

1 **Arabidopsis WALL-ASSOCIATED KINASES are not required for**  
2 **oligogalacturonide-induced signaling and immunity**

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15

16 **Abstract**

17 Carbohydrate-based cell wall signaling impacts plant growth, development, and stress  
18 responses; however, how cell wall signals are perceived and transduced remains poorly  
19 understood. Several cell wall breakdown products have been described as typical damage-  
20 associated molecular patterns (DAMPs) that activate plant immunity, including pectin-derived  
21 oligogalacturonides (OGs). Receptor kinases (RKs) of the WALL-ASSOCIATED KINASE  
22 (WAK) family have been shown to bind pectin and OGs, and were previously proposed as OG  
23 receptors. However, unambiguous genetic evidence for the role of WAKs in OG responses is  
24 lacking. Here, we investigated the role of *Arabidopsis* WAKs in OG perception using a novel  
25 deletion mutant of the clustered WAK family. Using a combination of immune assays for early  
26 and late pattern-triggered immunity (PTI), we show that WAKs are dispensable for OG-induced  
27 signaling and immunity, indicating that they are not *bona fide* OG receptors.

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29

30 **Introduction**

31 Plants are exposed to myriad potential pests and pathogens, against which they have evolved  
32 sophisticated defense mechanisms. The plant cell wall acts as the initial physical barrier  
33 against invasion, and alterations in this structure intricately interact with the plant immune  
34 system (Dora *et al.*, 2022; Wolf, 2022).

35 The plant cell wall is composed of cellulose, hemicellulose, pectin, polyphenolic lignin and a  
36 series of structural and enzymatically active proteins (Wolf, 2022; Cosgrove, 2023). Cell wall  
37 polysaccharides serve as extracellular sources for the generation of damage-associated

38 molecular patterns (DAMPs) that are thought to be released upon mechanical damage or  
39 pathogen infection (Pontiggia *et al.*, 2020). Several such carbohydrate DAMPs have been  
40 previously described, including cellulose-derived cellobiose and cellooligosaccharides, mixed linked  
41 glucans, and pectin-derived oligogalacturonides (OGs) (Bacete *et al.*, 2018; Oelmüller *et al.*,  
42 2023). OGs are generated from demethylesterified pectins and represent the best studied  
43 pectin-derived cell wall breakdown products. OGs with a degree of polymerization (DP) 10-15  
44 (OG<sub>10-15</sub>) have been shown to elicit canonical PTI signaling and confer plant protection against  
45 a range of pathogens (Bishop *et al.*, 1981; Hahn *et al.*, 1981; Ridley *et al.*, 2001; De Lorenzo  
46 *et al.*, 2011). More recently, shorter OGs such as trimers (GalA<sub>3</sub>/OG<sub>3</sub>) and tetramers have also  
47 been shown to trigger immune responses and defense (Davidsson *et al.*, 2017; Liu *et al.*, 2023;  
48 Xiao *et al.*, 2024).

49 In the model plant *Arabidopsis thaliana* (hereafter, Arabidopsis), demethylated pectin was  
50 shown to directly bind the extracellular domain (ECD) of several WALL-ASSOCIATED  
51 KINASES (WAKs) (Decreux and Messiaen, 2005; Decreux *et al.*, 2006; Kohorn *et al.*, 2009;  
52 Liu *et al.*, 2023). WAKs belong to a large family of receptor kinases (RKs) comprising 5 WAKs  
53 and at least 21 WAK-like (WAKLs) (Verica and He, 2002) that are characterized by epidermal-  
54 growth factor (EGF)-like domains and a galacturonan-binding domain in their ECD (He *et al.*,  
55 1996; Verica and He, 2002; Kohorn *et al.*, 2012; Stephens *et al.*, 2022). WAK1 was the first  
56 identified RK physically linking the plasma membrane (PM) to the cell wall, as isolation from  
57 fractions of proteolytically digested cell walls indicated a strong interaction of WAK1 with the  
58 cell wall and native pectin *in vivo* (He *et al.*, 1996; Anderson *et al.*, 2001; Wagner and Kohorn,  
59 2001). Further experiments suggested WAKs and their association with cell wall pectin are  
60 involved in cell expansion (Kohorn *et al.*, 2006), and, potentially the response to pathogens  
61 (He *et al.*, 1998; Kohorn *et al.*, 2012). Later, WAK1 was also shown to bind pectin and OG<sub>9-15</sub>  
62 with a high affinity *in vitro* (Decreux and Messiaen, 2005; Decreux *et al.*, 2006; Kohorn *et al.*,  
63 2009), with the WAK1-ECD preferentially interacting with de-esterified pectin through a binding  
64 site formed by cationic amino acids (Decreux and Messiaen, 2005; Decreux *et al.*, 2006).  
65 A chimeric approach using the ECD of WAK1 fused to the intracellular domain of the leucine-  
66 rich repeat RK ELONGATION FACTOR-TU RECEPTOR (EFR) served as evidence for a  
67 proposed role for WAK1 in OG perception (Brutus *et al.*, 2010). Although OG treatment of  
68 WAK1-EFR chimera-expressing plants induced an EFR cytosolic domain-mediated defense  
69 response, critical genetic evidence that WAKs are *bona fide* OG receptors is still lacking. Direct  
70 genetics of WAKs was previously hindered by the genetic clustering of the WAK family in  
71 Arabidopsis, the assumption that *wak1* null mutants were lethal, and expected functional  
72 redundancy among the five members of the WAK family (He *et al.*, 1999; Brutus *et al.*, 2010;  
73 Kohorn and Kohorn, 2012). Recently, however, a CRISPR deletion mutant for most of the  
74 chromosomal cluster carrying the five Arabidopsis WAK genes, *wakΔ*, was generated. This

75 mutant was shown to be less sensitive to the bacterial flagellin-derived epitope flg22, chitin  
76 and OGs in terms of reactive oxygen species (ROS) production (Kohorn *et al.*, 2021);  
77 suggesting that WAKs may generally regulate immune receptor complexes, rather than  
78 function specifically as OG receptors (Wang *et al.*, 2020; Zhang *et al.*, 2020). WAKs were also  
79 recently shown to be genetically required for GalA<sub>3</sub>-induced expression of the salicylic acid  
80 (SA) marker gene *PATHOGENESIS-RELATED 1 (PR1)*(Liu *et al.*, 2023).  
81 In this work, we directly investigated the genetic involvement of WAKs in OG-induced signaling  
82 in Arabidopsis. We generated a novel deletion of the entire *WAK1-5* region (*wakΔ2*) and tested  
83 this mutant for OG-induced responses. Surprisingly, we found that *wakΔ2* retained full  
84 responsiveness to OGs, as measured by both early and late outputs of immune signaling. In  
85 addition, *wakΔ2* plants were not affected in OG-induced resistance against both bacterial and  
86 fungal pathogens. Furthermore, we tested the genetic involvement of WAKs in response to  
87 flg22 and could observe that flg22-induced responses are not affected in the *wakΔ2* mutant.  
88 Together, our data indicate that WAKs are not genetically required for OG perception and  
89 ensuing immune signaling in Arabidopsis.

