Dai, F., Han, Y., Li, H., Li, X., Lin,**  
767 **C., McCooke, J.K., Tan, C., Wang, P., Wang, S., Yin, S., Zhou, G., Poland, J.A.,**  
768 **Bellgard, M.I., Borisjuk, L., Houben, A., Doležel, J., Ayling, S., Lonardi, S.,**  
769 **Kersey, P., Langridge, P., Muehlbauer, G.J., Clark, M.D., Caccamo, M.,**  
770 **Schulman, A.H., Mayer, K.F.X., Platzer, M., Close, T.J., Scholz, U., Hansson, M.,**  
771 **Zhang, G., Braumann, I., Spannagl, M., Li, C., Waugh, R., and Stein, N.** (2017). A  
772 chromosome conformation capture ordered sequence of the barley genome. *Nature*  
773 **544**, 427-433.
- 774 **Massalha, H., Korenblum, E., Tholl, D., and Aharoni, A.** (2017). Small molecules below-  
775 ground: the role of specialized metabolites in the rhizosphere. *The Plant Journal* **90**,  
776 788-807.
- 777 **Miedaner, T., and Juroszek, P.** (2021). Climate change will influence disease resistance  
778 breeding in wheat in Northwestern Europe. *Theor. Appl. Genet.*
- 779 **Monat, C., Padmarasu, S., Lux, T., Wicker, T., Gundlach, H., Himmelbach, A., Ens, J.,**  
780 **Li, C., Muehlbauer, G.J., Schulman, A.H., Waugh, R., Braumann, I., Pozniak, C.,**  
781 **Scholz, U., Mayer, K.F.X., Spannagl, M., Stein, N., and Mascher, M.** (2019).  
782 TRITEX: chromosome-scale sequence assembly of Triticeae genomes with open-  
783 source tools. *Genome Biology* **20**, 284.
- 784 **Muchlinski, A., Jia, M., Tiedge, K., Fell, J.S., Pelot, K.A., Chew, L., Davisson, D., Chen,**  
785 **Y., Siegel, J., Lovell, J.T., and Zerbe, P.** (2021). Furanoditerpenoid biosynthesis in  
786 the bioenergy crop switchgrass is catalyzed by an alternate metabolic pathway.  
787 *bioRxiv*, 2021.2003.2030.437764.
- 788 **Murphy, K.M., and Zerbe, P.** (2020). Specialized diterpenoid metabolism in monocot crops:  
789 Biosynthesis and chemical diversity. *Phytochemistry* **172**, 112289.
- 790 **Murphy, K.M., Edwards, J., Louie, K.B., Bowen, B.P., Sundaresan, V., Northen, T.R.,**  
791 **and Zerbe, P.** (2021). Bioactive diterpenoids impact the composition of the root-  
792 associated microbiome in maize (*Zea mays*). *Sci Rep* **11**, 333.
- 793 **Nakano, C., Okamura, T., Sato, T., Dairi, T., and Hoshino, T.** (2005). Mycobacterium  
794 tuberculosis H37Rv3377c encodes the diterpene cyclase for producing the halimane  
795 skeleton. *Chemical Communications*, 1016-1018.
- 796 **Nützmann, H.W., Huang, A., and Osbourn, A.** (2016). Plant metabolic clusters - from  
797 genetics to genomics. *New Phytol* **211**, 771-789.
- 798 **Otomo, K., Kanno, Y., Motegi, A., Kenmoku, H., Yamane, H., Mitsushashi, W., Oikawa,**  
799 **H., Toshima, H., Itoh, H., Matsuoka, M., Sassa, T., and Toyomasu, T.** (2004).  
800 Diterpene cyclases responsible for the biosynthesis of phytoalexins, momilactones A,  
801 B, and oryzalexins A-F in rice. *Biosci Biotechnol Biochem* **68**, 2001-2006.
- 802 **Pelot, K.A., Chen, R., Hagelthorn, D.M., Young, C.A., Addison, J.B., Muchlinski, A.,**  
803 **Tholl, D., and Zerbe, P.** (2018). Functional Diversity of Diterpene Synthases in the  
804 Biofuel Crop Switchgrass. *Plant Physiol* **178**, 54-71.
- 805 **Pelot, K.A., Mitchell, R., Kwon, M., Hagelthorn, L.M., Wardman, J.F., Chiang, A.,**  
806 **Bohlmann, J., Ro, D.K., and Zerbe, P.** (2017). Biosynthesis of the psychotropic  
807 plant diterpene salvinatorin A: Discovery and characterization of the *Salvia divinorum*  
808 clerodienyl diphosphate synthase. *Plant J* **89**, 885-897.
- 809 **Peters, R.J.** (2010). Two rings in them all: The labdane-related diterpenoids. *Natural Product*  
810 *Reports* **27**, 1521-1530.
