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1 **RESEARCH ARTICLE**

2 **A single amino acid transporter controls the uptake of priming-inducing beta-**
3 **amino acids and the associated trade-off between induced resistance and plant**
4 **growth.**

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14

15 **Key Words:** BABA, RBH, defense priming agents, amino acid transporter, LHT1,
16 *Arabidopsis*, defense-growth trade-off.

17

18 **Short title:** The cellular transporter of RBH and BABA

19 **One-sentence summary:** A forward genetic screen revealed the transporter of two
20 resistance-inducing beta-amino acids, BABA and RBH, which balance growth and induced
21 resistance

22

23 The author(s) responsible for distribution of materials integral to the findings presented in this
24 article in accordance with the policy described in the Instructions for Authors
25 (<https://academic.oup.com/plcell/pages/General-Instructions>) is: Jurriaan Ton
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28 ABSTRACT

29 Selected beta-amino acids, such as beta-aminobutyric acid (BABA) and R-beta-homoserine
30 (RBH), can prime plants for resistance against a broad spectrum of diseases. Here, we
31 describe a genome-wide screen of fully annotated *Arabidopsis thaliana* T-DNA insertion
32 lines for *impaired in RBH-induced immunity (iri)* mutants against the downy mildew
33 pathogen *Hyaloperonospora arabidopsidis*, yielding 104 lines that were partially affected and
34 four lines that were completely impaired in RBH-induced resistance. We confirmed the *iri1-1*
35 mutant phenotype with an independent T-DNA insertion line in the same gene, encoding the
36 high-affinity amino acid transporter LYSINE HISTIDINE TRANSPORTER 1 (LHT1).
37 Uptake experiments with yeast cells expressing *LHT1* and mass spectrometry-based
38 quantification of RBH and BABA in leaves of *lht1* mutant and *LHT1* overexpression lines
39 revealed that LHT1 acts as the main transporter for cellular uptake and systemic distribution
40 of RBH and BABA. Subsequent characterization of *lht1* mutant and *LHT1* overexpression
41 lines for induced resistance and growth responses revealed that the levels of LHT1-mediated
42 uptake determine the trade-off between induced resistance and plant growth by RBH and
43 BABA.

44

45 IN A NUTSHELL

46 **Background:** Specific chemicals can induce long-lasting disease resistance in plants. These
47 chemicals act by mediating a form of immune memory, called ‘priming’, which enables the
48 plant to activate a faster and/or stronger defence response upon future pathogen attack. The
49 beta-amino acids beta-aminobutyric acid (BABA) and R-beta-homoserine (RBH) are
50 particularly effective in priming taxonomically unrelated plants against a wide range of
51 diseases. Previous research from our lab has shown that BABA and RBH, despite their
52 structural similarity, are perceived and controlled by different receptors and pathways.
53 However, the transporter responsible for the cellular uptake of these two priming agents has
54 remained unknown.

55 **Question:** To identify new genes controlling RBH-induced resistance in Arabidopsis, we
56 carried out a genetic screen for Arabidopsis mutants that are impaired in RBH-induced
57 immunity against the downy mildew pathogen *Hyaloperonospora arabidopsidis* (*Hpa*). The
58 first mutant that we isolated turned out to be affected in the high-affinity amino acid
59 transporter LYSINE HISTIDINE TRANSPORTER 1 (LHT1).

60 **Findings:** Experiments to characterize the Arabidopsis *lht1* mutant demonstrated that LHT1
61 controls resistance induced by RBH and BABA by controlling the uptake of these chemicals
62 from the soil. Competition experiments with the LHT1 substrate L-alanine and yeast cells
63 expressing the *LHT1* gene confirmed that LHT1 acts as a high-affinity transporter of RBH
64 and BABA. Subsequent characterization of mutant and over-expression lines of Arabidopsis

65 revealed that the uptake level by LHT1 controls not only the resistance response to RBH and
66 BABA, but also the phytotoxic side-effects upon chemical overstimulation with higher
67 concentrations. Hence, LHT1 acts as a master regulator of the trade-off between induced
68 resistance and growth caused by RBH or BABA.

69 **Next steps:** An important take home message from our study is that the trade-off between
70 induced resistance and growth by resistance-inducing beta-amino acids like BABA and RBH
71 can be optimized by manipulating the *LHT1* gene. This conclusion offers major translational
72 opportunities for breeding programs that aim to exploit BABA- and/or RBH-induced
73 resistance in crops, but suffer from the phytotoxicity of these agents.

74

75 INTRODUCTION

76 The innate immune system enables plants to perceive and react to attacks by pathogens and
77 herbivores. The basal component of this regulatory system is under the control of pattern
78 recognition receptors (PRRs) that perceive molecular non-self-patterns from the attacker or
79 damaged-self patterns that form during an attack (Choi and Klessig, 2016). Following
80 recognition of these alarm signals, a signaling network is initiated that orchestrates the
81 induction of cellular defense mechanisms, including reactive oxygen species (ROS), callose-
82 rich cell wall depositions and the induction of defense-related genes (Chisholm et al., 2006;
83 Bigeard et al., 2015). Besides this pattern-triggered immunity (PTI), innate immunity can be
84 triggered by susceptibility-inducing pathogen effectors. If the challenged plant expresses a
85 resistance (R) gene that can recognize the activity of such a pathogen effector, the innate
86 immune response is referred to as effector-triggered immunity (ETI; Cui et al., 2015). In
87 addition to innate immunity, plants can acquire long-lasting resistance, which develops after
88 recovery from biotic stress. This induced resistance (IR) is typically based on the priming of
89 the innate immune system, which mediates a faster and/or stronger induction of inducible
90 defenses upon secondary attack (Wilkinson et al., 2019; De Kesel et al., 2021). Moreover, IR
91 can be triggered by root colonization of selected plant beneficial microbes or treatment with
92 specific chemical agents, such as microbe-associated molecular patterns, volatile organic
93 compounds and non-proteinogenic β -amino acids (Mauch-Mani et al., 2017; De Kesel et al.,
94 2021).

95 β -amino butyric acid-induced resistance (BABA-IR) has emerged as a popular model system
96 to study the molecular mechanisms controlling immune priming in plants. BABA-IR has
97 been reported in more than 40 plant species against different types of pathogens (Cohen,
98 1994; Cohen et al., 2016). In *Arabidopsis* (*Arabidopsis thaliana*), BABA primes both
99 salicylic acid (SA) dependent and independent defense mechanisms and protects plants

100 against biotrophic, hemibiotrophic and necrotrophic pathogens (Zimmerli et al., 2000; Ton et
101 al., 2005; Schwarzenbacher et al., 2020). Recent evidence suggests that BABA accumulates
102 during exposure to biotic and abiotic stress (Thevenet et al., 2017), which provides biological
103 relevance and supports previous evidence that an aspartyl tRNA aspartase, IMPAIRED IN
104 BABA-INDUCED DISEASE IMMUNITY 1 (IBI1), acts as a plant receptor for BABA
105 (Luna et al., 2014). BABA was also suggested to act as a microbial rhizosphere signal, based
106 on the finding that induced systemic resistance (ISR) upon root colonization by *Pseudomonas*
107 *simiae* WCS417 is blocked in the *ibi1-1* mutant (Luna et al., 2014). Despite the apparently
108 high efficiency by which plant roots are capable of taking up BABA from the soil (Zimmerli
109 et al., 2000; Ton et al., 2005), a cellular transporter for this well-known priming agent has not
110 been identified.

111 Although BABA-IR is effective against a broad spectrum of plant diseases, high doses of
112 BABA results in major growth reduction (Wu et al., 2010; Luna et al., 2014). This
113 undesirable side effect is partly caused by disruptive binding of R-BABA to the aspartic acid-
114 binding pocket of the IBI1 enzyme, causing the accumulation of uncharged tRNA^{Asp} and
115 GCN2 (GENERAL CONTROL NON-DEREPRESSIBLE 2)-dependent inhibition of
116 translation (Luna et al., 2014; Buswell et al., 2018). To search for less phytotoxic IR analogs
117 of BABA, we previously screened a small library of structurally related β -amino acids for IR
118 activity and phytotoxicity in Arabidopsis. This screen resulted in the identification of R- β -
119 homoserine (RBH), which induces resistance in Arabidopsis and tomato (*Solanum*
120 *lycopersicum* L.) cultivar Micro-Tom) against biotrophic and necrotrophic pathogens without
121 growth reduction (Buswell et al., 2018). A recent study comparing four IR agents for their
122 effectiveness in strawberry (*Fragaria* \times *ananassa*) against *Botrytis cinerea* also identified
123 RBH as the most effective IR agent without negative effects on plant growth (Badmi et al.,
124 2019). Like BABA, RBH primes defense activity of callose-rich papillae, which in
125 Arabidopsis are formed at relatively early stages of infection by the biotrophic oomycete
126 *Hyaloperonospora arabidopsidis* (*Hpa*). Interestingly, despite its structural similarity to
127 BABA, RBH does not require the IBI1 receptor to induce resistance in Arabidopsis (Buswell
128 et al., 2018). Furthermore, unlike BABA, RBH does not prime salicylic acid (SA)-dependent
129 induction of gene expression but primes camalexin production upon infection by *Hpa* and the
130 expression of jasmonic acid (JA)-dependent defense genes after infection by the necrotrophic
131 fungus *Plectosphaerella cucumerina* (Zimmerli et al., 2000; Ton et al., 2005; Buswell et al.,
132 2018). Hence, RBH-induced resistance (RBH-IR) is controlled by partially distinct pathways

133 relative to BABA-IR. Importantly, the molecular mechanisms responsible for the uptake and
134 perception of RBH are unknown.

135 In this study, we conducted a genome-wide screen of Arabidopsis T-DNA insertion mutants
136 for *impaired in RBH-induced immunity (iri)* phenotypes against *Hpa*, yielding 104 and four
137 lines that are partially and completely impaired in RBH-IR, respectively. Of the latter, we
138 characterized the *iri1* mutant, which is affected in the high-affinity amino acid transporter
139 LYSINE HISTIDINE TRANSPORTER 1 (LHT1). We provide evidence that the level of
140 LHT1-mediated uptake determines the balance between IR and plant tolerance by RBH and
141 BABA. Furthermore, mass spectrometry analysis of leaves from RBH- and BABA-treated
142 wild-type, *lht1* mutant and *LHT1*-overexpressing plants revealed that LHT1 is critical for the
143 uptake and systemic distribution of both RBH and BABA, while uptake experiments with
144 *LHT1*-expressing yeast cells demonstrated that LHT1 acts as a high-affinity transporter of
145 BABA and RBH. In support of other studies that have linked LHT1 to plant-microbe
146 interactions and plant immunity, we conclude that LHT1 acts as a master regulator of the
147 trade-off between growth and IR by priming-inducing beta-amino acids.

148

149 **RESULTS**

150 **Genome-wide screen for *impaired in RBH-immunity (iri)* mutants**

151 To search for new regulatory genes of RBH-induced resistance, we screened 23,547 T-DNA
152 insertion lines from the SALK and SAIL collections (Alonso and Ecker, 2006) for an
153 *impaired in RBH-induced immunity (iri)* phenotype against *Hpa*. This set of T-DNA insertion
154 lines covers >90% of all annotated protein-coding genes in the Arabidopsis genome. In
155 contrast to conventional ethyl methanesulfonate (EMS)-based mutant screens, which rely on
156 the selection of mutant phenotypes in individual plants, the collection of fully annotated
157 homozygous T-DNA insertion mutants allowed us to screen five genetically identical
158 seedlings per line for quantification of the *iri* mutant phenotype, including partial loss of
159 RBH-IR. To reduce false positives, we performed the screen in three successive stages. In the
160 first stage, we screened seedlings in 400-well trays, in which the soil was soaked to saturation
161 with RBH to a final soil concentration of ~0.5 mM, followed by inoculation with *Hpa*
162 conidiospores and scoring for visual sporulation at 5-7 days post inoculation (dpi; Figure 1A).
163 Each tray yielded ~1-2 lines displaying sporulation for at least two seedlings/well by 7 dpi;
164 these lines were selected and rescreened during stage 2, using the same 400-well tray

165 selection system. Stage 2 yielded 427 putative *iri* mutant lines (Figure 1A). These putative *iri*
166 mutant lines were taken forward for final validation in stage 3, which was based on
167 categorical scoring of *Hpa* colonization in trypan-blue-stained leaves from control- and RBH-
168 treated plants (0.5 mM) of each candidate line (Figure 1A). To validate the statistical
169 robustness of this screening stage, we conducted a pilot experiment that compared *Hpa*
170 colonization between 40 pots of Col-0 seedlings pre-treated with either water or RBH (0.5
171 mM). Categorical scoring of trypan blue-stained leaves confirmed statistically uniform
172 distributions of *Hpa* colonization within each treatment (Supplemental Figure 1A). Of the
173 427 putative *iri* lines from stage 2, we confirmed 104 lines as having partially impaired RBH-
174 IR in stage 3, as evidenced by statistically enhanced levels of *Hpa* colonization in RBH-
175 treated mutant plants compared to RBH-treated wild-type plants, while still showing a
176 statistically significant reduction in *Hpa* colonization by RBH treatment compared to the
177 water controls (Figure 1A, Supplemental Figure S1B and Supplemental Data Set S1). An
178 additional four lines, named *iri1-1* to *iri4-1*, showed a full impairment of RBH-IR, as
179 indicated by statistically identical levels of *Hpa* colonization between RBH- and water-
180 treated plants within each line (Figure 1A, Supplemental Figure S1B and Supplemental Data
181 Set S1).