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91

## 92 **Results**

### 93 **Generation of the *wakΔ2* mutant**

94 The Arabidopsis genome has five *WAK* genes located in cluster on chromosome 1 (FIGURE 1A). Recently, a partial deletion mutant was published, *wakΔ*, which lacks most of the cluster; however, this mutant still potentially expresses a fusion protein of the N-terminal region of WAK4 and the C-terminal region of WAK2 (FIGURE 1A) (Kohorn *et al.*, 2021). While the *wakΔ* mutant showed partially impaired flg22, chitin and OG responsiveness, it suffered from the presence of this potential WAK4-WAK2 fusion protein. Therefore, to explore if WAKs are genetically required for OG-induced responses, we generated a novel mutant using CRISPR/Cas9 that has a 23-kb deletion (*wakΔ2*), in which all *WAK* genes are absent (**Error! Reference source not found.**A-D). Lack of *WAK1-5* expression in *wakΔ2* seedlings was confirmed using RT-qPCR (

104 FIGURE 1D). In agreement with the previously published *WAK* deletion mutants (Kohorn *et al.*, 2021; Liu *et al.*, 2023), *wakΔ2* displayed no obvious growth phenotype when grown on soil (

107 FIGURE 1E).

108

### 109 **OG-induced immune signaling does not require the WAK family**

110 Given that WAKs are proposed as receptors for OGs (Brutus *et al.*, 2010), we investigated  
111 their genetic requirement for OG-induced immune responses using *wakΔ2*. Previous studies  
112 have extensively studied the immune responses in Arabidopsis triggered by exogenously  
113 applied OG<sub>10-15</sub> including extracellular ROS production, mitogen-activated protein kinase

114 (MAPK) activation, marker gene expression, ethylene production, callose deposition, seedling  
115 growth inhibition (SGI), and resistance against pathogens (Denoux *et al.*, 2008; Davidsson *et*  
116 *al.*, 2017; Gravino *et al.*, 2017; Bjornson *et al.*, 2021). Full loss-of-function mutants of a *bona*  
117 *fide* OG receptor should not be able to induce OG-induced responses, as shown for other  
118 ligand-perceiving receptors (Gómez-Gómez and Boller, 2000; Chinchilla *et al.*, 2006; Zipfel *et*  
119 *al.*, 2006; Miya *et al.*, 2007; Yamaguchi *et al.*, 2010; Cao *et al.*, 2014; Ranf *et al.*, 2015; Rhodes  
120 *et al.*, 2021).

121 To investigate if early immune signaling induced by OGs is dependent on WAKs, we measured  
122 extracellular ROS production in leaves of 3- to 4-week-old *Arabidopsis* plants. Surprisingly and  
123 in contrast to previous results (Kohorn *et al.*, 2021), OG<sub>10-15</sub>-induced ROS production was  
124 unaltered in *wakΔ2* in comparison to Col-0 grown in our conditions under short day (

125 FIGURE 2A,B). In addition to ROS, OGs induce rapid and transient MAPK phosphorylation  
126 (Gravino *et al.*, 2017). To determine if OG-induced MAPK activation is affected in the *wakΔ2*,  
127 MAPK phosphorylation was determined in *Arabidopsis* seedlings 5 and 15 min after elicitor  
128 treatment by western blot analysis using a commercial phosphorylation site-specific antibody.  
129 As with ROS production, OG<sub>10-15</sub>-triggered MAPK phosphorylation was unaltered in *wakΔ2*  
130 mutants (

131 FIGURE 2C). In addition to OG<sub>10-15</sub>, OG<sub>3</sub> (GalA<sub>3</sub>) was previously shown to trigger MAPK  
132 phosphorylation and WAKs have been shown to be required for OG<sub>3</sub>-induced *PR1* expression  
133 (Davidsson *et al.*, 2017; Liu *et al.*, 2023). We therefore additionally investigated if OG<sub>3</sub>-induced  
134 MAPK phosphorylation is dependent on WAKs. OG<sub>3</sub>-induced MAPK phosphorylation is  
135 comparatively weak but was still induced in *wakΔ2* mutant plants (

136 FIGURE 2D). OGs were also previously shown to induce synthesis of ethylene in  
137 *Arabidopsis* seedlings (Ferrari *et al.*, 2008; Brutus *et al.*, 2010; Gravino *et al.*, 2015). In line  
138 with other early induced PTI pathways, OG<sub>10-15</sub>-induced ethylene production was not  
139 compromised in *wakΔ2* mutants (

140 FIGURE 2E). Together, these results indicate that WAK1-5 are not required for OG-induced  
141 early immune outputs and thus the signaling initiation of OG-induced responses.

142 Aside from rapid signaling, PTI additionally involves longer-term responses such as callose  
143 deposition (Beffa *et al.*, 1996; Luna *et al.*, 2011; Wang *et al.*, 2021). To investigate the  
144 requirement of WAKs at later stages of OG-induced responses, OG<sub>10-15</sub>-induced callose  
145 deposition was measured in leaf discs of Col-0 and *wakΔ2* twenty-four hours after infiltration  
146 of either water or 100 µg/mL OG<sub>10-15</sub>. OG<sub>10-15</sub>-induced callose deposition in both Col-0 and  
147 *wakΔ2* (

148 FIGURE 3 3A,B). As is true of many elicitors, both OG<sub>3</sub> and long OGs can inhibit plant growth  
149 (Davidsson *et al.*, 2017). *Arabidopsis* seedlings grown in the presence of OG<sub>10-15</sub> showed a  
150 significant growth inhibition in comparison to mock-treated seedlings; however, no difference  
151 could be observed between Col-0 and *wakΔ2* (

152 FIGURE 3 3C). Another long-term measurement of plant immune signaling is the production  
153 of SA and ensuing signaling, which can be inferred through the accumulation of the PR1  
154 marker protein by immunoblotting (Tsuda *et al.*, 2009; Zhang and Li, 2019; Bender *et al.*,

155 2021). OG<sub>10-15</sub> and flg22 induced robust PR1 accumulation twenty-four hours after elicitor  
156 infiltration into leaves of Col-0 plants. Both flg22 and OG<sub>10-15</sub> -induced PR1 accumulation was  
157 not affected in *wakΔ2* (

158 FIGURE 3 3D). OG<sub>3</sub> induced very weak PR1 accumulation; however, no difference in PR1  
159 accumulation could be detected between Col-0 and *wakΔ2* (

160 FIGURE 3 3D).

161 Collectively, these results indicate that WAKs are not required for OG-induced immune  
162 signaling.

163

#### 164 **WAKs are not required for OG-induced immunity**

165 OGs have been shown to induce protection against the necrotrophic fungus *Botrytis cinerea*,  
166 the necrotrophic bacterium *Pectobacterium carotovorum* and the hemibiotrophic bacterium  
167 *Pseudomonas syringae* (Davidsson *et al.*, 2017; Gravino *et al.*, 2017; Howlader *et al.*, 2020).  
168 To investigate if WAKs are required for OG-induced immunity, we drop-inoculated *Arabidopsis*  
169 Col-0 and *wakΔ2* leaves with *B. cinerea* conidia 24 h after infiltration with water or 100 µg/mL  
170 OG<sub>10-15</sub>. Disease lesions on leaves were measured 48 h post inoculation. Plants pre-treated  
171 with water showed significantly larger lesions sizes in both Col-0 and *wakΔ2* than plants that  
172 were pretreated with OG<sub>10-15</sub> (

173 FIGURE 4 4A,B). OG-induced protection against *B. cinerea* was not affected in *wakΔ2* plants  
174 in comparison to wild-type plants. OG induced protection against *P. syringae* was similarly  
175 unaltered in *wakΔ2* in comparison to Col-0 (

176 FIGURE 4 4C). Overall, these results indicate that WAKs are not required for OG-induced  
177 immunity against these necrotrophic or hemi-biotrophic pathogens.

178

#### 179 **WAKs do not play a significant role in immune signaling triggered by other elicitors**

180 Aside from their role as potential OG receptors, WAKs were recently reported to function in  
181 immune signaling induced by bacterial flagellin in tomato and fungal chitin in cotton (Wang *et*  
182 *al.*, 2020; Zhang *et al.*, 2020). While in tomato only some flagellin-induced responses involved  
183 WAKs, e.g. callose deposition and anti-bacterial immunity, GhWAK7A was broadly required for  
184 full responsiveness to fungal chitin but not to OGs in cotton (Wang *et al.*, 2020; Zhang *et al.*,  
185 2020). In line with those observations, the *Arabidopsis* *wakΔ* mutant showed a reduction in  
186 ROS production induced by flg22, chitin and OGs (Kohorn *et al.*, 2021). Intrigued by these  
187 findings, we also tested whether flg22-induced responses are affected by the full deletion of  
188 WAKs in *Arabidopsis*. In contrast to previous results, flg22-induced ROS production in leaves  
189 of 3- to 4-week-old *Arabidopsis* plants were not affected in *wakΔ2* in comparison to Col-0 under  
190 our conditions (

191 FIGURE 5A,B). As expected, flg22-induced ROS production was dependent on the receptor  
192 FLAGELLIN-SENSING 2 (FLS2) and its co-receptor BRASSINOSTEROID-INSENSITIVE 1  
193 (BRI1)-ASSOCIATED KINASE 1 (BAK1). In line with this, flg22-induced MAPK activation,

194 ethylene production and induced resistance against *P. syringae* were not reduced in *wakΔ2*  
195 in comparison to Col-0 (

196 FIGURE 5C-E). These results indicate that the deletion of WAKs does not affect flg22-induced  
197 responses under our growth conditions.