- 811 **Pichersky, E., and Lewinsohn, E.** (2011). Convergent Evolution in Plant Specialized  
812 Metabolism. *Annual Review of Plant Biology* **62**, 549-566.

- 813 **Pichersky, E., Noel, J.P., and Dudareva, N.** (2006). Biosynthesis of Plant Volatiles:  
814 Nature's Diversity and Ingenuity. *Science* **311**, 808-811.
- 815 **Riehl, C.A.S., and Pinto, A.C.** (2000). A cleistanthane diterpene lactone from *Vellozia*  
816 *compacta*. *Phytochemistry* **53**, 917-919.
- 817 **Riehl, S.** (2019). *Barley in Archaeology and Early History* (Oxford University Press).
- 818 **Rontein, D., Onillon, S., Herbette, G., Lesot, A., Werck-Reichhart, D., Sallaud, C., and**  
819 **Tissier, A.** (2008). CYP725A4 from yew catalyzes complex structural rearrangement  
820 of taxa-4(5),11(12)-diene into the cyclic ether 5(12)-oxa-3(11)-cyclotaxane. *J Biol*  
821 *Chem* **283**, 6067-6075.
- 822 **Rosyara, U.R., Subedi, S., Duveiller, E., and Sharma, R.C.** (2010). The effect of spot  
823 blotch and heat stress on variation of canopy temperature depression, chlorophyll  
824 fluorescence and chlorophyll content of hexaploid wheat genotypes. *Euphytica* **174**,  
825 377-390.
- 826 **Sallaud, C., Giacalone, C., Töpfer, R., Goepfert, S., Bakaher, N., Rösti, S., and Tissier,**  
827 **A.** (2012). Characterization of two genes for the biosynthesis of the labdane diterpene  
828 Z-abienol in tobacco (*Nicotiana tabacum*) glandular trichomes. *Plant J* **72**, 1-17.
- 829 **Sarkar, D., Rovenich, H., Jeena, G., Nizam, S., Tissier, A., Balcke, G.U., Mahdi, L.K.,**  
830 **Bonkowski, M., Langen, G., and Zuccaro, A.** (2019). The inconspicuous  
831 gatekeeper: endophytic *Serendipita vermifera* acts as extended plant protection  
832 barrier in the rhizosphere. *New Phytol* **224**, 886-901.
- 833 **Scheler, U., Brandt, W., Porzel, A., Rothe, K., Manzano, D., Božić, D., Papaefthimiou, D.,**  
834 **Balcke, G.U., Henning, A., Lohse, S., Marillonnet, S., Kanellis, A.K., Ferrer, A.,**  
835 **and Tissier, A.** (2016). Elucidation of the biosynthesis of carnosic acid and its  
836 reconstitution in yeast. *Nat Commun* **7**, 12942.
- 837 **Schmelz, E.A., Kaplan, F., Huffaker, A., Dafeo, N.J., Vaughan, M.M., Ni, X., Rocca, J.R.,**  
838 **Alborn, H.T., and Teal, P.E.** (2011). Identity, regulation, and activity of inducible  
839 diterpenoid phytoalexins in maize. *Proceedings of the National Academy of Sciences*  
840 **108**, 5455-5460.
- 841 **Sekido, H., Endo, T., Suga, R., Kodama, O., Akatsuka, T., Kono, Y., and Takeuchi, S.**  
842 (1986). Oryzalexin D (3, 7-Dihydroxy-(+)-sandaracopimaradiene), a New Phytoalexin  
843 Isolated from Blast-infected Rice Leaves. *Journal of Pesticide Science* **11**, 369-372.
- 844 **Shimura, K., Okada, A., Okada, K., Jikumaru, Y., Ko, K.-W., Toyomasu, T., Sassa, T.,**  
845 **Hasegawa, M., Kodama, O., Shibuya, N., Koga, J., Nojiri, H., and Yamane, H.**  
846 (2007). Identification of a Biosynthetic Gene Cluster in Rice for Momilactones. *Journal*  
847 *of Biological Chemistry* **282**, 34013-34018.
- 848 **Shiono, Y., Ogata, K., Koseki, T., Murayama, T., and Funakoshi, T.** (2010). A  
849 Cleistanthane Diterpene From a Marine-derived *Fusarium* Species Under Submerged  
850 Fermentation. *Zeitschrift für Naturforschung B* **65**, 753-756.
- 851 **Spielmeier, W., Ellis, M., Robertson, M., Ali, S., Lenton, J.R., and Chandler, P.M.** (2004).  
852 Isolation of gibberellin metabolic pathway genes from barley and comparative  
853 mapping in barley, wheat and rice. *Theor Appl Genet* **109**, 847-855.
- 854 **Tang, H., Bomhoff, M.D., Briones, E., Zhang, L., Schnable, J.C., and Lyons, E.** (2015).  
855 SynFind: Compiling Syntenic Regions across Any Set of Genomes on Demand.  
856 *Genome Biology and Evolution* **7**, 3286-3298.