182

183 **Identification of *IRI1/LHT1* as a critical regulator of RBH-IR against *Hpa***

184 Since SALK/SAIL lines can carry multiple T-DNA insertions and/or T-DNA-induced
185 mutations (Alonso and Ecker, 2006), it is possible that the *iri* mutant phenotypes are caused
186 by mutations in genes other than those identified and annotated by PCR border recovery
187 analysis. To address this possibility, we quantified RBH-IR in independent T-DNA insertion
188 lines in the annotated genes for each of the four complete *iri* lines (Figure 1B,C and
189 Supplemental Figure S2A,B). Since RBH-IR against *Hpa* in Arabidopsis is associated with
190 greater effectiveness of callose-rich papillae (Buswell et al., 2018), we quantified the
191 effectiveness of callose-mediated cell wall defense at 3 dpi, as detailed previously
192 (Schwarzenbacher et al., 2020). All original *iri* lines consistently lacked RBH-IR and
193 concomitantly failed to augment callose-mediated defense upon RBH treatment (Figure 1D,
194 Supplemental Figure 2C), confirming the importance of this post-invasive defense barrier in
195 RBH-IR against *Hpa*. However, independent T-DNA insertions in the annotated genes
196 inactivated in *iri2-1*, *iri3-1* or *iri4-1* did not affect RBH-IR and showed wild-type levels of

197 callose-mediated defense against *Hpa* (Supplemental Figure 2C), indicating that their *iri*
198 phenotypes are caused by T-DNA-induced mutations in other genes. By contrast, an
199 independent T-DNA insertion mutant (*iri1-2*) in the annotated gene disrupted in *iri1-1*
200 displayed a complete *iri* phenotype (Figures 1B and 1C) and was concomitantly impaired in
201 RBH-induced priming of callose defense (Figure 1D). The *iri1-1* and *iri1-2* mutants carry a
202 T-DNA insertion in the 5th intron and the 2nd intron of *LYSINE HISTIDINE TRANSPORTER1*
203 (*LHT1*; At5g40780; Figure 1B; Supplemental Figures S3A and S3B), respectively. *LHT1*
204 encodes a high-affinity amino acid transporter for acidic and neutral amino acids in roots and
205 mesophyll cells (Chen and Bush, 1997; Hirner et al., 2006; Svennerstam et al., 2007). We
206 will therefore refer to *IRI1* as *LHT1* thereafter.

207

208 **LHT1 controls RBH uptake from the soil.**

209 Since *LHT1* was characterized as an amino acid transporter (Chen and Bush, 1997), we
210 hypothesized that the lack of RBH-IR in *lht1* mutants (*lht1-5*, for *iri1-1*; and *lht1-4*, for *iri1-2*)
211 might be caused by impaired RBH uptake from the soil. To test this hypothesis, we
212 determined RBH concentrations after saturating the soil with increasing RBH concentrations
213 in the leaves of Col-0, *iri1-1* and a previously characterized *LHT1* overexpression line
214 (Hirner et al., 2006; *35Spro:LHT1*), which shows a 27-fold higher *LHT1* expression level
215 than Col-0 plants under our experimental conditions (Supplemental Figure S3C). At 2 days
216 after soil treatment, we harvested replicate leaf tissues for RBH quantification by hydrophilic
217 interaction liquid chromatography coupled to quadrupole time-of-flight mass spectrometry
218 (HILIC-Q-TOF; Figure 2A) or challenged the leaves with *Hpa* to quantify RBH-IR (Figure
219 2B). The three genotypes differed statistically in their RBH shoot concentrations after soil
220 treatment with increasing RBH concentrations, as evidenced by a highly statically significant
221 interaction between soil treatment and genotype (two-way ANOVA; $p < 0.001$; Figure 2A).
222 For both Col-0 and *35Spro:LHT1*, RBH shoot accumulation showed a dose-dependent rise
223 with increasing RBH concentrations in the soil. The *35Spro:LHT1* seedlings accumulated
224 statistically higher RBH concentrations in their shoots than Col-0 after saturating the soil to a
225 final concentration of 0.15 mM or 0.5 mM RBH, whereas RBH concentrations in the shoot of
226 *lht1-5* were hardly detectable by HILIC-Q-TOF and failed to show a dose-dependent increase
227 with RBH soil treatment (Figure 2A). The observed variation in RBH shoot concentrations
228 correlated with RBH-IR intensity against *Hpa* (Figure 2B); while RBH failed to induce

229 statistically significant levels of resistance in *lht1-5* at all concentration tested, *35Spro:LHT1*
230 plants showed increased levels of RBH-IR compared to Col-0 at all RBH concentrations
231 tested. Notably, the relatively low concentration of 0.05 mM RBH failed to protect Col-0
232 against *Hpa* seedlings, whereas the same RBH concentration induced a statistically
233 significant reduction in *Hpa* colonization in *35Spro:LHT1* (Figure 2B). Thus, RBH uptake
234 from the soil by LHT1 increases by overexpression of *LHT1*, which in turn boosts RBH-IR
235 against *Hpa*.

236

237 **Tolerance to RBH depends on LHT and not on catabolism**

238 In contrast to BABA, RBH induces resistance in Arabidopsis without concomitant growth
239 inhibition (Buswell et al., 2018). To examine whether LHT1 controls tolerance to RBH, we
240 quantified seedling growth of Col-0, *lht1-5*, and *35Spro:LHT1* on Murashige and Skoog (MS)
241 agar medium. To strengthen the evidence that RBH-induced phytotoxicity in *35Spro:LHT1*
242 depends on LHT1 uptake, we conducted this experiment in the presence of increasing
243 concentrations of L-Ala, a high-affinity substrate of LHT1 (Hirner et al., 2006), expecting
244 that if tolerance is controlled by LHT1-dependent uptake, the L-Ala in the medium would
245 outcompete RBH for uptake and antagonize RBH-induced phytotoxicity. Indeed, while green
246 leaf areas (GLA) of Col-0 and *lht1-5* were unaffected by increasing concentrations of RBH
247 after 1 week of growth, growth of the *35Spro:LHT1* overexpression line showed a dose-
248 dependent repression with increasing RBH concentrations, which was antagonized by L-Ala
249 in a dose-dependent manner (Figure 3). Together with our earlier finding that RBH uptake
250 increased in the *35Spro:LHT1* line (Figure 2A), these results indicate that natural tolerance of
251 Arabidopsis to RBH (Buswell et al., 2018) is determined by RBH uptake capacity of LHT1.

252 To exclude a role for catabolism in RBH tolerance, we repeated the experiment on
253 MS medium without inorganic nitrogen (N_{inorg} ; NO_3^- and NH_4^+), supplemented with
254 increasing concentrations of RBH and L-Ala. Importantly, Arabidopsis failed to grow on agar
255 medium without N_{inorg} (Supplemental Figure S4), and increasing RBH concentrations in the
256 growth medium failed to rescue growth. Hence, Arabidopsis cannot metabolize RBH as a N
257 source, which rules out metabolic breakdown (catabolism) as a mechanism of RBH tolerance.
258 By contrast, increasing L-Ala concentrations added to the agar medium rescued seedling
259 growth of all genotypes, albeit to varying degrees. While *35Spro:LHT1* seedlings showed the
260 strongest growth response to increasing L-Ala concentrations, Col-0 displayed an

261 intermediate growth response, followed by a relatively weak growth response in *lht1-5*
262 (Supplemental Figure S4), thus confirming the contribution of LHT1 to L-Ala uptake (Hirner
263 et al., 2006; Svennerstam et al., 2007; Svennerstam et al., 2011). Notably, increasing RBH
264 concentrations in the presence of L-Ala caused a dose-dependent growth reduction in
265 *35Spro:LHT1* seedlings but not in Col-0 or *lht1-5* (Supplemental Figure S4), which supports
266 our conclusion that increased RBH uptake through *LHT1* overexpression renders Arabidopsis
267 sensitive to RBH-induced stress due to accumulation of phytotoxic RBH concentrations that
268 cannot be catabolized. Thus, tolerance of Arabidopsis to RBH is controlled by LHT1-
269 dependent uptake of RBH, rather than catabolism of RBH.

270

271 **LHT1 also controls BABA uptake, BABA-IR and BABA tolerance.**

272 Given the published broad substrate range of the LHT1 transporter for acidic and neutral
273 amino acids (Hirner et al., 2006; Svennerstam et al., 2007), we examined whether LHT1 also
274 plays a role in the uptake of BABA. To this end, we harvested replicate shoot tissues of Col-0
275 and *lht1-5* seedlings to quantify *in planta* concentrations of BABA at 2 days after saturating
276 the soil with increasing concentrations of the chemical (0, 0.025, 0.05, 0.15 and 0.5 mM),
277 using HILIC-Q-TOF (Figure 4A). While saturating the soil on which Col-0 seedlings grew
278 with increasing BABA concentrations resulted in a dose-dependent increase of BABA
279 concentrations in the shoot (Figure 4A), a similar treatment of the *lht1-5* mutant failed to
280 increase shoot BABA concentrations (Figure 4A), indicating that BABA uptake is dependent
281 on LHT1. To corroborate this, we saturated the soil of Col-0, *lht1-5* and *35Spro:LHT1*
282 seedlings with increasing BABA concentrations and scored BABA-IR against *Hpa* (Figure
283 4B). As reported previously, BABA was more efficient than RBH in protecting Col-0 against
284 *Hpa* (Buswell et al., 2018), already reducing *Hpa* colonization at 0.025 mM BABA and
285 reaching maximum levels of resistance at concentrations of 0.05 mM and higher (Figure 4B).
286 The *35Spro:LHT1* line showed even higher levels of resistance at 0.025 mM BABA
287 compared to Col-0, indicating that these seedlings are sensitized to respond to BABA. By
288 contrast, the *lht1-5* mutant was severely compromised in its effectiveness of BABA-IR, and
289 only displayed weak levels of IR at soil BABA concentrations of 0.25 mM and 0.5 mM
290 (Figure 4B). Thus, like RBH-IR, BABA-IR depends on a functional LHT1 transporter and is
291 enhanced by overexpression of *LHT1*.

292 To determine whether LHT1 also controls BABA-induced phytotoxicity, we
293 quantified the growth of Col-0, *lht1-5* and *35Spro:LHT1* seedlings growing on MS agar
294 plates supplemented with phytotoxic concentrations of BABA. As shown in Figure 5, GLA
295 values of Col-0 after 1 week of growth declined with increasing BABA concentrations. This
296 BABA-induced stress increased dramatically in *35Spro:LHT1* seedlings and decreased in
297 *lht1-5* seedlings (Figure 5). The fact that *lht1-5* seedlings still showed growth repression at
298 higher BABA concentrations suggests that additional mechanisms contribute to BABA-
299 induced phytotoxicity. To compare the severity of RBH- and BABA-induced phytotoxicity,
300 we cultivated Col-0, *lht1-5* and *35Spro:LHT1* seedlings on MS agar plates containing the
301 same doses of RBH or BABA (0.25 mM, 0.5 mM, 1 mM or 2.5 mM). Of the three genotypes
302 tested, only *35Spro:LHT1* seedlings were affected in growth by both chemicals at
303 concentrations of 0.25 mM and above (Supplemental Figure S5A), with BABA causing more
304 severe growth repression than RBH (Supplemental Figure S5B). Quantification of green leaf
305 areas of *35Spro:LHT1* across all inhibitor concentrations confirmed that BABA is more
306 potent in repressing growth than RBH (Supplemental Figure S5B). Collectively, our results
307 indicate that LHT1 is the dominant transporter for BABA uptake from the soil, controlling
308 both BABA-IR and BABA-induced stress.