198 The *wakΔ2* mutant had no obvious growth phenotype when grown on soil (FIGURE 1E). The  
199 only *wak*-related growth phenotype previously observed was reduced root length when  
200 seedlings were grown on MS medium lacking sucrose, most pronounced on 1/6 MS (Kohorn  
201 *et al.*, 2006, 2021). Therefore, to investigate if elicitor-induced root-growth inhibition is affected  
202 in the *wakΔ2* mutant, both Col-0 and *wakΔ2* plants were grown in the presence of 10 nM  
203 Atpep1, 100 nM flg22 or without elicitor for 5 days. Elicitor-induced root-growth inhibition was  
204 similar in Col-0 and *wakΔ2* in the same extent to Col-0 for both flg22 and Atpep1 (

205 FIGURE 5F).

206

207

## 208 Discussion

209 PTI is achieved by the recognition of diverse elicitor molecules as ligands for plasma  
210 membrane-resident pattern recognition receptors (PRRs) (DeFalco & Zipfel, 2021). Cell walls  
211 are the first layer of defense against invading pathogens, many of which have evolved arsenals  
212 of enzymatic and mechanical means to degrade or penetrate the cell wall (Bacete *et al.*, 2018;  
213 Dora *et al.*, 2022). Thus, the integrity of the cell wall needs to be carefully monitored by sensor  
214 proteins. Several RKs have been proposed as PRRs that perceive cell wall breakdown  
215 products, including WAKs based on their ability to interact with pectin and its breakdown  
216 products (He *et al.*, 1996; Decreux and Messiaen, 2005; Decreux *et al.*, 2006; Kohorn *et al.*,  
217 2009; Brutus *et al.*, 2010). Yet, genetic evidence that WAKs function as *bona fide* OG  
218 receptor(s) was missing. Here, we have used the *wakΔ2* mutant, which lacks all five members  
219 of the WAK family, to demonstrate that none of the WAKs are required for responses to either  
220 short or long chain demethylated OGs in Arabidopsis.

221 Previously, the galacturonan-binding domain of WAKs was shown to bind both pectins and  
222 demethylesterified OG<sub>9-15</sub> (Decreux and Messiaen, 2005; Decreux *et al.*, 2006). Additionally,  
223 chimeric WAK-EFR receptors were able to induce EFR-like responses upon OG-treatment  
224 (Brutus *et al.*, 2010). While our results indicate that WAKs are not genetically required for OG-  
225 induced responses, they do not contradict the ability of WAK ECDs to bind pectins or pectin  
226 breakdown products. Interestingly, the ECD of the malectin-like RK FERONIA was also  
227 recently reported to bind to pectin and pectin breakdown products (Feng *et al.* 2018; Tang *et*  
228 *al.* 2022; Lin *et al.* 2022), suggesting that this biochemical property might be true for several  
229 cell wall-anchored RKs without necessarily functioning as the true receptors for these  
230 carbohydrates.

231 OG<sub>10-15</sub> were suggested to be produced during pathogen infection and to subsequently induce  
232 immune signaling (Ferrari *et al.*, 2013; Xiao *et al.*, 2024). Although demethylesterified OG<sub>10-15</sub>  
233 are active as elicitors, recent evidence challenges their production *in planta* as most Ogs  
234 produced during infection with *B. cinerea* or *Fusarium oxysporum* were acetyl- and  
235 methylesterified (Voxeur *et al.*, 2019; Gámez-Arjona *et al.*, 2022). While pectic fractions of  
236 various sizes and modifications show elicitor activity, the complexity of those *in planta*-  
237 produced Ogs as well as the profile of crude extracts produced in the lab complicates the  
238 attribution of individual OG species to the elicitor activity (Liu *et al.*, 2023). Regardless of the  
239 exact nature of *in planta* OG species, WAKs have been previously proposed as the receptors  
240 for demethylesterified OG<sub>10-15</sub> based on *in vitro* binding studies and chimeric approaches, and  
241 we are here unable to confirm any corresponding genetic requirement for WAKs in OG<sub>10-15</sub>-  
242 induced signaling. Interestingly, electrostatic analysis of the WAK1 ECD predicted by AlphaFold  
243 revealed a negatively charged galacturonan-binding domain at apoplastic pH, contradicting  
244 the suggested binding of polyanionic de-esterified pectins (Lee and Santiago, 2023).  
245 While our data demonstrate that members of the WAK family are not required for OG-induced  
246 responses, it is possible that quantitative phenotype(s) are masked by persistent functional  
247 redundancy. WAKs are characterized by a galacturonan-binding domain and many WAKs  
248 contain one or more copies of EGF-like domains in their ECD (Verica and He, 2002; Stephens  
249 *et al.*, 2022). In addition to 5 WAKs, there are at least 21 WAK-likes (WAKLs) in Arabidopsis.  
250 To date, clear evidence is missing that WAKLs are also able to bind cell wall fragments  
251 (Kohorn, 2016), with the exception of WAKL22/RESISTANCE TO FUSARIUM OXYSPORUM  
252 1 (RFO1) and WAKL14 (Huerta *et al.*, 2023; Ma *et al.*, 2024). However, based on their  
253 phylogenetic relationship, WAKLs are obvious candidates to test for further genetic  
254 redundancy.  
255 Aside from a role in OG perception, WAKs were recently suggested to be involved in the  
256 regulation of other RK complexes during immunity, indicating that they might serve as  
257 accessory RKs of PRR complexes. In tomato and cotton, WAKs interact with and positively  
258 regulate PRR complexes and are required for full responsiveness to the corresponding elicitors  
259 (Wang *et al.*, 2020; Zhang *et al.*, 2020). In Arabidopsis, the *wakΔ* mutant was less sensitive to  
260 multiple elicitors in terms of ROS production (Kohorn *et al.*, 2021). In contrast with these  
261 previous results, no quantitative reduction in OG- or flg22-induced ROS production could be  
262 observed in the *wakΔ2* mutant. Although this difference is striking, it might further underline  
263 the role of WAKs as accessory RKs under certain growth conditions rather than OG-perceiving  
264 receptors. While WAKs appear to interact with multiple elicitor-perceiving RKs, the exact  
265 mechanisms by which WAKs regulate immunity seem to differ between plant species or  
266 different WAKs.