- 857 **Thiel, T., Graner, A., Waugh, R., Grosse, I., Close, T.J., and Stein, N.** (2009). Evidence  
858 and evolutionary analysis of ancient whole-genome duplication in barley predating the  
859 divergence from rice. *BMC Evolutionary Biology* **9**, 209.
- 860 **Ube, N., Nishizaka, M., Ichyanagi, T., Ueno, K., Taketa, S., and Ishihara, A.** (2017).  
861 Evolutionary changes in defensive specialized metabolism in the genus *Hordeum*.  
862 *Phytochemistry* **141**, 1-10.
- 863 **Ube, N., Katsuyama, Y., Kariya, K., Tebayashi, S., Sue, M., Tohnooka, T., Ueno, K.,**  
864 **Taketa, S., and Ishihara, A.** (2021). Identification of methoxylchalcones produced in  
865 response to CuCl<sub>2</sub> treatment and pathogen infection in barley. *Phytochemistry* **184**,  
866 10.
- 867 **Urban, P., Mignotte, C., Kazmaier, M., Delorme, F., and Pompon, D.** (1997). Cloning,  
868 yeast expression, and characterization of the coupling of two distantly related

- 869 Arabidopsis thaliana NADPH-cytochrome P450 reductases with P450 CYP73A5. J  
870 Biol Chem **272**, 19176-19186.
- 871 **Wang, Q., Hillwig, M.L., and Peters, R.J.** (2011). CYP99A3: functional identification of a  
872 diterpene oxidase from the momilactone biosynthetic gene cluster in rice. The Plant  
873 Journal **65**, 87-95.
- 874 **Watanabe, M., Sakai, Y., Teraoka, T., Abe, H., Kono, Y., Uzawa, J., Kobayashi, K.,**  
875 **Suzuki, Y., and Sakurai, A.** (1990). Novel C19-Kaurane Type of Diterpene  
876 (Oryzalide A), a New Antimicrobial Compound Isolated from Healthy Leaves of a  
877 Bacterial Leaf Blight-resistant Cultivar of Rice Plant. Agricultural and Biological  
878 Chemistry **54**, 1103-1105.
- 879 **Wawra, S., Fesel, P., Widmer, H., Timm, M., Seibel, J., Leson, L., Kessler, L., Nostadt,**  
880 **R., Hilbert, M., Langen, G., and Zuccaro, A.** (2016). The fungal-specific  $\beta$ -glucan-  
881 binding lectin FGB1 alters cell-wall composition and suppresses glucan-triggered  
882 immunity in plants. Nature Communications **7**, 13188.
- 883 **Weber, E., Gruetzner, R., Werner, S., Engler, C., and Marillonnet, S.** (2011). Assembly of  
884 Designer TAL Effectors by Golden Gate Cloning. Plos One **6**.
- 885 **Wu, Y., Zhou, K., Toyomasu, T., Sugawara, C., Oku, M., Abe, S., Usui, M., Mitsuhashi,**  
886 **W., Chono, M., Chandler, P.M., and Peters, R.J.** (2012). Functional characterization  
887 of wheat copalyl diphosphate synthases sheds light on the early evolution of labdane-  
888 related diterpenoid metabolism in the cereals. Phytochemistry **84**, 40-46.
- 889 **Yadav, H., Dreher, D., Athmer, B., Porzel, A., Gavrin, A., Baldermann, S., Tissier, A.,**  
890 **and Hause, B.** (2019). Medicago TERPENE SYNTHASE 10 Is Involved in Defense  
891 Against an Oomycete Root Pathogen. Plant Physiol **180**, 1598-1613.
- 892 **Zheng, X.-H., Yang, J., Lv, J.-J., Zhu, H.-T., Wang, D., Xu, M., Yang, C.-R., and Zhang,**  
893 **Y.-J.** (2018). Phyllaciduloids A–D: Four new cleistanthane diterpenoids from  
894 Phyllanthus acidus (L.) Skeels. Fitoterapia **125**, 89-93.
- 895 **Zhou, K., Xu, M., Tiernan, M., Xie, Q., Toyomasu, T., Sugawara, C., Oku, M., Usui, M.,**  
896 **Mitsuhashi, W., Chono, M., Chandler, P.M., and Peters, R.J.** (2012). Functional  
897 characterization of wheat ent-kaurene(-like) synthases indicates continuing evolution  
898 of labdane-related diterpenoid metabolism in the cereals. Phytochemistry **84**, 47-55.
- 899 **Zi, J.C., Matsuba, Y., Hong, Y.J., Jackson, A.J., Tantillo, D.J., Pichersky, E., and Peters,**  
900 **R.J.** (2014). Biosynthesis of Lycosantalanol, a cis-Prenyl Derived Diterpenoid. J. Am.  
901 Chem. Soc. **136**, 16951-16953.  
902