309

310 **LHT1 transports both RBH and BABA**

311 Having established that LHT1 is responsible for the uptake of RBH and BABA, we next
312 examined the kinetics by which LHT1 transports these β -amino acids. To this end, we
313 heterologously expressed the Arabidopsis *LHT1* coding sequence in the yeast
314 (*Saccharomyces cerevisiae*) 22 Δ 10 α strain, which lacks ten amino acid transporter genes and
315 is completely deficient in the uptake of amino acids (Besnard et al., 2016). In contrast to
316 empty vector (EV)-transformed 22 Δ 10 α cells, the *LHT1*-expressing 22 Δ 10 α strain was
317 capable of growing on agar plates containing 1 mM L-Ala as the only nitrogen (N) source
318 (Figure 6A), while supplementing liquid growth medium without inorganic (NH₄)₂SO₄ with
319 increasing L-Ala concentrations steadily improved planktonic growth by *LHT1*-expressing
320 22 Δ 10 α cells (Figure 6B). Increasing RBH and BABA concentrations in liquid growth
321 medium with 1 mM L-ala repressed growth by *LHT1*-expressing 22 Δ 10 α cells completely
322 (Supplemental Figures S6A and S6B, respectively), despite the fact that both chemicals only
323 marginally repressed 22 Δ 10 α growth in liquid medium with 10 mM (NH₄)₂SO₄ as an N

324 source (Supplemental Figure S7). These results not only show that yeast fails to metabolize
325 RBH and BABA, but they also suggest that increasing RBH and BABA concentrations
326 outcompete L-Ala for cellular uptake.

327 To study the kinetics of RBH and BABA uptake, we carried out experiments with ^{14}C -labeled
328 L-Ala in the absence and presence of RBH or BABA. To this end, we incubated EV- and
329 *LHT1*-expressing 22 Δ 10 α cells for 2, 5 and 10 min in buffer containing 50 μM or 500 μM L-
330 Ala with a fixed amount of ^{14}C -L-Ala for incubation, after which we quantified cellular L-
331 Ala uptake by ^{14}C scintillation. In contrast to EV-transformed cells, *LHT1*-expressing cells
332 showed a linear uptake for L-Ala over time (Supplemental Figure S8), confirming the
333 functionality of the transporter in yeast. To determine whether RBH and BABA
334 competitively inhibit the LHT1 transporter for L-Ala uptake, we incubated *LHT1*-expressing
335 cells for 5 min in buffer containing increasing concentrations L-Ala and a fixed amount of
336 ^{14}C - L-Ala in the presence or absence of 500 μM RBH or 500 μM BABA (Figure 6C, D).
337 Plotting the uptake velocity (V_{uptake} ; fmol L-Ala/cell) against L-Ala concentration revealed a
338 dose-dependent increase until saturation (V_{max} ; Figure 6C, D). Based on these data, we
339 calculated that LHT1 has a K_m value of 9.4 μM for L-Ala-uptake, which is in line with
340 previously reported K_m values for acidic and neutral amino acids (Hirner et al., 2006).
341 Although V_{uptake} in the presence of either 500 μM RBH or 500 μM BABA decreased across a
342 lower range L-Ala concentration, it still reached similar V_{max} values at higher L-Ala
343 concentrations, indicating that RBH and BABA are competitive inhibitors of L-Ala uptake by
344 LHT1. To calculate the inhibition constants (K_i) of RBH and BABA, we conducted further
345 uptake experiments in the presence of multiple inhibitor concentrations (0, 250, and 1,000
346 μM RBH/BABA) and increasing L-Ala concentrations. We generated Dixon plots of the
347 inverse uptake velocity ($1/V_{\text{uptake}}$) against inhibitor concentration (Cornish-Bowden, 1974;
348 Yoshino & Murakami, 2009) to determine K_i values at the intersecting lines of the different
349 L-Ala concentrations (1, 5, 25, 50, 250 μM ; Figure 6E,F). Predicted intersects were called at
350 modeled RBH/BABA concentrations that had the smallest $1/V_{\text{uptake}}$ range between the various
351 L-Ala concentrations (Supplemental Figure S9), revealing a K_i of 87.9 μM for RBH and a K_i
352 of 68.9 μM for BABA (Figure 6E, F). Hence, LHT1 is a transporter of both beta-amino acids
353 and shows a higher affinity for BABA than for RBH.

354

355 **DISCUSSION**

356 Using annotated T-DNA insertion lines for a genome-saturating mutant screen

357 We used a genome-covering collection of Arabidopsis T-DNA insertion lines in a forward
358 mutant screen for regulatory genes of IR. The availability of homozygous T-DNA insertions
359 with high genomic coverage (Alonso and Ecker, 2006) facilitates a near genome-saturating
360 screen. The use of this resource has several benefits compared to conventional mutant screens.
361 First, the availability of T-DNA flanking sequences mapped to the Arabidopsis genome
362 allows for immediate identification of gene candidates without having to commit to a time-
363 consuming generation of mapping populations and linkage analysis. Second, the collection of
364 homozygous mutant lines enables the screening of small populations that all carry the same
365 mutant allele, which facilitates the identification of partial (leaky) mutant phenotypes, as
366 illustrated by the identification of 104 *iri* lines that are partially affected in RBH-IR (Figure
367 1A; Supplemental Figure S1, Supplemental Data Set S1). This relatively high number of
368 partial *iri* mutants supports the notion that IR is a highly quantitative form of resistance,
369 relying on the additive contribution of multiple genes (Ton et al. 2006; Ahmad et al. 2010,
370 Wilkinson et al. 2019). Thus, the within-genotype replication of this screen enables selection
371 for genes that make a quantitative contribution to complex multigenic traits. A disadvantage
372 of using annotated T-DNA insertion lines in a forward mutant screen is that a single T-DNA
373 insertion line can carry multiple mutations (O'Malley et al., 2015). These mutations are not
374 necessarily covered by the annotated T-DNA flanking sequences, since they can be caused by
375 truncated T-DNA elements or mis-repairs of integration sites from abortive T-DNA
376 integrations (leaving mutational footprints; Gelvin, 2021). Indeed, several other studies have
377 reported that mutant phenotypes in this collection of T-DNA insertion lines do not always co-
378 segregate with the annotated T-DNA insertion (De Muyt et al., 2009; Dobritsa et al., 2011;
379 Wilson-Sánchez et al., 2014). To account for this issue, we validated the mutant phenotypes
380 of the four complete *iri* mutants in independent T-DNA insertion lines of their disrupted
381 annotated genes for both RBH-IR and augmented cell wall defense against *Hpa* (Figure 1C,
382 1D and Supplemental Figure S2). Even though the *iri* phenotypes of the four original mutant
383 lines were robust and reproducible (Figure 1C, D and Supplemental Figure S2), only the
384 phenotype of the *lht1-5 (iri1-1)* mutant could be confirmed in an independent T-DNA
385 insertion line in the annotated disrupted gene. Identifying the causal mutation in the other
386 three *iri* lines will require thermal asymmetric interlaced PCR (TAIL-PCR) to identify
387 flanking sequences of alternative T-DNA insertions or conventional linkage analysis in
388 segregating mapping populations.

390 **The role of LHT1 in plant-biotic interactions**

391 *IR11* encodes the broad-range amino acid transporter LHT1. Cellular transporters play
392 important roles in the control of plant-pathogen interactions by facilitating pathogen feeding
393 (Elashry et al., 2013; Marella et al., 2013), secretion of antibiotic compounds (Lu et al., 2015;
394 Khare et al., 2017), transporting defense plant hormones (Serrano et al., 2013), or
395 contributing to plant defense responses (Liu et al., 2010; Yang et al., 2014). Furthermore, the
396 *LHT1* ortholog *LjLHT1.2* in birdsfoot trefoil (*Lotus japonicus*) is transcriptionally induced by
397 arbuscular mycorrhizal fungi (AMF; Guether et al., 2011), suggesting that it facilitates AMF-
398 dependent uptake of organic nitrogen. Given the role of LHT1 in IR, it is tempting to
399 speculate that LHT1 also plays a role in mycorrhiza-IR (Cameron et al., 2013). In
400 *Arabidopsis*. LHT1 has been implicated in the direct regulation of SA-dependent disease
401 resistance. Liu et al. (2010) reported that *lht1* mutant lines had increased basal resistance
402 against the hemibiotrophic bacterium *Pseudomonas syringae* pv. *tomato*, the hemibiotrophic
403 fungus *Colletotrichum higginsianum*, and the biotrophic fungus *Erysiphe cichoracearum*.
404 The study furthermore provided evidence that LHT1 controls plant immunity by cellular
405 uptake of L-glutamine (L-Gln), which is a precursor of the redox-buffering compound
406 glutathione. Liu et al. (2010) proposed that the lower L-Gln uptake capacity in *lht1* mutants
407 suppresses cellular redox buffering capacity, thereby enabling augmented elicitation of ROS
408 and SA-dependent defenses upon pathogen attack. Our experiments did not reveal
409 statistically significant differences in basal defense against the biotrophic oomycete *Hpa*
410 between wild-type and *lht1* mutant plants (Figures 1 and 2), in contrast to the results shown
411 by Liu et al. (2010). This discrepancy may be explained by the fact that we used relatively
412 young plants (2- to 3-week-old seedlings), which do not express SA-dependent age-related
413 resistance (ARR; Kus et al., 2002). Indeed, other studies have reported that *lht1* seedlings
414 display normal growth phenotypes without the enhanced SA levels observed in older plants
415 (Liu et al., 2010; Zhang et al., 2022). Accordingly, it is possible that glutamine-dependent
416 redox regulation contributes to age-related resistance in older plants. Since *LHT1* expression
417 is lower in seedlings (Hirner et al., 2006), it is also possible that other amino transporters
418 contribute to the cellular delivery of glutamine in these younger seedlings, such as AMINO
419 ACID PERMEASE 1 (AAP1; Boorer et al., 1996) or CATIONIC AMINO ACID
420 TRANSPORTER 8 (CAT8; Yang et al., 2010). Interestingly, in contrast to the negative role
421 of LHT1 in innate immunity reported by Liu et al. (2010), a recent study by Yoo et al. (2020)

422 revealed that LHT1 contributes positively to ETI-related resistance in Arabidopsis against
423 *Pseudomonas syringae* pv. *maculicola* carrying the avirulence gene *AvrRpt2*. Moreover,
424 Zhang et al. (2022) showed that LHT1 is the dominant transporter responsible for increased
425 amino acid uptake during early PTI against pathogenic *Pseudomonas syringae*, when it has a
426 positive contribution to resistance by restricting bacterial colonization. Hence, LHT1 has
427 been reported to have both positive and negative roles in innate plant resistance. It should be
428 noted, however, that the immune-related function of LHT1 described in our study is related to
429 IR by priming-inducing β -amino acids, rather than innate resistance.