267 WAKs are found across land plants, with the WAK/WAKL family expanded in monocots (de  
268 Oliveira *et al.*, 2014; Kanyuka and Rudd, 2019; Stephens *et al.*, 2022; Zhang *et al.*, 2023a;  
269 Ngou *et al.*, 2024). Several *WAKs* or *WAKLs* have been identified as resistance genes and are  
270 required for basal resistance to pathogens in a variety of different crop plants (Diener and  
271 Ausubel, 2005; Zuo *et al.*, 2015; Hurni *et al.*, 2015; Hu *et al.*, 2017; Saintenac *et al.*, 2018; Bot  
272 *et al.*, 2019; Larkan *et al.*, 2020; Li *et al.*, 2020; Stephens *et al.*, 2022; Zhang *et al.*, 2023b; Dai  
273 *et al.*, 2024; Zhong *et al.*, 2024). While diverse roles and mechanisms for WAKs in plant  
274 immunity have been proposed, a clear possibility is that WAKs perceive pathogen-derived  
275 molecules. Indeed, *Arabidopsis* WAK3 was recently shown to be required for immune  
276 responses induced by bacterial harpins (Held *et al.*, 2024) indicating that WAKs might indeed  
277 perceive microbial molecules. Additionally, three WAKs have also been demonstrated to exhibit  
278 a gene-for-gene interaction with specific pathogenic effectors in crops (Stephens *et al.*, 2022).  
279 The WAK proteins Stb6 and Rlm9 provide resistance against *Zymoseptoria tritici* isolates  
280 expressing AvrSbt6 in wheat and *Leptosphaeria maculans* expressing AvrLm5-9 in oilseed  
281 rape, respectively (Brading *et al.*, 2002; Larkan *et al.*, 2016; Larkan *et al.*, 2020). While no  
282 direct interaction could be detected between these fungal effectors and corresponding WAK  
283 resistance proteins (Saintenac *et al.*, 2018; Larkan *et al.*, 2020), intriguingly, a direct interaction  
284 has been observed between the maize WAK protein Snn1 and the *Phaeosphaeria nodorum*  
285 effector protein SnTox1. Unlike most other WAKs studied thus far, Snn1 serves as susceptibility  
286 factor for *P. nodorum* leading to disease in Snn1-expressing plants (Liu *et al.*, 2012; Stephens  
287 *et al.*, 2022; Shi *et al.*, 2023). Maize qRgls1/WAKL<sup>Y</sup> was also recently shown to confer  
288 quantitative disease resistance against gray leaf spot caused by the fungi *Cercospora zeae-*  
289 *maydis* and *C. zeina* (Zhong *et al.*, 2024). Notably, an aqueous extract of *C. zeina* hyphae and  
290 spores was sufficient to induce WAKL<sup>Y</sup>-dependent ROS production suggesting that WAKL<sup>Y</sup>  
291 perceives a fungal ligand.  
292 Altogether, there is emerging evidence that WAKs may perceive diverse molecules of microbial  
293 origin and orchestrate both broad-spectrum and race-specific resistance (Kanyuka and Rudd,  
294 2019), which is consistent with our evidence that they do not function as *bona fide* OG  
295 receptor(s) in *Arabidopsis*. However, the mechanisms by which WAKs contribute to immunity  
296 and their true ligand(s) remain to be definitively characterized.

297

298

## 299 **Material and methods**

300

### 301 **Plant growth**

302 *Arabidopsis* seeds were surface-sterilized using ethanol, plated on 0.5 MS medium (1 %  
303 sucrose, pH 5.8, 0.9 % phytoagar), stratified for 48 hours in the dark at 4 °C, and grown at 22

304 °C under a 16-hour photoperiod (120  $\mu\text{mol} * \text{s}^{-1} \text{m}^{-2}$  illumination). For assays in adult plants,  
305 including ROS production, pathogen infection and callose deposition, seedlings were  
306 transferred to soil after 7-10 days growth on plates. Plants were grown in short-day cycles (10  
307 h light/14 h dark, 60 % humidity, 20 °C) for an additional 2-3 weeks. For assays with seedlings,  
308 including MAPK activation, RNA extraction, seedling growth inhibition and root growth  
309 inhibition, these were transferred 5 days after exposure to light to liquid MS and grown there  
310 for 10 days. Mutants were generated in the *A. thaliana* Columbia (Col-0) ecotype and primers  
311 for genotyping are found in SUPPLEMENTAL TABLE 1.

312

### 313 **CRISPR-Cas9 mutagenesis**

314 The *WAK4* and *WAK2* oligonucleotides used as templates for SgRNA-targeted sites  
315 (GCTGT TTCGTTATTGTTAAATGG) 432 bp 5' to the *WAK4* ATG start codon, and  
316 (GGGGAGATTGAACAC TTGCTCGG) 77 bp 5' to the *WAK2* stop codon were each cloned  
317 into pSkAtu26 (Feng et al., 2013). These two expression Sg cassettes were then cloned into  
318 pCambia1302 that also had a pOLE1-RFP cassette inserted into the ASN718 site by PCR  
319 cloning (Shimada et al., 2010).

320 The T1, RFP<sup>+</sup> (expressing linked CAS9 and sgRNAs) were screened for a deletion by PCR  
321 using primers flanking the deletion, and then T2 RFP<sup>-</sup> plants were screened again by PCR to  
322 isolate a plant with a deletion but not expressing CAS9 or the sgRNAs. These isolates were  
323 self-crossed to generate a homozygous deletion.

324

### 325 **RNA extraction and real-time (RT) quantitative PCR analysis**

326 Total RNA was extracted from 2-week-old liquid-grown seedlings. Total RNA was extracted  
327 using TRI reagent (Sigma-Aldrich). To remove genomic DNA, samples were treated with  
328 TURBO DNA-free Kit (Thermo Fisher Scientific). cDNA synthesis was performed using 1  $\mu\text{g}$  of  
329 DNA-free RNA sample with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher  
330 Scientific) according to the manufacturer's protocol. RT-qPCR analysis was performed using  
331 diluted cDNA as template for PowerUp SYBR Green (Applied biosystems) with the primers  
332 provided in SUPPLEMENTAL TABLE 1.

333

### 334 **MAPK activation**

335 MAPK activation was performed as previously described (Mühlenbeck et al., 2023). Five-day-  
336 old seedlings were transferred into 24-well plates containing 1 mL of liquid 0.5 MS (1 %  
337 sucrose). Two seedlings per well were grown there for another 10-12 days. Seedlings were  
338 treated with 100  $\mu\text{g/mL}$  OG<sub>10-15</sub> (Elicityl, GAT114), 100  $\mu\text{g/mL}$  OG<sub>3</sub> (GalA3, Megazyme) or 1  
339  $\mu\text{M}$  flg22, and harvested at each time point as indicated in figure captions. Total proteins were  
340 extracted using extraction buffer (50 mM Tris pH 7.5 (HCl), 150 mM NaCl, 10 % (v/v) glycerol,

341 2 mM EDTA, 1 mM homemade PPI (equivalent to Sigma-Aldrich Protease-inhibitor cocktail  
342 P9599), 1 mM NaF, 1 mM sodium-orthovanadate, 2 mM sodium-molybdate, 4 mM sodium-  
343 tartrate, 1 % (v/v) IGEPAL CA630, 5 mM DTT). Proteins were analyzed by SDS-PAGE and  
344 immunoblotting using p44/42 MAPK antibody (Cell Signaling Technology).

345

### 346 **Seedling growth inhibition**

347 Seedling growth inhibition assays was performed as previously described (Abarca *et al.*, 2021).  
348 Briefly, 5-day-old *Arabidopsis* seedlings were transferred to 48-well plate with one seedling per  
349 well. Each well contained either 500  $\mu$ L 0.5 liquid MS with or without 200  $\mu$ g/mL OG<sub>10-15</sub> (Elicityl,  
350 GAT114). After 10 days of growth in the presence of the respective elicitor, individual seedling  
351 weight as assessed using an analytical balance.

352

### 353 **Root growth Inhibition**

354 Five-day-old seedlings were transferred from solid MS plates to 12-well plate with 6 seedling  
355 per well. Each well contained 4 mL of liquid MS supplemented with mock (sterile ddH<sub>2</sub>O), 10  
356 nM Atpep1 or 100 nM flg22. After 5 days of treatment, seedlings were transferred to MS plates  
357 and imaged. Root lengths were quantified with ImageJ.

358

### 359 **Ethylene production**

360 Four- to six-week-old *Arabidopsis* leaves were cut into 3-mm slices and floated on water  
361 overnight. For each sample, three leaf slices were transferred to a 6-mL glass tube containing  
362 200  $\mu$ L MES buffer (pH 5.7), followed by adding either water control or the elicitor to a final  
363 concentration of 1  $\mu$ M. Vials were closed with a rubber septum and ethylene production in the  
364 free air space was measured by gas chromatography (Shimadzu, GC-14A) after 3 hours of  
365 incubation.

366

### 367 **ROS production**

368 Leaf-discs of 3- to 4-week-old plants were taken (4 mm Ø) and placed with the abaxial side  
369 down into a well of a white polystyrene 96-well plate containing 100  $\mu$ L ddH<sub>2</sub>O and recovered  
370 overnight. The next day, the water was replaced by a solution containing 20  $\mu$ g/mL horseradish  
371 peroxidase (HRP, sigma), luminol (17  $\mu$ g/mL) and elicitor (100 nM for flg22, 100  $\mu$ g/mL OG<sub>10-</sub>  
372 <sub>15</sub> (Elicityl, GAT114), as stated). Luminescence was immediately measured for 60 minutes  
373 using a charge-coupled device camera (Photek Ltd, East Sussex UK).

374

### 375 **Callose deposition**

376 Callose deposition assays were performed as described previously (Mason *et al.*, 2020).  
377 Briefly, four leaves of 4- to 5-week-old plants were syringe-infiltrated with either mock (ddH<sub>2</sub>O),

378 1  $\mu$ M flg22 or 100  $\mu$ g/mL OG<sub>10-15</sub> (Elicityl, GAT114). Twenty-four hours after infiltration, leaf  
379 discs were taken and collected in 24-well plates filled with 1 mL 100 % EtOH until completely  
380 destained. Leaf discs were equilibrated in 1 mL 67 mM K<sub>2</sub>HPO<sub>4</sub> (pH 12) for 60 min. Afterwards,  
381 the tissue was stained using aniline blue (Acros Organics) staining solution (0.01 % (w/v) aniline  
382 blue in 67 mM K<sub>2</sub>HPO<sub>4</sub> (pH 12) for 60 min and washed in 67 mM K<sub>2</sub>HPO<sub>4</sub> (pH=12) for 60  
383 minutes. Stained tissue was mounted in mounting solution (80 % glycerol, 67 mM K<sub>2</sub>HPO<sub>4</sub>, pH  
384 12) on microscope slides. Callose deposits were imaged using a Leica DM6000B and  
385 quantified in ImageJ.

386

### 387 **PR1 protein abundance**

388 PR1 accumulation was assayed as previously described (Bender *et al.*, 2021). Briefly, three  
389 leaves of 3-week-old plants were infiltrated with mock (sterile ddH<sub>2</sub>O), 1  $\mu$ M flg22, 100  $\mu$ g/mL  
390 OG<sub>10-15</sub> (Elicityl, GAT114) or 100  $\mu$ g/mL OG<sub>3</sub> (GalA3, Megazyme). Twenty-four hours after  
391 infiltration, leaves were harvested in 1.5-mL tubes and snap-frozen in liquid nitrogen and  
392 pulverized. Extraction buffer (50 mM Tris pH7.5 (HCl), 150 mM NaCl, 10 % (v/v) glycerol, 2  
393 mM EDTA, 1x plant protease inhibitor cocktail) was added and protein concentration was  
394 adjusted by Bradford assay. Normalized protein extracts were analyzed by SDS-PAGE (15 %)  
395 and immunoblotting using PR1-antibodies (Agrisera).

396

### 397 **Induced resistance against *Pseudomonas syringae***

398 Two leaves of 4- to 5-week-old plants were infiltrated with 1  $\mu$ M flg22 or 100  $\mu$ g/mL OG<sub>10-15</sub>  
399 (Elicityl, GAT114) or mock (sterile ddH<sub>2</sub>O). Freshly restreaked *P. syringae* pv tomato DC3000  
400 was grown in liquid Kings B overnight and refreshed in a subculture the next morning for  
401 additional 1-2 hours. Bacteria were infiltrated into pretreated leaves with an OD<sub>600</sub> of 0.0002.  
402 Plants were covered for two days, after which 1 leaf disc was harvested per treated leaf (8 mm  
403 Ø) and pooled per plant. Leaf discs were ground in 10 mM MgCl<sub>2</sub>, thoroughly mixed and diluted  
404 in a 1:10 series until 1:10<sup>-6</sup>. Samples were plated on LB plates. After two days of growth at 28  
405 °C, colony forming units were counted. Statistics were performed on log<sub>10</sub> (CFU/cm<sup>2</sup>).

406

### 407 **Induced resistance against *Botrytis cinerea***

408 Four leaves of 4- to 5-week-old plants were infiltrated with 1  $\mu$ M flg22 or 100  $\mu$ g/mL OG<sub>10-15</sub>  
409 (Elicityl, GAT114) or mock (sterile ddH<sub>2</sub>O) in the morning. The next day, spores of *B. cinerea*  
410 BMM were collected in sterile ddH<sub>2</sub>O and the spores were counted using a counting chamber.  
411 At least 1 hour prior infection, infection solutions were prepared with a final concentration 5x10<sup>5</sup>  
412 spores/mL in 0.5 Potato Dextrose Broth and incubated at RT. Five microliters of the *Botrytis*  
413 infection solution were dropped on the adaxial site next to the middle vein. Plant solid trays  
414 were filled with water, covered with a lid, and sealed with parafilm to produce high humidity.

415 After 2 days after infection at dimmed light, leaves were detached, images were taken, and  
416 lesion size was measured using Image J.

417

418

419

420 **Author Contributions**

421 L.H. and C.H. performed the experiments and analyzed the data. B.K. generated the genetic  
422 material. T.N., T.A.D and C.Z designed and supervised the project. L.H. wrote the first draft of  
423 the manuscript. All authors contributed to the final version of the manuscript.

424

425

426 **Acknowledgments**

427 We thank all the members of the Zipfel group for fruitful discussions during the project. We  
428 thank also Jiashu Chu, John Haidoulis and Jana Ordon for feedback on the manuscript. L.H.  
429 was funded by a Zurich-Basel Plant Science Center-Syngenta Fellowship. B.D.K. was  
430 supported by National Science Foundation grant IOS 1556057. T.N. was supported by DFG-  
431 TRR356 (B5). T.A.D. was supported by a Discovery Grant from the Natural Sciences and  
432 Engineering Council of Canada (NSERC RGPIN-2023-04222). C.Z. was supported by the  
433 University of Zürich and the Swiss National Science Foundation grants no. 31003A\_182625  
434 and 310030\_212382.