430

431 **The role of LHT1 in beta-amino acid-IR**

432 Our results have shown that LHT1 is the dominant transporter for cellular uptake of RBH and
433 BABA from the soil (Figures 2 and 4). LHT1 localizes to the cell membrane (Hirner et al.,
434 2006), which enables cellular import of RBH and BABA from the apoplast. *LHT1* is
435 expressed in root tips, lateral roots and mature leaves (Hirner et al., 2006), enabling cellular
436 uptake of RBH and BABA in both roots and leaves. Since *LHT1* is not expressed in the leaf
437 vein, we propose that the activity of RBH and BABA in leaves is preceded by long-distance
438 transport via the xylem and apoplastic distribution in the leaves. While BABA was applied
439 exogenously in our experiments, recent studies have reported that biotic and abiotic stresses
440 can elicit low concentrations of endogenous BABA in Arabidopsis (Thevenet et al., 2017;
441 Balmer et al., 2019). Under these conditions, BABA only accumulates in locally stressed
442 tissues and not systemically in non-stressed tissues (Balmer et al., 2019), indicating that
443 stress-induced accumulation of BABA does not contribute to systemic defense signaling.
444 Although the biosynthesis pathway of stress-induced BABA remains unknown, it seems
445 plausible that this local biosynthesis occurs inside the cell. The K_i values of RBH (87.9 μ M)
446 and BABA (68.9 μ M) indicate that these beta amino acids have marginally lower affinities
447 for LHT1 than endogenous alpha-amino acids (Hirner et al., 2006). Since alpha-amino acids
448 typically reach apoplastic concentrations between 1 μ M to 10 μ M (Zhang et al., 2022), it
449 would be difficult for BABA to compete with these substrates. Moreover, *Hpa*-induced
450 BABA concentrations do not exceed 25 ng/g fresh weight (242.7 nM; Thevenet et al. 2017),
451 which seems too low to be a competitive substrate for LHT1. Hence, cellular uptake of
452 BABA by LHT1 does not appear to play a major role in *Hpa*-induced BABA accumulation,
453 which would also explain why the *lht1* mutant and *35Spro:LHT1* overexpression lines were

454 not majorly affected in basal resistance to *Hpa* (Figure 2). Nevertheless, we cannot exclude
455 that *Hpa* locally induces much higher BABA concentrations in the cells directly interacting
456 with the parasite, and that LHT1 plays a role in countering diffusion of this intracellular
457 BABA into the apoplast. In this context, it is interesting to note that *Hpa* infection induces
458 *LHT1* expression (Sonawala et al. 2018; Supplemental Figure S10), which could play a role
459 in upholding defense-inducing intracellular concentrations of BABA in *Hpa*-challenged cells
460 and would also explain why stress-induced BABA is not distributed systemically (Balmer et
461 al. 2019).

462 While our results provide strong evidence that LHT1 is the dominant transporter for
463 the uptake of RBH and BABA (Figures 2-6), they do not necessarily mean that the
464 contribution of LHT1 to RBH-IR or BABA-IR solely depends on its uptake activity. For
465 instance, while treatment with 0.05 mM RBH resulted in similar foliar concentrations in both
466 *35Spro:LHT1* and wild-type plants (Figure 2A), this relatively low RBH concentration only
467 triggered a significant IR response in *35Spro:LHT1* plants and not in wild-type plants. This
468 uncoupling of RBH concentration from IR suggests that the function of LHT1 in RBH-IR
469 may involve an additional defense signaling activity that becomes active at low RBH
470 concentrations. Such a transporter-receptor co-functionality (transceptor activity) has been
471 reported for NITRATE TRANSPORTER 1.1 (NRT1.1) for nitrate uptake and signaling.
472 Replacing Pro-492 with Leu-492 in NRT1.1 disabled the nitrate transport activity of this
473 protein but not its ability to induce *NRT2.1* expression (Ho et al., 2009), which is a nitrate-
474 responsive gene that has concomitantly been linked to the regulation of disease resistance
475 (Camanes et al., 2012). Although no amino acid transporters have been reported with receptor
476 co-functionality (Dinkeloo et al., 2018), it is tempting to speculate that LHT1 might act as a
477 transceptor of β -amino acids. Site-directed mutagenesis of LHT1 and testing whether its RBH
478 and BABA transport activity can be uncoupled from its role in RBH-IR and BABA-IR would
479 be required to test this attractive hypothesis.

480 Since the *lht1* mutant still displayed residual levels of BABA-IR and BABA-induced
481 stress after treatment with high BABA doses (Figures 4B, 5B), we cannot exclude the
482 possibility that other amino acid transporters have a minor contribution to BABA uptake. A
483 recent study reported that LHT2 has a similar substrate specificity as LHT1, including several
484 D-amino acids and 1-aminocyclopropane-1-carboxylate (ACC) (Choi et al., 2019), and could
485 thus have a complementary contribution to BABA uptake.

486

487 **RBH and BABA compete with proteinogenic amino acids for uptake by LHT1**

488 We used *LHT1*-expressing yeast cells to assess competitive inhibition of L-Ala uptake by
489 RBH and BABA. Our uptake essays revealed a K_m for LHT1 of 9.4 μM for L-Ala (Figure
490 6C), which supports previously reported K_m values of LHT1 for proteinogenic amino acids
491 (Hirner et al., 2006). Furthermore, the inhibitory kinetics of RBH or BABA on L-Ala uptake
492 confirmed competitive inhibition, as evidenced by the fact that L-Ala uptake in the presence
493 of RBH or BABA still reached maximum velocities at higher L-Ala concentrations (Figure
494 6C,D). Of the two beta-amino acids, BABA had a lower K_i than RBH (68.9 μM vs 87.9 μM),
495 suggesting that LHT1 has a higher affinity for BABA than RBH (Figure 6E,F). This
496 difference in affinity is consistent with our observation that BABA has a stronger inhibitory
497 effect on growth of *35Spro:LHT1* than RBH (Supplemental Figure 5). Since the affinity of
498 LHT1 has been reported to be similar or higher for a range of acidic and neutral amino acids,
499 including L-Gln (Hirner et al., 2006; Svennerstam et al., 2007), our results also explain
500 previous findings by Wu et al. (2010), who showed that BABA-induced phytotoxicity in
501 *Arabidopsis* can be alleviated by co-application with L-Gln.

502

503 **LHT1: not just a transporter for proteinogenic amino acids**

504 Although LHT1 was initially identified as a transporter for proteinogenic amino acids (Chen
505 and Bush, 1997), subsequent studies have shown that it transports a much wider range for
506 non-proteinogenic amino acids, such as the ethylene precursor ACC (Shin et al., 2015) and
507 xenobiotic amino acid conjugates (Chen et al., 2018; Jiang et al., 2018). Consistent with this
508 broad-spectrum uptake activity, we showed that LHT1 is the main transporter of the β -amino
509 acids RBH and BABA. Of particular interest is the regulatory function of LHT1 in the trade-
510 off between beta-amino acid-IR and plant growth. For BABA, overexpression of *LHT1* in
511 *Arabidopsis* increased BABA-IR at the relatively low concentration of 0.025 mM BABA
512 (Figure 4) but it also dramatically increased plant sensitivity to BABA-induced growth
513 repression (Figure 5 and Supplemental Figure S5). However, RBH elicited high levels of IR
514 in wild-type plants at soil concentrations of 0.15 mM RBH and above (Figure 2B) but did not
515 repress growth across all concentrations tested (Figure 3), supporting our earlier conclusion
516 that RBH induces disease resistance without costs on plant growth (Buswell et al. 2018).
517 Interestingly, *35Spro:LHT1* overexpression plants increased the level of IR at relatively low

518 RBH concentrations (Figure 2B), but also repressed growth in a dose-dependent manner
519 (Figure 3 and Supplemental Figure 5). Direct comparison of RBH- and BABA-induced
520 growth repression in *35Spro:LHT1* plants confirmed that BABA is more active than RBH
521 (Supplemental Figure S5B), which is also apparent from the IR response (Figures 2B, 4B). It
522 is worth noting that the molecular mechanisms of RBH-induced stress remain unclear, and its
523 lower toxicity in plants might come from a combination of uptake and intracellular modes of
524 action.

525 The observed trade-offs between beta-amino acid-IR and plant growth reveal two
526 important conclusions. First, like BABA, RBH can repress plant growth, but this
527 phytotoxicity depends on LHT1-dependent uptake capacity rather RBH catabolism. Second,
528 our results show that the trade-off between beta-amino acid-IR and growth can be optimized
529 in favor of the IR response by manipulating the *LHT1* gene. This conclusion holds major
530 translational value for breeding programs aiming to exploit BABA-IR in vegetable crops that
531 are protected by BABA but also suffer from BABA-induced phytotoxicity (Cohen et al.,
532 2016; Yassin et al., 2021).

533

534

535 **MATERIALS AND METHODS**

536 **Biological material**

537 All *Arabidopsis* (*Arabidopsis thaliana*) genotypes were in accession Columbia-0 (Col-0). The
538 *iri1-1* mutant (*lht1-51*) and *iri1-2* mutant (*lht1-4*) were described previously by Svennerstam
539 et al. (2007) and Liu et al. (2010); the *35Spro: LHT1* overexpression lines were described by
540 Hirner et al. (2006). The *iri* mutant screen was performed with fully annotated T-DNA
541 insertion lines from the SALK and SAIL collections (Alonso *et al.*, 2003) and purchased
542 from the Nottingham Arabidopsis Stock Centre (sets N27941, N27951, N27942, N27943,
543 N27944, N27945). The annotated T-DNA insertions in *iri1-1* (SALK_115555), *iri1-2*
544 (SALK_036871), *iri2-1* (SALK_204380), SAIL_902_B08, *iri3-1* (SALK_118654),
545 SALK_078838, *iri4-1* (SALK_076708) and SALK_046376 were confirmed by PCR before
546 further testing (Supplemental Table S1), as described below. *Hyaloperonospora*
547 *arabidopsidis* strain WACO9 was maintained in its asexual cycle by alternate conidiospore
548 inoculations of Col-0 and Ws NahG plants.

549 **Plant growth conditions**

550 For soil-based IR experiments, seeds were sown in a 2:1 (v/v) Scott's Levington M3
551 compost/sand mixture and stratified for 2-4 days in the dark at 4°C. Plants were subsequently
552 cultivated under short-day conditions (8-h light (Sylvania GroLux T8 36W or Valoya NS1
553 LED); 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; 21°C; and 16-h dark; 18°C) with a ~60% relative humidity
554 (RH). Plants for seed propagation were grown in long-day growth conditions (16-h light
555 (Sylvania GroLux T8 36W); 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; 21°C; and 8-h dark; 18°C) with
556 ~60% RH. For plate assays, seeds were surface sterilized (vapor-phase sterilization method)
557 prior to sowing on half-strength Murashige and Skoog (MS) medium (pH = 5.7 and 1%
558 sucrose), solidified with 1.5% (w/v).

559 **Mutant screen**

560 Approximately 10-15 seeds for each seed line were sown in individual wells of 400-well
561 trays (Teku JP 3050/230 H). Each tray was filled with ~2.4 L of compost/sand mixture. After
562 sowing, stratification of seeds and seed germination, seedlings were thinned to five
563 seedlings/well. Two-week-old seedlings were treated with RBH by watering each tray with
564 1.5 L of 2x concentrated RBH solution (1 mM), which was left overnight to saturate the soil.
565 Excess RBH solution (~300 mL) was removed the next morning, resulting in a final soil
566 concentration of ~0.5 mM RBH. Challenge inoculation was performed at 2 days after RBH
567 treatment by spraying seedlings with a suspension of *Hpa* conidiospores (10^5 spores/mL).
568 Trays were sealed with clingfilm after inoculation to maintain 100% RH and promote
569 infection. To verify RBH-IR, each tray contained three randomly distributed wells with Col-0
570 seedlings. Furthermore, to verify favorable conditions for *Hpa* disease, three additional wells
571 with Col-0 seedlings were cut out from each tray and left outside during RBH-uptake to
572 prevent RBH-IR prior to inoculation. At 5-7 dpi, trays were visually inspected for *Hpa*
573 sporulation when sporulation on Col-0 seedlings in the untreated wells of the tray became
574 apparent. Lines developing sporulation within 7 dpi were scored as stage 1 *impaired in RBH-*
575 *induced-immunity* (S1 *iri*) lines, while nongerminated lines were scored as stage 1
576 nongerminated (S1 *ug*). All S1 *iri* and S1 *ug* lines were pooled for the stage 2 screen in 400-
577 well trays, as described above. S1 *iri* lines allowing visible sporulation in two screens time
578 were scored as Stage 2 *iri* (S2 *iri*). S1 *ug* lines that germinated upon rescreening and showed
579 sporulation were re-tested for S2 *iri* phenotypes. Of the 26,631 T-DNA insertion lines,
580 23,547 lines germinated and could be screened for *iri* mutant phenotypes. The 427 putative

581 *iri1* lines selected after stage 2 were pooled for seed bulking and validated by controlled IR
582 assays in stage 3 (S3) of the screen, as described below.