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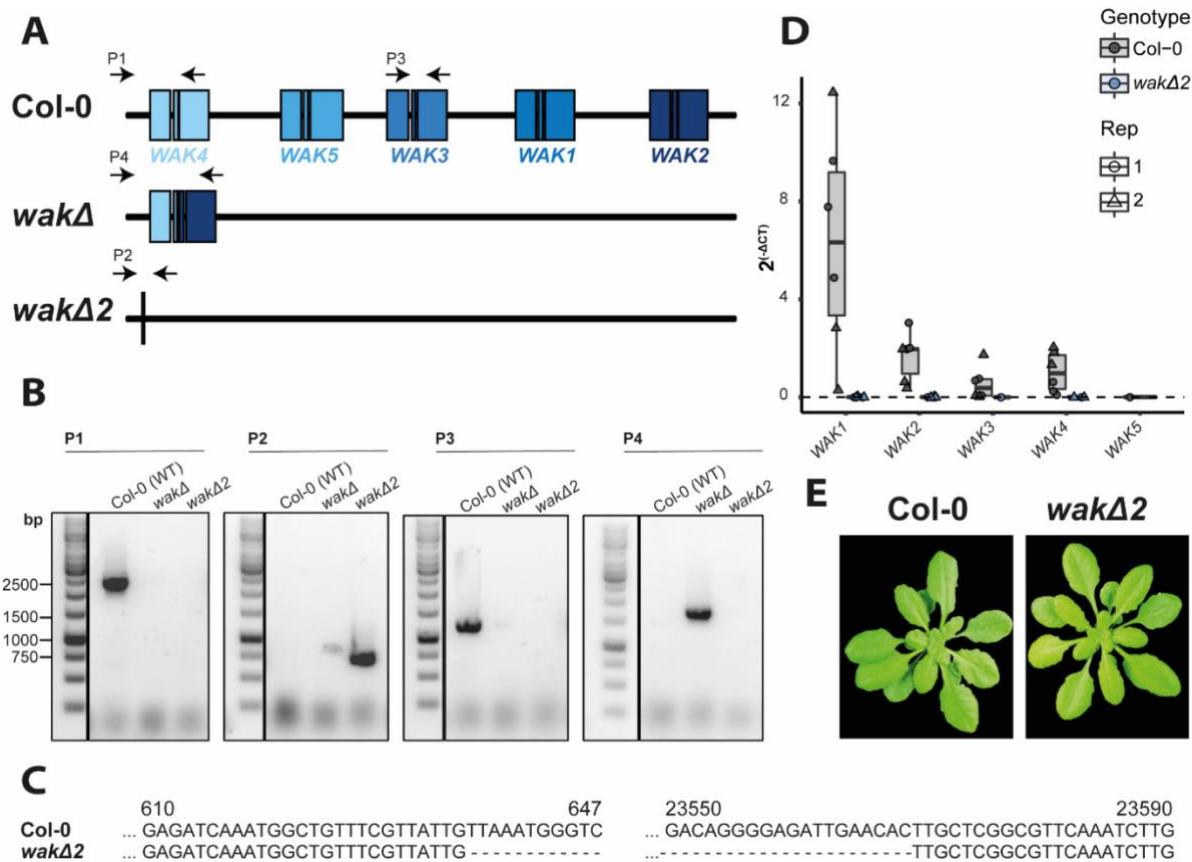
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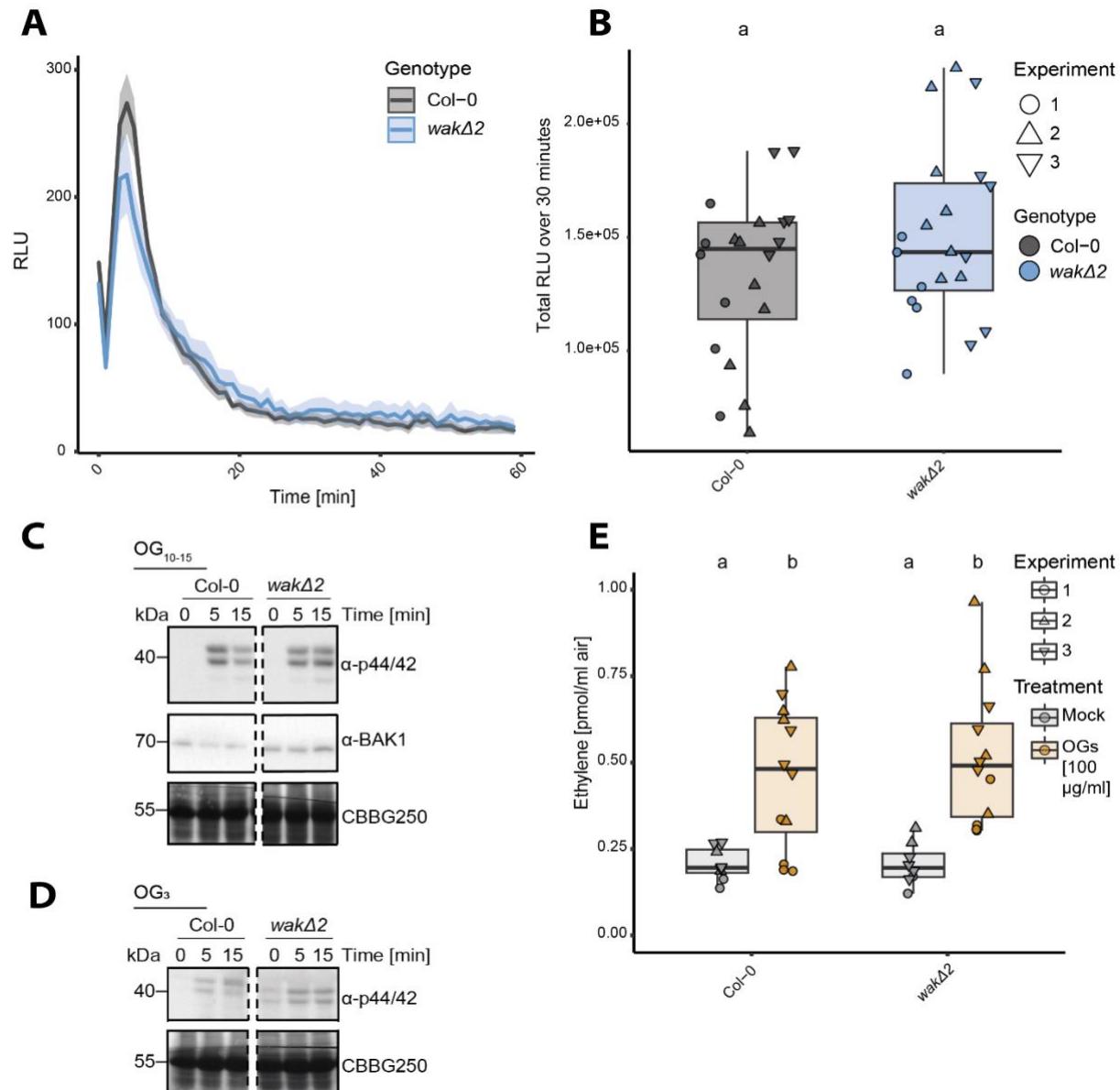
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454 **Figures and legends**  
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459 **FIGURE 1 – wakΔ2 is a full WAK deletion mutant.**  
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**A**) Schematic representation of the genomic arrangement of *WAK1-5* in *Arabidopsis*. Middle cartoon shows the genomic deletion in *wakΔ* and the consequent fusion of *WAK4* and *WAK2*. The lower cartoon shows the genomic region of the *WAK* cluster in *wakΔ2*. Black arrows indicate primer pairs (P) used in B. **B**) Genotyping gel of Col-0, *wakΔ* and *wakΔ2*. Ethidium bromide-stained PCR products for parts indicated in A on agarose gel. P1-P4 refer to the primer pairs shown in A. **C**) Sequencing results from the *wakΔ2* aligned against the 5'UTR of *WAK4* and part of the third exon of *WAK2* of Col-0. **D**) Transcript levels of *WAK1-5* in Col-0 and *wakΔ2* determined by RT-qPCR. RNA was extracted from 14-day-old *Arabidopsis* seedlings grown in liquid culture. Transcripts were normalized to the house-keeping gene *UBOX*. Three biological replicates per experiment (Rep) were used. **E**) Representative images of four-week-old *Arabidopsis* plants grown on soil. These experiments were performed two times.



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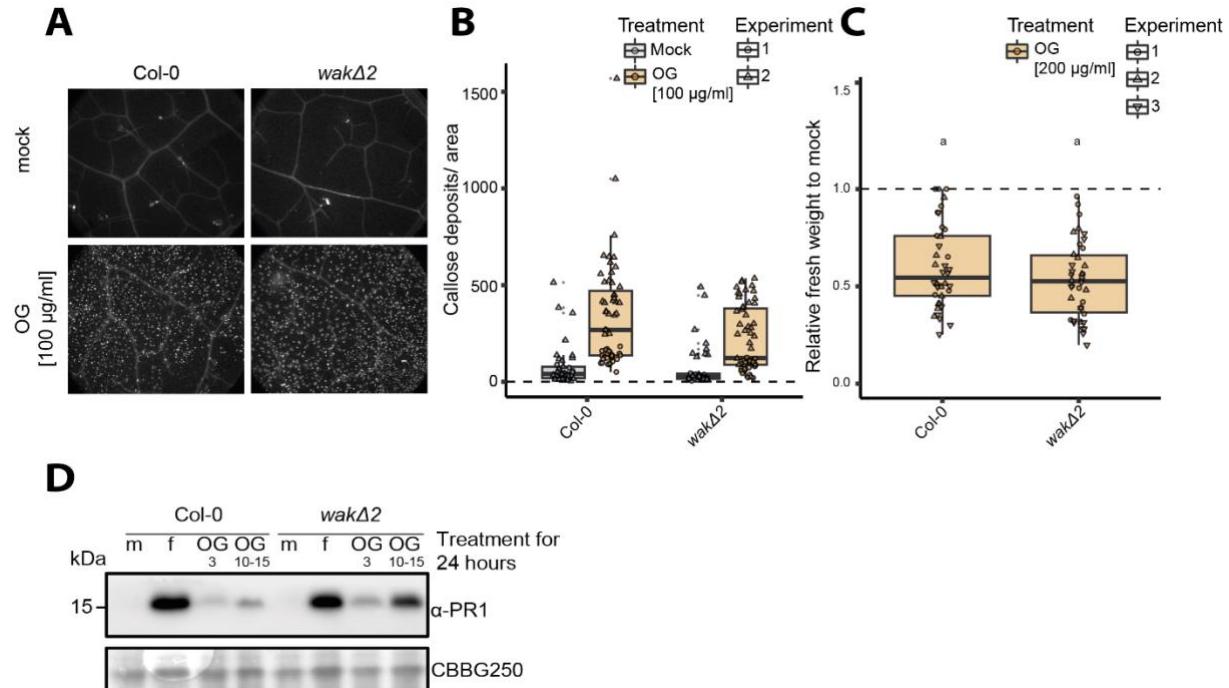
475 **FIGURE 2 – WAKs are not required for OG-induced early immune responses.**

476 **A-B)** ROS production in response to OG $_{10-15}$  in leaf discs of 3- to 4-week-old *Arabidopsis* plants  
477 (n = 12 leaf discs of 6 plants). 100  $\mu$ g/mL – 1 mg/mL of OG $_{10-15}$  were used as concentration  
478 dependent of the experiment. Mean  $\pm$  standard errors are plotted. RLU=relative luminescent  
479 units. **A)** Representative graph of the kinetics of one replicate. 1 mg/mL OG $_{10-15}$  was used as  
480 concentration. **B)** Values are means of total photon counts over 30 min. Data from three  
481 independent experiments (Rep) are shown. Shapes indicate different replicates. Outliers are  
482 included in statistical analysis. Statistical test: Kruskal-Wallis test ( $p < 2.62 \times 10^{-8}$ ), Dunn's post-  
483 hoc test with Benjamin-Hochberg correction ( $p \leq 0.05$ ). Groups with like letter designations  
484 are not statistically different. **C-D)** MAPK activation assay with 2-week-old seedlings in  
485 response to 100  $\mu$ g/mL OG $_{10-15}$  (C) or 100  $\mu$ g/mL OG $_3$  (D). Samples were collected 0, 5 and 15  
486 min after elicitation as indicated. Blot was probed with  $\alpha$ -p44/42 and  $\alpha$ -BAK1 was used as  
487 loading control. CBB=commassie brilliant blue was used as loading control as well. **E)** Ethylene

488 accumulation after treatment with 100  $\mu$ g/mL OG<sub>10-15</sub> or water as control in Arabidopsis  
489 seedlings. Box plots represent means  $\pm$  SE of three replicates. Equal letters at the top of the  
490 panel indicate  $p > 0.05$ , two-way ANOVA and a post hoc Tukey test. These experiments were  
491 performed three times.

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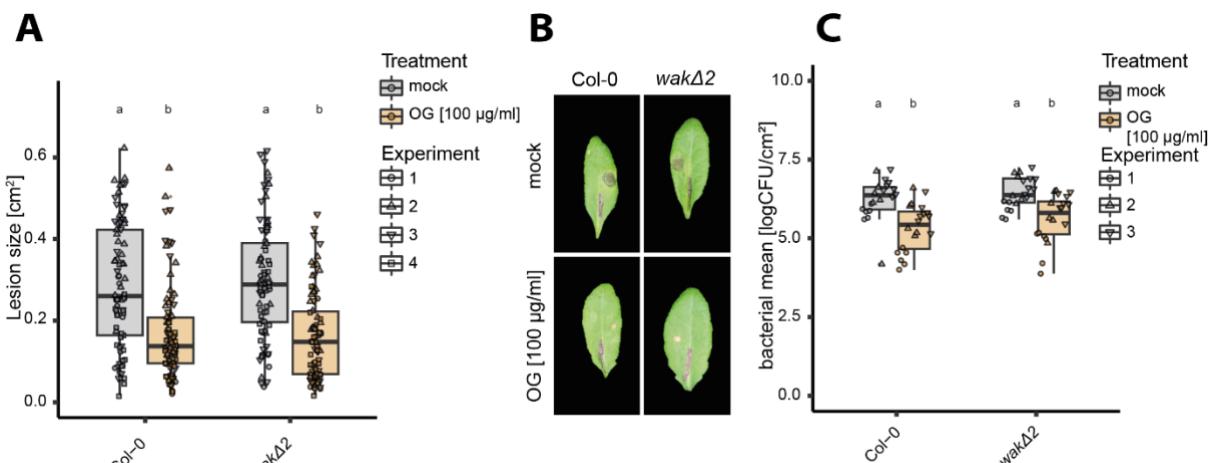
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### 496 **FIGURE 3 WAKs are not required for OG-induced late immune responses.**

497 **A -B)** Callose deposition visualized by aniline blue staining in response to 100  $\mu$ g/mL OG<sub>10-15</sub>  
498 or water 24 hours after infiltration into leaves of 3- to 4-week-old Arabidopsis plants. n=16-32  
499 leaf discs from 4 different plants were taken per independent experiment. The experiment was  
500 performed two times with similar results. **A)** Representative images of OG-induced callose  
501 deposition in the presented genotypes stained with aniline blue. **B)** Callose deposits induced  
502 by OGs and water infiltration. **C)** Relative weight of seedlings grown in liquid media for 10 days  
503 in the presence of 200  $\mu$ g/mL OG<sub>10-15</sub> or in the absence of neither (mock). Means  $\pm$  SE are  
504 shown with individual values for each plant and experiment (n = 12-14 seedlings per  
505 experiment). Outliers are included in statistical analysis. Equal letters at the top of the panel  
506 indicate  $p > 0.05$ , one-way ANOVA and a post hoc Tukey test. Groups with like letter  
507 designations are not statistically different. The experiment was repeated three times with  
508 similar results. **D)** PR1 accumulation assessed by immunoblotting with PR1 antibodies. Leaves  
509 from 3-week-old Arabidopsis plants were infiltrated with water (m = mock), 1  $\mu$ M flg22 (f) or  
510 100  $\mu$ g/mL OG<sub>10-15</sub> or 50  $\mu$ g/mL OG<sub>3</sub> and harvested after 24 hours. The experiment was  
511 repeated three times with similar results.