583 **Induced resistance (IR) assays**

584 Two-week-old seedlings were grown in 60-mL pots, after which the soil was saturated with
585 water, (*R*)- β -homoserine (Sigma-Aldrich; #03694), or R/S-BABA (Sigma-Aldrich, #A44207)
586 to the indicated concentrations, as described previously (Buswell et al., 2018). Two days after
587 chemical treatment, seedlings were spray-inoculated with a suspension of *Hpa* conidiospores
588 (10^5 spores/mL) and maintained in 100% RH to promote infection. Leaves were collected at
589 6-7 dpi for trypan blue staining for microscopy scoring of *Hpa* colonization by categorizing
590 them into four classes, ranging from healthy leaves (I) to heavily colonized leaves (IV), as
591 described in detail by Schwarzenbacher et al. (2020). To investigate augmented induction of
592 cell wall defense by chemical priming treatment, leaves were harvested at 3 dpi for aniline
593 blue/calcofluor staining and analysis by epifluorescence microscopy (Leica DM6B; light
594 source: CoolLED pE-2; 365 nm excitation filter, L 425 nm emission filter, 400 nm dichroic
595 filter). For each genotype/treatment combination, germinated conidiospores on 10 leaves
596 from independent seedlings were scored either as arrested (spores or germ tubes fully encased
597 in callose), or non-arrested by callose depositions (no callose or lateral callose deposition
598 along the germ tube/hyphae), as detailed by Schwarzenbacher *et al.* (2020). Statistical
599 differences in *Hpa* colonization or callose defense were analyzed by pairwise Fisher's
600 exact tests, using R software (v 3.5.1). For multiple comparisons, an additional Bonferroni
601 multiple correction was applied, using the R package 'fifer' (fifer_1.1.tar.gz).

602 **Plant growth assays**

603 Surface-sterilized seeds were sown onto half-strength MS agar plates and cultivated for 2
604 weeks under standard plant growth conditions, as indicated above. Photographs were taken
605 after 1 and 2 weeks of growth with a Nikon D5300 digital camera. Green leaf areas (GLA)
606 were quantified from digital photographs of 1- or 2-week-old seedlings, using Fiji/ImageJ
607 software (Rueden et al., 2017). Statistical differences in the natural logarithm of (1+GLA)
608 were analyzed by two-way ANOVA, using R software (v 3.5.1).

609 **Genotyping verification by PCR and gene expression analysis by reverse transcription 610 quantitative PCR (RT-qPCR)**

611 Genomic T-DNA insertions of all *iri1*, *iri2*, *iri3* and *iri4* lines were confirmed by PCR using
612 LP+RP and LBb1.3/ LB3+RP primers (Supplemental Table S2) To quantify *LHT1*

613 expression levels by RT-qPCR, shoot tissues from five 2-week-old seedlings were collected
614 and combined as one biological replicate. A total of five replicates were collected at the same
615 time and snap-frozen in liquid nitrogen and homogenized. Total RNA was extracted using an
616 RNeasy Plant Mini Kit (Qiagen, cat. no. 74904) and first-strand cDNA was synthesized from
617 800 ng total RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher, cat. no.
618 K1641). The cDNA was diluted 20 times in nuclease-free water before qPCR. All qPCR
619 reactions were performed with 2 μ L diluted cDNA and primer concentrations at a final
620 concentration of 250 nM in a Rotor-Gene Q real-time PCR cycler (Qiagen, Q-Rex v1.0),
621 using a Rotor-Gene SYBR Green PCR Kit (Qiagen, cat. no. 204074). The qPCR
622 amplification of *LHT1* was performed with gene-specific primers (FP: ATCTCCGGCGTTTCTCTTGCTG,
623 RP: GCCCATGCGATTGTTGAGTAGCTG) and
624 normalized to the transcript levels of two housekeeping genes (At1g13440
625 [*GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C2*, *GAPC2*], and At2g28390
626 [*MONENSIN SENSITIVITY 1*, *MON1*]), as detailed previously (Schwarzenbacher et al.,
627 2020).

628 **Quantification of *in planta* RBH and BABA concentrations by hydrophilic interaction** 629 **liquid chromatography coupled to quadrupole time-of-flight mass spectrometry**

630 Shoot tissues were collected at 2 days after soil-drenching and divided into four replicate
631 tubes per treatment (five plants per tube, from separate trays), frozen at -80°C , freeze-dried
632 and weighed. Dry tissue was crushed and extracted into 1 mL of cold extraction buffer
633 (methanol: water: formic acid, 10:89.99:0.01, v/v/v). Extracts were centrifuged at 16,000 g
634 for 5 min at 4°C , after which each supernatant was divided between three aliquots. RBH and
635 BABA standards were prepared as individual standards from 0.1 to 100 μM . Separation was
636 performed with a Waters Acquity HILIC BEH C18 analytical column, 1.7-mm particle size,
637 2.1 x 50 mm. The mobile phase was 20 mM ammonium formate with 0.1% (v/v) formic acid
638 (A) and acetonitrile with 0.1% (v/v) formic acid (B). The gradient started at 99% (v/v) A and
639 reached 65% (v/v) A in 4 min. The gradient changed to 1% (v/v) A up to 6 min and was held
640 there for 1.5 min and then returned to initial conditions. The solvent flow rate was 0.3 mL
641 min^{-1} , with an injection volume of 4 μL . Mass spectra were recorded in positive electro-spray
642 ionization mode, using a Waters UPLC system interfaced to a Waters quadrupole time-of-
643 flight mass spectrometer (Q-TOF; G2Si Synapt). Nitrogen was used as the drying and
644 nebulizing gas. Desolvation gas flow was adjusted to approximately 150 L/h and the cone gas
645 flow was set to 20 L/h with a cone voltage of 5 V and a capillary voltage of 2.5 kV. The

646 nitrogen desolvation temperature was 280°C and the source temperature was 100°C. The
647 instrument was calibrated in 20-1,200 m/z range with a sodium formate solution. Leucine
648 enkephalin (Sigma-Aldrich, St. Louis MO, USA) in methanol: water (50:50, v/v) with 0.1%
649 (v/v) formic acid was simultaneously introduced into the qTOF instrument via the lock-spray
650 needle for recalibrating the m/z axis. Quantification of amino acids in tissues was based on
651 the standard curves, using MassLynx v4.1 software (Waters, Elstree UK). Amino acids
652 identities were confirmed by co-elution of product fragment ions with parent ions and
653 matching peak retention times to individual amino acid standards. Statistical differences in
654 RBH and BABA between genotypes and soil-drench treatments were tested by two-way
655 ANOVA followed by Welch t-tests to test cross-genotype differences at each RBH/BABA
656 concentration, using R software (v 3.5.1).

657 **Yeast transformation**

658 The *LHT1* (At5g40780) coding sequence with stop codon was amplified from wild-type Col-
659 0 cDNA with Phusion High-Fidelity DNA Polymerase (New England Biolabs, #M0530L)
660 and cloned into the pENTR plasmid (Invitrogen). *LHT1* was then subcloned into pDR196
661 (Meyer et al., 2006) by restriction (EcoRI and XhoI) and ligation (T4 DNA ligase). Empty
662 vector (EV)- and *LHT1*-harboring plasmids were confirmed by Sanger sequencing and
663 introduced into competent cells of the 22Δ10α strain (Besnard et al., 2016), using heat shock
664 transformation (Gietz and Schiestl, 2007).

665 **Yeast growth assays**

666 To assess the growth of *LHT1*- and EV-transformed 22Δ10α yeast strains, cells were first
667 cultivated in liquid Yeast Nitrogen Base (YNB) medium (Alfa Aesar, #H26271, without
668 amino acids and ammonium sulfate) supplemented with 10 mM ammonium sulfate at 30°C
669 and 220 rpm for 2 days. Cells were washed by centrifugation at room temperature (3,000 g; 5
670 min) and resuspended in distilled water to an OD₆₀₀ of 0.3-0.5. To assess whether yeast can
671 metabolize RBH and BABA, 5 μL of the cell suspension was added to 2 mL Yeast Nitrogen
672 Base and increasing concentrations of RBH or BABA (0.2–5 mM). To assess toxicity of
673 RBH and BABA, 5 μL of the suspension was added to 2 mL YNB medium with 10 mM
674 ammonium sulfate and increasing concentrations of RBH or BABA (0.2–5 mM). To assess
675 competition between L-Ala and RBH or BABA, 5 μL of the suspension was added to 2 mL
676 YNB medium supplemented with 1 mM L-Ala and increasing concentrations of RBH or
677 BABA (0.2–5 mM). cultures were incubated at 30°C with 220 rpm shaking for 3 days, after

678 which the OD₅₉₅ was determined in a plate reader (FLUOstar OPTIMA; BMG LABTECH;
679 Germany).

680

681 **Assessment of uptake and inhibition kinetics of LHT1 in yeast**

682 Transformed 22Δ10α cells were grown in YNB medium supplemented with 10 mM
683 (NH₄)₂SO₄ at 30°C with shaking at 220 rpm for 2 days. Yeast cells were collected by
684 centrifugation at room temperature (3000 g; 5 min), washed in distilled water, and
685 resuspended in ice-cold washing buffer (0.6 M sorbitol, 50 mM sodium phosphate, pH 4.5) to
686 OD₆₀₀ of 5. Before the uptake assay, cells were energized by adding 1 M glucose (final
687 concentration 50 mM) to the growth medium for 10 min. To assess time-dependent uptake of
688 L-[¹⁴C] Ala in EV- and *LHT1*-transformed cells (Supplemental Figure S8), 1.5-mL of the
689 energized cell culture was added to 1.5 mL uptake buffer, containing 50 nCi L-[¹⁴C]Ala (158
690 mCi/mmol; Perkin Elmer; NEC856) with unlabeled L-Ala (50 or 500 μM). After 2, 5 or
691 10 min of incubation in a thermomixer (Grant bio ES-20; Grant Instruments; UK; 30°C,
692 220 rpm), the cell suspensions were mixed with 2 mL ice-cold water and kept on ice to
693 inhibit L-Ala uptake. Cells were then centrifuged (3000 g; 5 min; 4°C) and washed four
694 times with 2 mL ice-cold water, after which pellets were stored at -20°C for
695 quantification of radioactivity the following day. To determine uptake and inhibition
696 kinetics (Figure 6C,D), *LHT1*-transformed cells were incubated in the same uptake medium,
697 containing 50 nCi L-[¹⁴C] Ala with increasing concentrations (1–3,000 μM) of unlabeled
698 L-Ala and/or 500 μM inhibitory RBH or BABA. After 5 min of incubation, cells were
699 washed, collected, and stored as described above. To assess radioactivity, frozen pellets
700 were resuspended in 750 μL distilled water, from which 200 μL was loaded onto
701 Combusto-Pads (Perkin Elmer, part number 5067034) and combusted in a sample
702 oxidizer (Model 307 Sample Oxidizer; Perkin Elmer; USA). Trapped ¹⁴CO₂ was
703 quantified by liquid scintillation counting (Tri-Carb 3100TR; Perkin Elmer; USA). L-
704 Ala uptake velocities over the 5-min time window (V_{uptake}) were expressed as fmol L-
705 Ala/cell and plotted against the L-Ala concentration, using the R package drc (Ritz et al.,
706 2015) to determine the kinetics of L-Ala uptake in the absence and presence of RBH or
707 BABA.

708 To estimate inhibition constants (K_i) of RBH and BABA (Figure 6E,F), L-Ala uptake
709 velocities were determined in the presence of 0, 250 and 1,000 μM RBH or BABA, using a
710 medium containing increasing concentrations of L-Ala (1, 5, 25, 50, 250 μM) with a fixed
711 quantity of 50 nCi L- ^{14}C Ala. Dixon plots were created by plotting inverse L-Ala uptake
712 velocities ($1/V_{\text{uptake}}$) against inhibitor concentration (RBH or BABA), after which five linear
713 models for each L-Ala concentration were generated using the *lm* function (R base). Exact K_i
714 values of RBH and BABA were determined by modeling 1,200 $1/V_{\text{uptake}}$ values in the range
715 between -200 to 1,000 μM of the inhibitor concentration using the *predict()* function (R base),
716 after which K_i values were selected by calculating the inhibitor concentration yielding the
717 minimum range in $1/V_{\text{uptake}}$.

718 Accession numbers

719 *LHT1 (IRI1)*: At5g40780

720

721 Supplemental Data

722 **Supplemental Figure S1.** Validation of putative *iri* mutants at stage 3 of the mutant screen.

723 **Supplemental Figure S2.** Characterization of RBH-IR in mutants carrying independent T-
724 DNA insertions in the annotated genes disrupted by the SALK/SAIL lines in *iri2-1*, *iri3-1*
725 and *iri4-1*.

726 **Supplemental Figure S3.** Genetic characterization of two independent *lht1* mutant lines and
727 the *LHT1* overexpression line.