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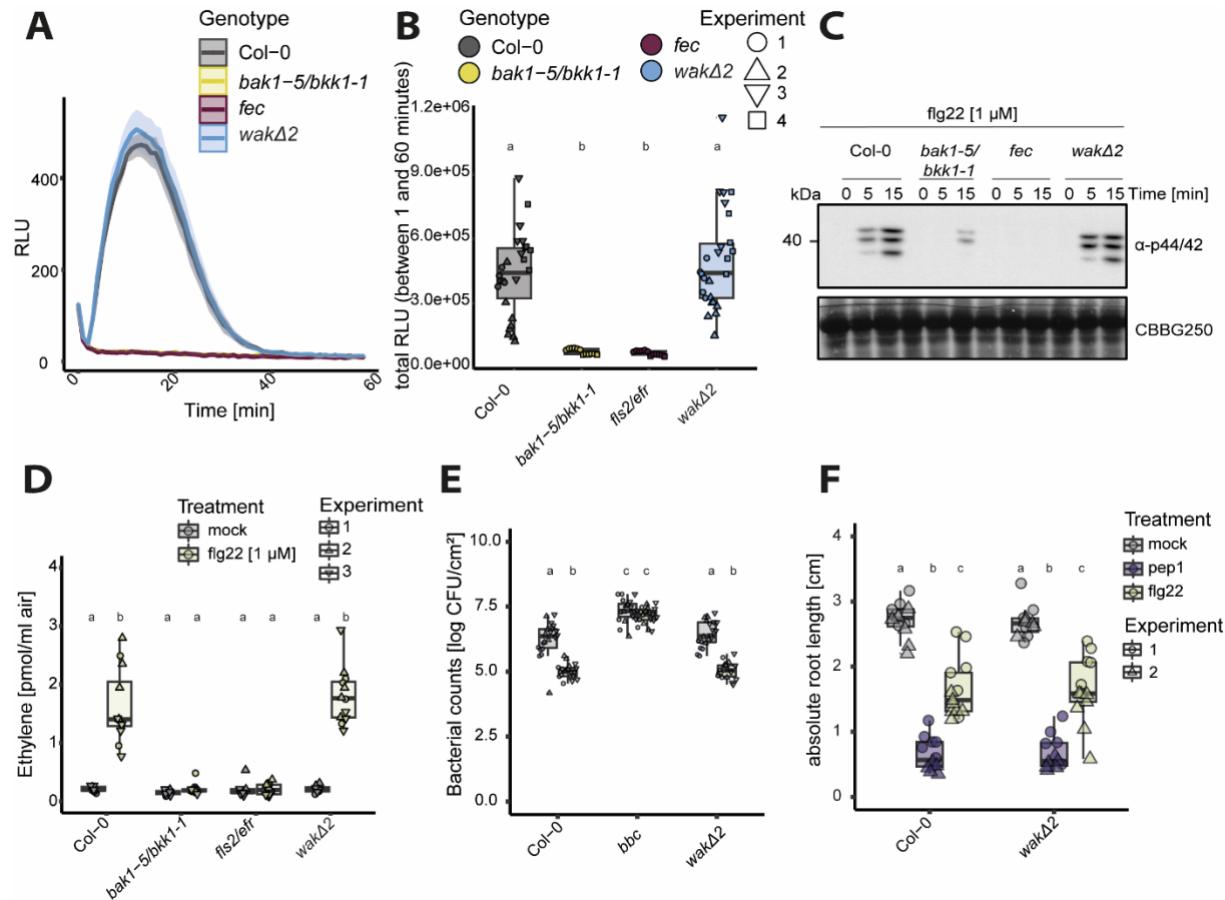
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515 **FIGURE 4 OG-induced immunity is not affected in wakΔ2.**

516 **A -B)** OG-induced resistance against *B. cinerea*. 4- to 5-week-old Col-0 or *wakΔ2* plants were  
517 infiltrated with water or 100 µg/mL OG<sub>10-15</sub> 24 hours prior drop-inoculation with *B. cinerea* strain  
518 BMM spores (5 µL; 5x10<sup>5</sup> spores/mL). Lesion areas were measured 48 hours post inoculation.  
519 The experiment was performed four times. **A)** Quantification of lesion sizes. Results show  
520 mean ± SE (n = 18-24 per experiment). Equal letters at the top of the panel indicate p > 0.05,  
521 two-way ANOVA and a post hoc Tukey test. Groups with like letter designations are not  
522 statistically different. **B)** Representative images of OG-induced immunity in the different  
523 genotypes. Images were taken 48 hours post inoculation. **C)** OG-induced resistance against  
524 *P. syringae* pv tomato DC3000. Plants were pretreated with water or 100 µg/mL OG<sub>10-15</sub> for 24  
525 hours before infiltration with *P. syringae*. 48 hours after *P. syringae* infiltration, bacteria were  
526 extracted and plated. Results show means ± SE and individual data points from the three  
527 pooled experiments (n = 6 per experiment). Equal letters at the top of the panel indicate p >  
528 0.05, two-way ANOVA and a post hoc Tukey test. Groups with like letter designations are not  
529 statistically different. The experiment was performed three times.

530

531



532

533 **FIGURE 5 – flg22-induced immunity is not affected by the loss of WAK1-5.**

534 **A-B)** ROS production in leaf discs of 3- to 4-week-old plants using 100 nM flg22 in Col-0, *bak1-5/bkk1-1*, *fls2/efr/cerk1* (*fec*) and *wakΔ2*. The experiment was repeated at least three times (A-B). Mean  $\pm$  standard errors are plotted. RLU=relative luminescent units. A) Kinetics of three representative independent replicates over 40-60 minutes. B) Values are means of total photon counts over 60 minutes as stated in the graph. Individual data points show ROS production in individual plants ( $n = 6-8$  plants with each two leaf discs). Outliers are included in statistical analysis. Kruskal-Wallis Test flg22 ( $p$ -value =  $9.364 \cdot 10^{-15}$ ), Dunn's post-hoc test with Benjamin-Hochberg correction ( $p \leq 0.05$ ). Groups with like letter designations are not statistically different. **C)** MAPK activation assay with 2-week-old seedlings in response to 1  $\mu$ M flg22. Samples were collected 0, 5 and 15 min after elicitation as indicated. Blot was probed with  $\alpha$ -p44/42. CBB = commassie brilliant blue was used as loading control as well. **D)** Ethylene accumulation after treatment with 1  $\mu$ M flg22 or water as control in *Arabidopsis* seedlings. Box plots represent means  $\pm$  SE of three replicates. Equal letters at the top of the panel indicate  $p > 0.05$ , two-way ANOVA and a post hoc Tukey. **E)** OG-induced resistance against *P. syringae* pv. tomato DC3000. Plants were pretreated with water or 1  $\mu$ M flg22 for 24 hours before infiltration with *P. syringae*. 48 hours after *P. syringae* infiltration, bacteria were extracted and plated. Results show means  $\pm$  SE and individual data points from the three pooled experiments ( $n = 6$  per experiment). Equal letters at the top of the panel indicate  $p > 0.05$ , two-way ANOVA

552 and a post hoc Tukey test. Groups with like letter designations are not statistically different. F)  
553 Primary root length of Col-0 and *wakΔ2* seedlings. Plants were grown on ½ MS +1 % sucrose  
554 plates for 5 days and then transferred to liquid ½ MS +1 % sucrose without elicitor (mock), 10  
555 nM pep1 or 100 nM flg22. Root-growth was determined after 4 days in liquid culture. 6 plants  
556 were measured per experiment. Values correspond to length of each root in cm. Equal letters  
557 at the top of the panel indicate  $p > 0.05$ , two-way ANOVA and a post hoc Tukey test. All  
558 experiments were performed three times with similar results, only primary root length was only  
559 measured twice.

560

561

## 562 **Supplemental table**

563 *SUPPLEMENTAL TABLE 1 – Primers used in this study.*

Target	Primer forward	Primer reverse
P1 – pair ( <i>WAK4</i> )	ACTATCTTTGAGCGGGCTCC	TTGGTATCAGCCTTGAAGCAC
P2 – pair (big deletion)	ACTATCTTTGAGCGGGCTCC	ACTATCTTTGAGCGGGCTCC
P3 - pair ( <i>WAK3</i> )	CCAAGACGCCGATTATGATTAC	AGAAGATGAAGTTCCAGGGAGGG
P4 - pair	CAAGCATGATAACCGAGAACG	GATATGATGAAGAGCCCTGCG
<i>WAK1-</i> qPCR	ACCTTCAGCTGGTTGCCAAGAC	TGGTGGTATCTAACGCGTAACCAG
<i>WAK2-</i> qPCR	TGCCCATCTGGTTACCGCAAAG	AGAAGCCGATGGTGGTTCCAAG
<i>WAK3-</i> qPCR	ACCGTTCAGAGGGTTGCAAAGAC	ACTTACAATCGAAGCCTCCATCCC
<i>WAK4-</i> qPCR	CTTGCCTCAGCCACGAAAGAG	TCTCGTTCATCACTTGGCCATC
<i>WAK5-</i> qPCR	TCGGACGGTTGCCAAGACATC	GGGCACTGACAATGGAAGCTTCC

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