728 **Supplemental Figure S4.** Transgenic overexpression of *LHT1* improves Arabidopsis growth
729 on medium with L-alanine as the only N source, which is antagonized by co-application of
730 RBH.

731 **Supplemental Figure S5.** Comparison of growth repression by low concentrations of BABA
732 and RBH.

733 **Supplemental Figure S6.** RBH and BABA compete with L-alanine for *LHT1* uptake and
734 inhibit yeast growth.

735 **Supplemental Figure S7.** RBH and BABA have minimal effects on yeast growth but cannot
736 be used as N source by yeast.

737 **Supplemental Figure S8.** Transformation of the yeast $22\Delta 10\alpha$ mutant with *LHT1* rescues
738 uptake of L- ^{14}C alanine.

739 **Supplemental figure S9.** Modeling exact inhibitor constants (K_i) of RBH (A) and BABA.

740 **Supplemental Figure S10.** Induction of *LHT1* expression by *Hpa*.

741 **Supplemental Table 1.** Primers used for characterization of T-DNA insertion lines.

742 **Supplemental Data Set S1.** Annotated genomic T-DNA insertions of the 108 confirmed *iri*
743 lines, RBH-IR phenotypes, and expression profiles of the associated T-DNA-tagged genes.

744 **Supplemental Data Set S2.** Details of statistical tests and results used in the manuscript.

745

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758

759 **AUTHOR CONTRIBUTIONS**

760 J.T. conceived the research; C.-N.T, W.B., P.Z., R.S., and J.T. designed the experiments;
761 C.-N.T, W.B., P.Z., H.W., I.J., and K.F. conducted the experiments; C.-N.T, W.B., P.Z.,
762 and J.T. analyzed the data; C.-N.T, W.B., and J.T. wrote the paper.

763

764

765 **Figure legends**

766 **Figure 1. Mutant screen for *impaired in RBH-induced immunity (iri)* phenotypes and**
767 **characterization of the *iri1* mutant in Arabidopsis.**

768 **(A)** Schematic diagram of the three successive selection stages of the *iri* mutant screen on
769 23,547 T-DNA insertion lines from the SALK/SAIL collection. Small populations of ~five

770 seedlings were screened per line (stage 1) and rescreened (stage 2) for sporulation by
 771 *Hyaloperonospora arabidopsidis* WACO9 (*Hpa*) upon saturating the soil to a final
 772 concentrations of 0.5 mM R- β -homoserine (RBH) and subsequent inoculation with *Hpa*
 773 conidiospores (top). Putative *iri* lines were validated in controlled RBH-induced resistance
 774 (RBH-IR) assays by scoring leaves from water- and RBH-treated (0.5 mM) plants into four
 775 *Hpa* colonization classes at 5-7 days post inoculation (dpi; bottom; Supplemental Figure 1).
 776 Representative photographs of trypan blue-stained leaves on the bottom left indicate the *Hpa*
 777 colonization classes, ranging from healthy leaves (I), hyphal colonization without
 778 conidiospores (II), hyphal colonization with conidiophores (III), to extensive hyphal
 779 colonization with conidiophores and deposition of sexual oospores (IV).

780 **(B)** Gene model of the *IRI1* gene (At5g40780) encoding LYSINE HISTIDINE
 781 TRANSPORTER1 (LHT1). Red triangles indicate two independent T-DNA insertions in the
 782 *lht1-5 (iri1-1)* and *lht1-4 (iri1-2)* mutants, respectively, to confirm the involvement of *LHT1* in
 783 RBH-IR against *Hpa*.

784 **(C)** Quantification of RBH-IR against *Hpa* in leaves of Col-0, *lht1-4* and *lht1-5*. Shown are
 785 frequency distributions of trypan blue-stained leaves across the four *Hpa* colonization classes
 786 (see A). Different letters indicate statistically significant differences between samples at 6 dpi
 787 (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n = 70-80$ leaves).

788 **(D)** Quantification of arrested *Hpa* colonization by callose. *Hpa*-induced callose was
 789 analyzed in aniline blue/calcofluor-stained leaves by epifluorescence microscopy. Shown are
 790 percentages of callose-arrested and non-arrested conidiospores at 3 dpi, as detailed by
 791 Schwarzenbacher et al. (2020). Different letters indicate statistically significant differences in
 792 frequencies between samples (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n > 100$
 793 conidiospores).

794

795 **Figure 2. LHT1 controls RBH-uptake and RBH-induced resistance against *Hpa*.**

796 **(A)** Quantification of RBH in leaves of Col-0 (wild-type), *lht1-5* (mutant) and *35Spro: LHT1*
 797 (overexpression) plants after soaking the soil to saturation with increasing RBH
 798 concentrations. Leaves were collected at 2 days after soil treatment with RBH and analyzed
 799 by HILIC-Q-TOF. Boxplots show median (middle bar), interquartile range (IQR; box), 1.5 x
 800 IQR (whiskers) and replication units (single dots) of leaf RBH concentrations (nmol/g dry
 801 weight [DW]). Inset shows p -values of statistically significant effects on RBH concentration
 802 by genotype, soil treatment and their interaction (two-way ANOVA). Asterisks indicate
 803 statistically significant differences relative to Col-0 for each soil treatment (Welch t-test; **,
 804 $p < 0.01$; *, $0.01 < p < 0.05$).

805 **(B)** Quantification of RBH-induced resistance against *Hpa* Col-0, *lht1-5* and *35Spro:LHT1*.
 806 Two-week-old seedlings had the soil of their pots saturated with increasing concentrations of
 807 RBH and challenge-inoculated with *Hpa* conidiospores 2 days later. Shown are frequency
 808 distributions of trypan blue-stained leaves across four *Hpa* colonization classes at 6 dpi (see
 809 Figure 1A). Different letters indicate statistically significant differences between samples
 810 (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n = 70-90$ leaves).

811

812 **Figure 3. Overexpression of *LHT1* renders *Arabidopsis* susceptible to growth repression
 813 by RBH, which is antagonized by co-application of L-alanine**

814 **(A)** *LHT1*-dependent effects of RBH and L-alanine on plant growth. Shown are 2-week-old
 815 seedlings of Col-0 (upper left), *lht1-5* (upper right), and *35Spro: LHT1* (bottom) grown on
 816 MS agar plates, supplemented with 10 mM (NH₄)₂SO₄ and increasing concentrations of RBH
 817 and/or L-alanine.

818 **(B)** Quantification of green leaf area (GLA \pm SEM; $n=7-19$) in 1-week-old Col-0, *lht1-5*, and
 819 *35Spro:LHT1* seedlings from the same experiment. Inset shows p -values of effects on GLA

820 by RBH concentration, L-alanine concentration and their interaction inside each genotype
821 (two-way ANOVA).

822

823 **Figure 4. LHT1 controls BABA-uptake and BABA-induced resistance against *Hpa***

824 **(A)** Quantification of BABA in leaves of Col-0 (wild-type) and *lht1-5* (mutant) plants after
825 soaking the soil to saturation with increasing BABA concentrations. Leaves were collected at
826 2 days after soil treatment and analyzed by HILIC-Q-TOF. Boxplots show median (middle
827 bar), interquartile range (IQR; box), 1.5 x IQR (whiskers) and replication units (single dots)
828 of leaf BABA concentrations (nmol/g DW). Inset shows *p*-values of statistically significant
829 effects on BABA concentration by genotype, soil treatment and their interaction (two-way
830 ANOVA). Asterisks indicate statistically significant differences to Col-0 for each soil
831 treatment (Welch t-test; **, $p < 0.01$; *, $0.01 < p < 0.05$).

832 **(B)** Quantification of BABA-induced resistance against *Hpa* in Col-0, *lht1-5* and
833 *35Spro:LHT1* seedlings. Two-week-old seedlings had the soil of their pots saturated with
834 increasing concentrations of BABA and challenge-inoculated with *Hpa* conidiospores 2 days
835 later. Shown are frequency distributions of trypan blue-stained leaves across four *Hpa*
836 colonization classes at 6 dpi (see Figure 1A). Different letters indicate statistically significant
837 differences between samples (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n = 70-80$
838 leaves).

839

840 **Figure 5. LHT1 controls stress tolerance to BABA**

841 **(A)** Effects of BABA on growth by Col-0, *lht1-5*, *35Spro:LHT1* Shown are 2-week-old
842 seedlings of Col-0 (upper left), *lht1-5* (upper right), and *35Spro:LHT1* (bottom) grown on MS
843 agar plates, supplemented with increasing concentrations of BABA.

844 **(B)** Average green leaf areas (GLA \pm SEM; $n=14-20$) of 1-week-old Col-0, *lht1-5*,
845 *35Spro:LHT1* plants from the same experiment. Asterisks indicate statistically significant
846 differences compared to Col-0 at each BABA concentration (Welch t-tests + Bonferroni FDR;
847 $p < 0.05$).

848

849 **Figure 6. Characterization of RBH and BABA uptake kinetics by LHT1 via
850 heterologous expression in yeast**

851 **(A, B)** Transformation of the yeast mutant $22\Delta 10\alpha$ (Besnard et al., 2016) with Arabidopsis
852 *LHT1* rescues growth on agar **(A)** or liquid medium **(B)** with L-alanine (L-Ala) as the only
853 nitrogen source. Shown in **(A)** are growth phenotypes of empty vector (EV)- and *LHT1*-
854 transformed $22\Delta 10\alpha$ cells on agar medium supplemented with inorganic nitrogen (10 mM
855 $(\text{NH}_4)_2\text{SO}_4$; top) or 1 mM L-alanine (bottom). **(B)** Growth of EV- and *LHT1*-transformed
856 $22\Delta 10\alpha$ in liquid medium supplemented with increasing L-Ala concentrations. Data points
857 and lines represent individual measurements and means of ΔOD_{595} values ($n=4$),
858 respectively.

859 **(C, D)** Competitive inhibition of LHT1-dependent uptake of L-Ala by RBH **(C; blue)** and
860 BABA **(D; red)**. Uptake velocities by LHT1 were determined in the presence of increasing L-
861 Ala concentrations containing 50 nCi ^{14}C -labeled L-Ala with and without 500 μM RBH **(C)**
862 or BABA **(D)**. Data points represent L-Ala uptake velocities (fmol L-Ala/cell; $n=3$) over a 5-
863 min time window. In the absence of RBH or BABA, the K_m for L-Ala-uptake by LHT1 was
864 9.4 μM . Competitive inhibition by RBH and BABA is shown by a decrease in K_m but not
865 V_{max} .

866 **(E, F)** Dixon plots to determine the inhibition constants (K_i) of RBH **(E)** and BABA **(F)**. K_i
867 values were determined in the presence of increasing L-Ala concentrations containing a fixed
868 amount of 50 nCi ^{14}C -labeled L-Ala and 0, 250 and 1,000 μM of RBH or BABA. Data points
869 represent values of inverse L-Ala uptake velocities over a 5-min time window (cell/fmol L-

870 Ala; n=3). Dotted vertical lines indicate intercepts at Ki values of RBH and BABA (see also
 871 Supplemental Figure S9).
 872

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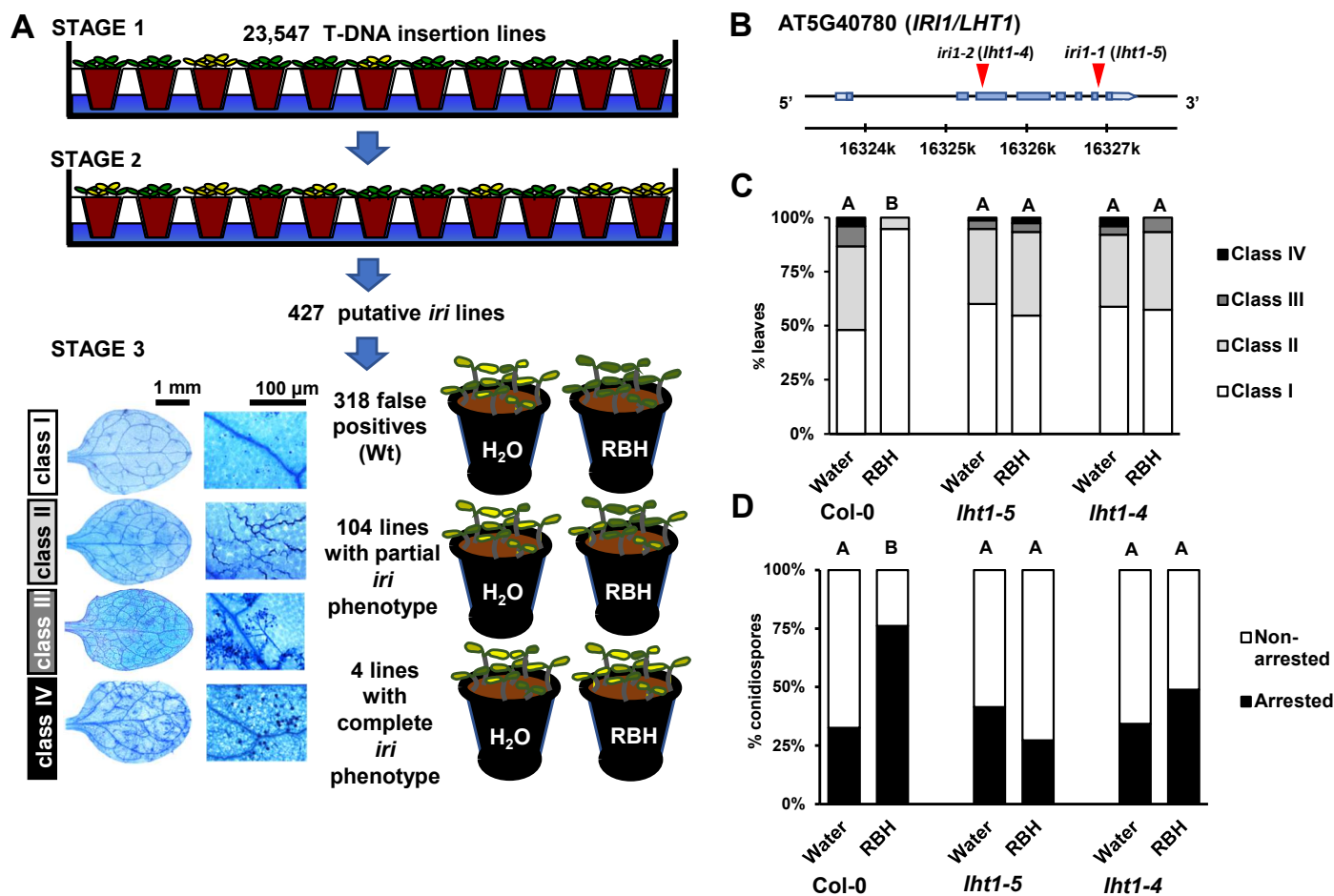


Figure 1. Mutant screen for *impaired in RBH-induced immunity (iri)* phenotypes and characterization of the *iri1* mutant in Arabidopsis.

(A) Schematic diagram of the three successive selection stages of the *iri* mutant screen on 23,547 T-DNA insertion lines from the SALK/SAIL collection. Small populations of ~five seedlings were screened per line (stage 1) and rescreened (stage 2) for sporulation by *Hyaloperonospora arabidopsidis* WACO9 (*Hpa*) upon saturating the soil to a final concentrations of 0.5 mM R- β -homoserine (RBH) and subsequent inoculation with *Hpa* conidiospores (top). Putative *iri* lines were validated in controlled RBH-induced resistance (RBH-IR) assays by scoring leaves from water- and RBH-treated (0.5 mM) plants into four *Hpa* colonization classes at 5-7 days post inoculation (dpi; bottom; Supplemental Figure 1). Representative photographs of trypan blue-stained leaves on the bottom left indicate the *Hpa* colonization classes, ranging from healthy leaves (I), hyphal colonization without conidiospores (II), hyphal colonization with conidiophores (III), to extensive hyphal colonization with conidiophores and deposition of sexual oospores (IV).

(B) Gene model of the *IRI1* gene (At5g40780) encoding LYSINE HISTIDINE TRANSPORTER1 (LHT1). Red triangles indicate two independent T-DNA insertions in the *lht1-5* (*iri1-1*) and *lht1-4* (*iri1-2*) mutants, respectively, to confirm the involvement of *LHT1* in RBH-IR against *Hpa*.

(C) Quantification of RBH-IR against *Hpa* in leaves of Col-0, *lht1-4* and *lht1-5*. Shown are frequency distributions of trypan blue-stained leaves across the four *Hpa* colonization classes (see A). Different letters indicate statistically significant differences between samples at 6 dpi (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n = 70-80$ leaves).

(D) Quantification of arrested *Hpa* colonization by callose. *Hpa*-induced callose was analyzed in aniline blue/calcofluor-stained leaves by epifluorescence microscopy. Shown are percentages of callose-arrested and non-arrested conidiospores at 3 dpi, as detailed by Schwarzenbacher et al. (2020). Different letters indicate statistically significant differences in frequencies between samples (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n > 100$ conidiospores).

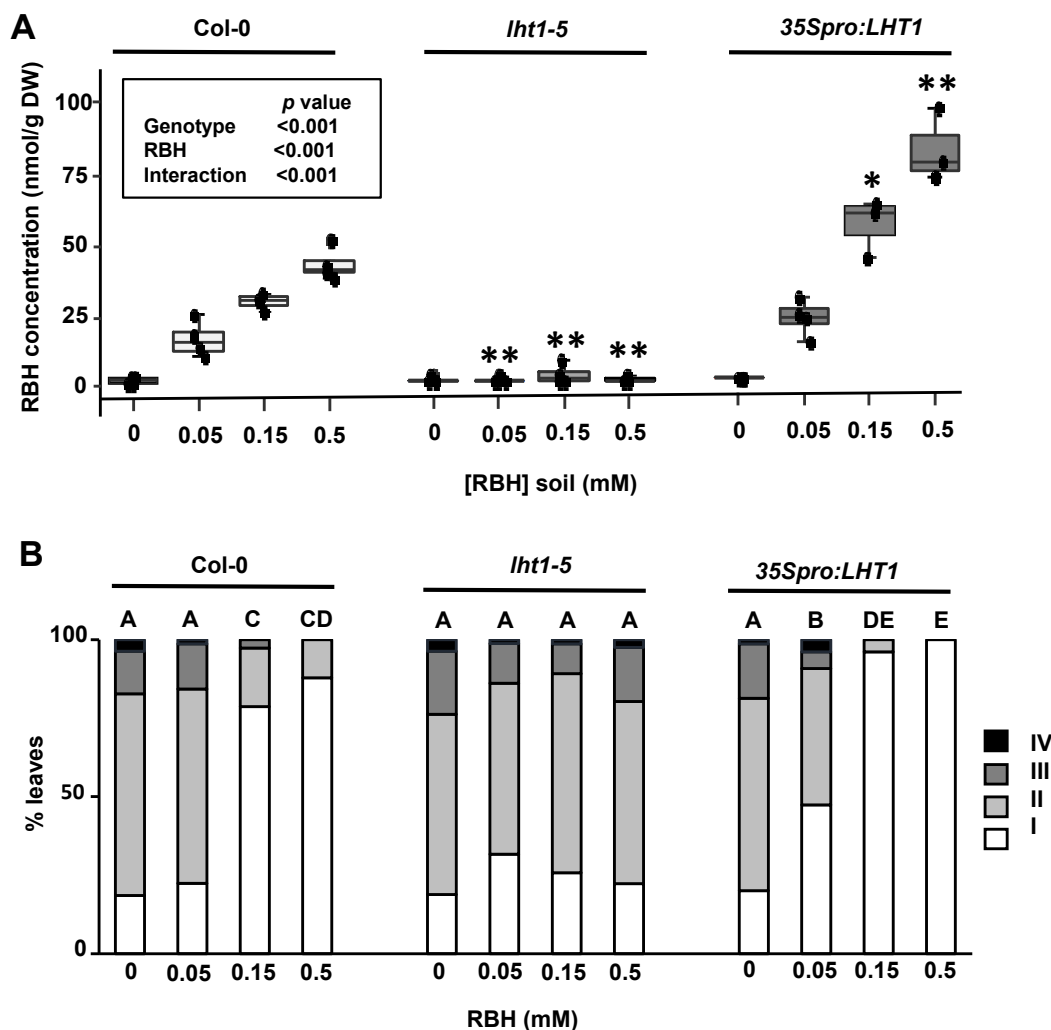


Figure 2. LHT1 controls RBH-uptake and RBH-induced resistance against *Hpa*.

(A) Quantification of RBH in leaves of Col-0 (wild-type), *lht1-5* (mutant) and *35Spro:LHT1* (overexpression) plants after soaking the soil to saturation with increasing RBH concentrations. Leaves were collected at 2 days after soil treatment with RBH and analyzed by HILIC-Q-TOF. Boxplots show median (middle bar), interquartile range (IQR; box), 1.5 x IQR (whiskers) and replication units (single dots) of leaf RBH concentrations (nmol/g dry weight [DW]). Inset shows p -values of statistically significant effects on RBH concentration by genotype, soil treatment and their interaction (two-way ANOVA). Asterisks indicate statistically significant differences relative to Col-0 for each soil treatment (Welch t-test; **, $p < 0.01$; *, $0.01 < p < 0.05$).

(B) Quantification of RBH-induced resistance against *Hpa* Col-0, *lht1-5* and *35Spro:LHT1*. Two-week-old seedlings had the soil of their pots saturated with increasing concentrations of RBH and challenge-inoculated with *Hpa* conidiospores 2 days later. Shown are frequency distributions of trypan blue-stained leaves across four *Hpa* colonization classes at 6 dpi (see Figure 1A). Different letters indicate statistically significant differences between samples (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n = 70-90$ leaves).

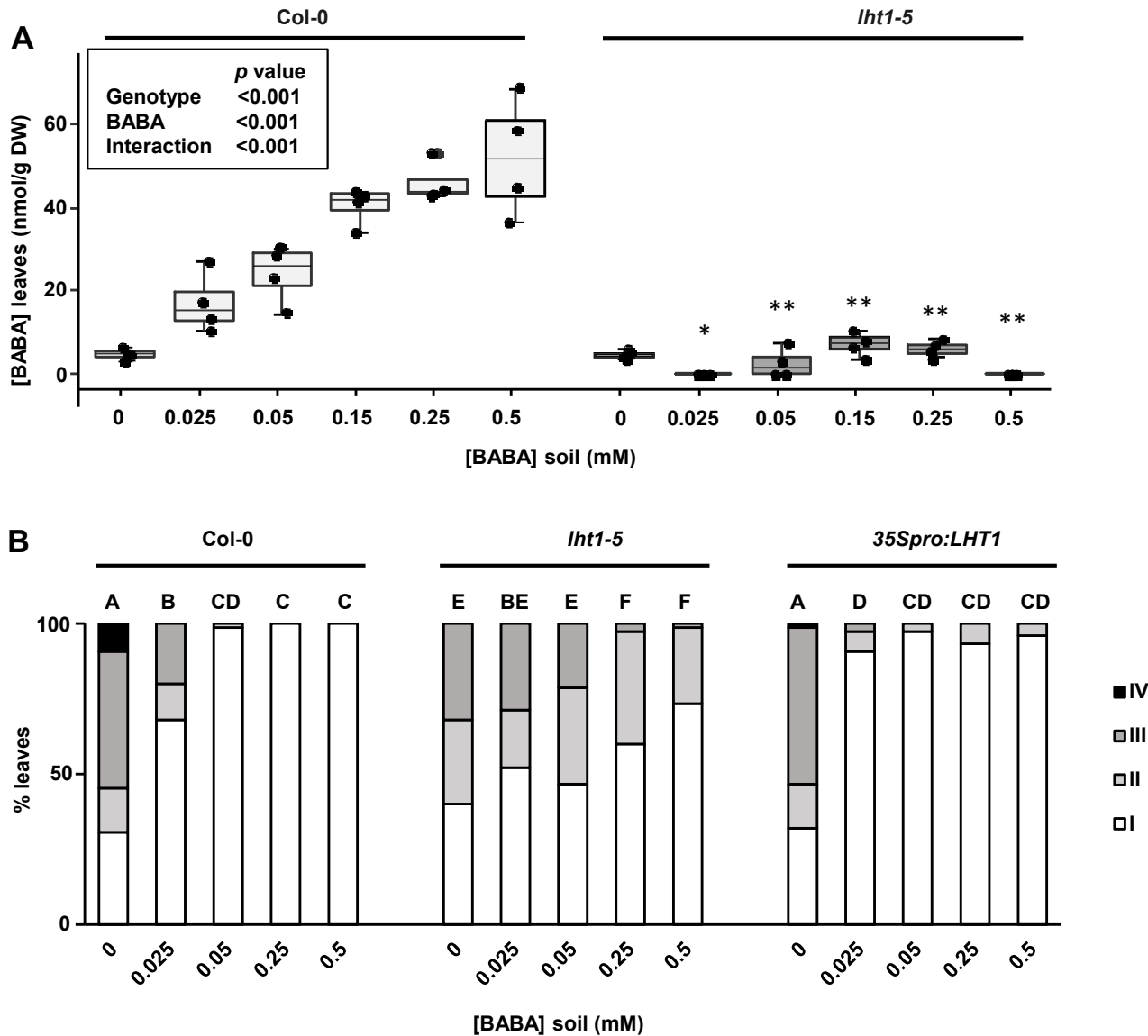


Figure 4. LHT1 controls BABA-uptake and BABA-induced resistance against *Hpa*

(A) Quantification of BABA in leaves of Col-0 (wild-type) and *lht1-5* (mutant) plants after soaking the soil to saturation with increasing BABA concentrations. Leaves were collected at 2 days after soil treatment and analyzed by HILIC-Q-TOF. Boxplots show median (middle bar), interquartile range (IQR; box), 1.5 x IQR (whiskers) and replication units (single dots) of leaf BABA concentrations (nmol/g DW). Inset shows p -values of statistically significant effects on BABA concentration by genotype, soil treatment and their interaction (two-way ANOVA). Asterisks indicate statistically significant differences to Col-0 for each soil treatment (Welch t-test; **, $p < 0.01$; *, $0.01 < p < 0.05$).

(B) Quantification of BABA-induced resistance against *Hpa* in Col-0, *lht1-5* and 35Spro:LHT1 seedlings. Two-week-old seedlings had the soil of their pots saturated with increasing concentrations of BABA and challenge-inoculated with *Hpa* conidiospores 2 days later. Shown are frequency distributions of trypan blue-stained leaves across four *Hpa* colonization classes at 6 dpi (see Figure 1A). Different letters indicate statistically significant differences between samples (Fisher's exact tests + Bonferroni FDR; $p < 0.05$; $n = 70-80$ leaves).

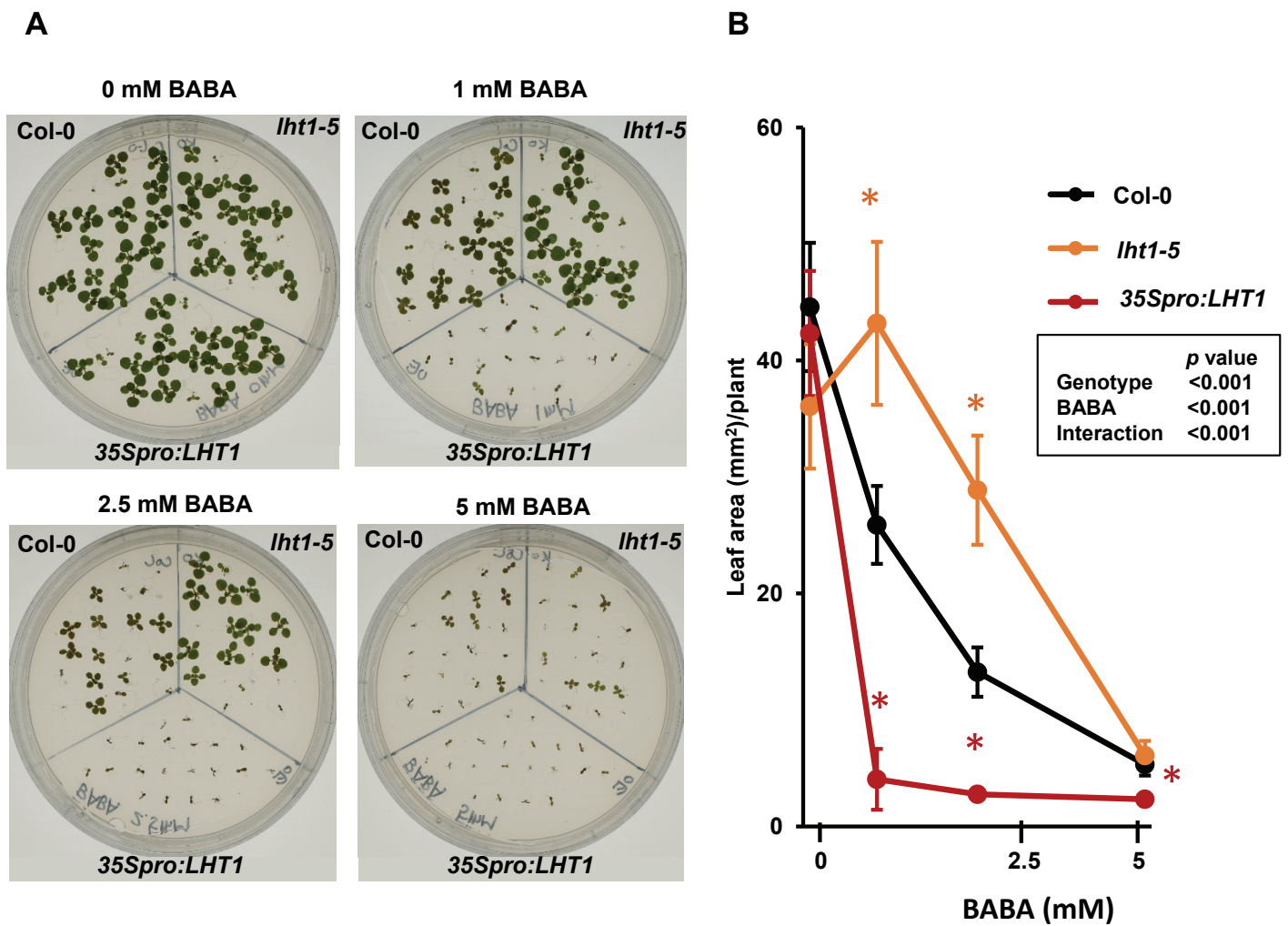


Figure 5. LHT1 controls stress tolerance to BABA

(A) Effects of BABA on growth by Col-0, *lht1-5*, *35Spro:LHT1* Shown are 2-week-old seedlings of Col-0 (upper left), *lht1-5* (upper right), and *35Spro:LHT1* (bottom) grown on MS agar plates, supplemented with increasing concentrations of BABA.

(B) Average green leaf areas (GLA \pm SEM; $n=14-20$) of 1-week-old Col-0, *lht1-5*, *35Spro:LHT1* plants from the same experiment. Asterisks indicate statistically significant differences compared to Col-0 at each BABA concentration (Welch t-tests + Bonferroni FDR; $p < 0.05$).

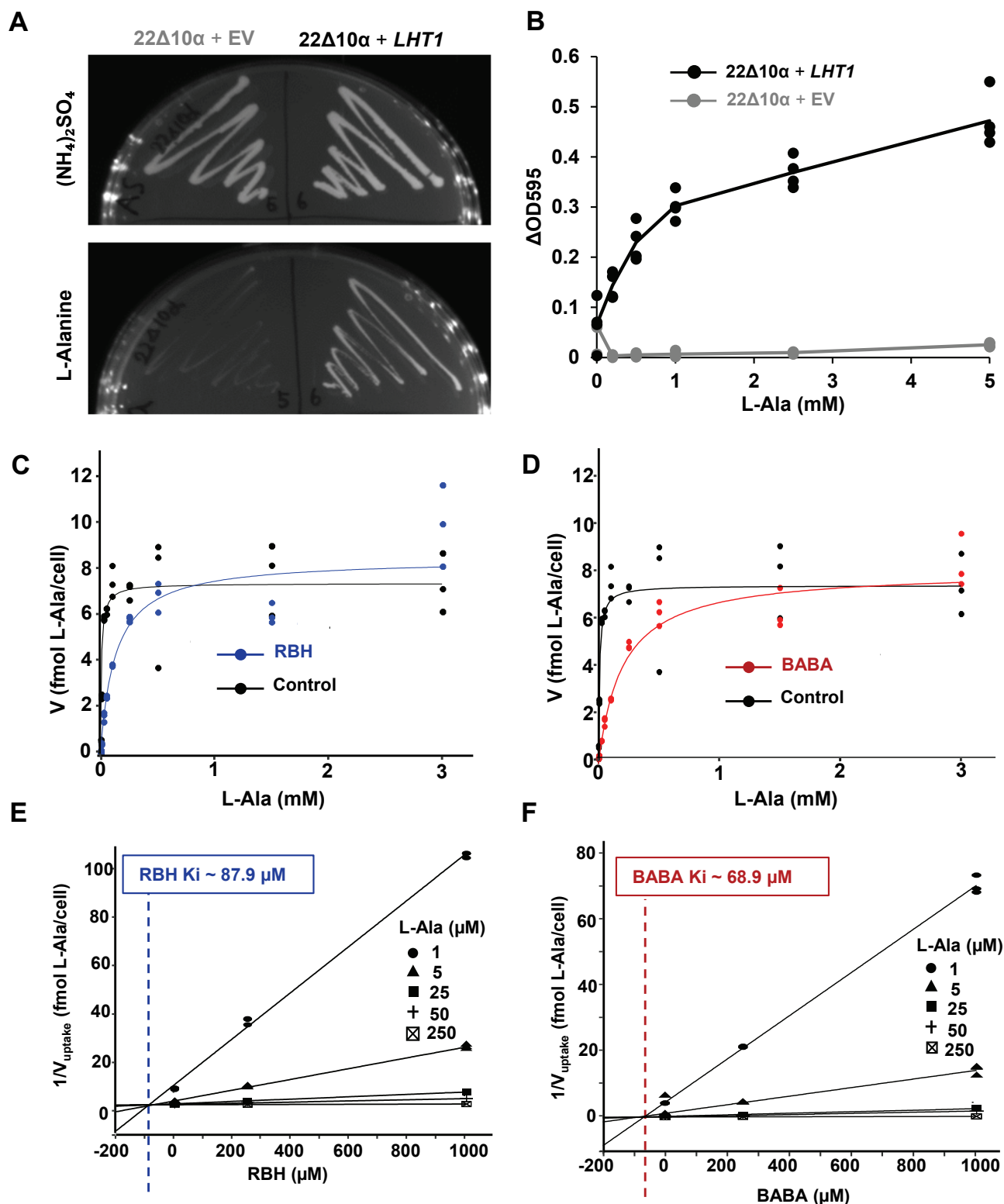


Figure 6. Characterization of RBH and BABA uptake kinetics by LHT1 via heterologous expression in yeast

(A, B) Transformation of the yeast mutant *22Δ10α* (Besnard et al., 2016) with *Arabidopsis LHT1* rescues growth on agar (A) or liquid medium (B) with L-alanine (L-Ala) as the only nitrogen source. Shown in (A) are growth phenotypes of empty vector (EV)- and *LHT1*-transformed *22Δ10α* cells on agar medium supplemented with inorganic nitrogen (10 mM (NH₄)₂SO₄; top) or 1 mM L-alanine (bottom). (B) Growth of EV- and *LHT1*-transformed *22Δ10α* in liquid medium supplemented with increasing L-Ala concentrations. Data points and lines represent individual measurements and means of ΔOD_{595} values ($n=4$), respectively.

(C, D) Competitive inhibition of LHT1-dependent uptake of L-Ala by RBH (C; blue) and BABA (D; red). Uptake velocities by LHT1 were determined in the presence of increasing L-Ala concentrations containing 50 nCi ¹⁴C-labeled L-Ala with and without 500 μM RBH (C) or BABA (D). Data points represent average L-Ala uptake velocities (fmol L-Ala/cell; $n=3$) over a 5-min time window. In the absence of RBH or BABA, the K_m for L-Ala-uptake by LHT1 was 9.4 μM . Competitive inhibition by RBH and BABA is shown by a decrease in K_m but not V_{max} .

(E, F) Dixon plots to determine the inhibition constants (K_i) of RBH (E) and BABA (F). K_i values were determined in the presence of increasing L-Ala concentrations containing a fixed amount of 50 nCi ¹⁴C-labeled L-Ala and 0, 250 and 1,000 μM of RBH or BABA. Data points represent mean values of inverse L-Ala uptake velocities over a 5-min time window (cell/fmol L-Ala; $n=3$). Dotted vertical lines indicate intercepts at K_i values of RBH and BABA (see also Supplemental Figure S9).

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