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1 **Title:**

2 OXI1 protein kinase is required for plant immunity against *Pseudomonas*
3 *syringae* in *Arabidopsis*

4

5 **Running Title:**

6 Role for OXI1 in plant immunity

7

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32

33

Abstract

Expression of the Arabidopsis Oxidative Signal-Inducible1 (OXI1) serine/threonine protein kinase gene (At3g25250) is induced by oxidative stress. The kinase is required for root hair development and basal defence against the oomycete pathogen *Hyaloperonospora parasitica*, two separate H_2O_2-mediated processes. In this study, the role of OXI1 during pathogenesis was further characterised. Null *oxi1* mutants are more susceptible to both virulent and avirulent strains of the biotrophic bacterial pathogen *Pseudomonas syringae* compared to wild type, indicating that OXI1 positively regulates both basal resistance triggered by the recognition of pathogen-associated molecular patterns, as well as effector-triggered immunity. The level of *OXI1* expression appears to be critical in mounting an appropriate defence response since *OXI1* overexpressor lines also display increased susceptibility to biotrophic pathogens. The induction of *OXI1* after *P. syringae* infection spatially and temporally correlates with the oxidative burst. Furthermore, induction is reduced in *atrbohD* mutants and after application of DPI (an inhibitor of NADPH oxidases) suggesting that reactive oxygen species produced through NADPH oxidases drives *OXI1* expression during this plant-pathogen interaction.

Key words

Reactive oxygen species

Hyaloperonospora parasitica

Pseudomonas syringae

Plant defence

59 Signal transduction

60 **Introduction**

61 Plant immunity to the wide variety of potential pathogens involves a
62 complicated web of components ranging from preformed defence barriers to
63 signalling molecules such as reactive oxygen species (ROS), protein kinases
64 and hormones to elicit appropriate end responses (Thomma et al., 2001;
65 Ingle et al., 2006; Torres et al., 2006). The current viewpoint is that there are
66 two major branches of plant immunity as reviewed by Jones and Dangl
67 (2006). The first encompasses a general immune response triggered by the
68 recognition of evolutionary conserved pathogen-associated molecular
69 patterns (PAMPs), for example, bacterial flagellin, lipopolysaccharides and
70 fungal chitin. This PAMP-triggered immunity (PTI) activates a series of
71 inducible basal defence mechanisms such as callose deposition and
72 defence gene expression and is successful against nonhost pathogens.
73 Virulent pathogens suppress PTI via pathogen effector molecules which can
74 target components of the basal defence mechanism and induce effector
75 triggered susceptibility (ETS). This enables virulent pathogens to cause
76 disease on susceptible host plants (Jones and Dangl, 2006). The second
77 layer of immunity occurs when the host plant harbours a resistance protein
78 to detect either the presence and/or activity of one or more effectors
79 resulting in the rapid activation of plant defence responses and disease
80 resistance known as effector triggered immunity (ETI) (Mackey et al., 2002;
81 Jones and Dangl, 2006). Although, ETI responds faster to pathogen
82 infection, PTI and ETI share many regulatory components (Ingle et al.,
83 2006).

84

85 Central to plant immunity against biotrophic pathogens is the accumulation
86 of ROS, which apart from direct functions in toxicity (Keppler et al., 1989)
87 and oxidative cross-linking of plant cell walls (Bradley et al., 1992; Fry et al.,
88 2000) serve a signalling role in mounting the defence response (Grant and
89 Loake, 2000). A key feature of ROS signalling is regulation of the
90 hypersensitive response (HR) characterised by the rapid localised cell death
91 at the infection site as well as the induction of defence related genes (Levine
92 et al., 1994; Lamb and Dixon, 1997; Grant and Loake, 2000). Chemical
93 inhibition of ROS accumulation following pathogen challenge in Arabidopsis
94 led to a reduction in the HR and inhibited expression of the defence gene
95 *glutathione-S-transferase1* (Alvarez et al., 1998). Conversely, elevation of
96 H_2O_2 levels either through suppression of antioxidant enzyme activity, such
97 as in transgenic tobacco plants deficient in peroxisomal catalase activity
98 (Chamnongpol et al., 1998), or expression of enzymes required for ROS
99 production, such as in transgenic potato plants expressing glucose oxidase
100 (Wu et al., 1997), resulted in a primed immune response with accumulation
101 of salicylic acid (SA), expression of defence related genes and enhanced
102 resistance to a broad range of pathogens. More recently Arabidopsis
103 ascorbate-deficient mutants were found to exhibit microlesions, constitutive
104 *Pathogenesis Related (PR)* gene expression and increased resistance to
105 *Pseudomonas syringae* infection (Pavet et al., 2005) providing further
106 evidence for the role of ROS accumulation in disease resistance responses.

107

108 Genetic evidence points to a role for the respiratory burst NADPH oxidase as
109 the principal source of ROS production during pathogen challenge (Torres et
110 al., 2002). Arabidopsis mutants lacking either or both of the respiratory burst
111 oxidase genes, *AtrbohD* and *AtrbohF*, which encode catalytic subunits of the
112 NADPH oxidase, displayed a reduction in H_2O_2 accumulation and the HR in
113 response to avirulent *P. syringae* pv. tomato DC3000 *avrRpm1* infection
114 compared to wild type Arabidopsis (Torres et al., 2002). However, following
115 challenge with a virulent *Hyaloperonospora parasitica* (formerly known as
116 *Peronospora parasitica* (Constantinescu and Fatehi, 2002)) strain, the
117 *atrbohF* mutant displayed an enhanced HR and increased resistance to this
118 pathogen (Torres et al., 2002) indicating that the HR is differentially
119 regulated by ROS accumulation depending on the invading pathogen.
120 Alternative mechanisms for ROS production during pathogen attack have
121 been demonstrated, for example, pharmacological inhibition of peroxidase
122 activity during pathogen treatment resulted in a significant decrease in *GST1*
123 expression, a marker of ROS accumulation, compared to pathogen
124 treatment alone (Grant et al., 2000). More recently, overexpression of the
125 pepper extracellular peroxidase *CaPO2* gene in Arabidopsis conferred
126 enhanced disease resistance against *P. syringae* and increased H_2O_2 levels
127 following infection (Choi et al., 2007). The increased H_2O_2 production was
128 sensitive to chemical inhibition of peroxidase activity but unaffected by
129 inhibition of NADPH oxidase.
130
131 Despite the strong correlation between ROS accumulation and disease
132 resistance, current understanding of the discriminators of ROS signalling is

133 sorely limiting. The *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*) protein kinase
134 has emerged as a potential player linking ROS accumulation to disease
135 resistance in response to virulent *H. parasitica* attack (Rentel et al., 2004).
136 *OXI1* is not only induced by exogenous application of H_2O_2 and challenge
137 with virulent *H. parasitica* Emco5 but the *oxi1* null mutant also displayed
138 increased susceptibility in comparison to wild-type Arabidopsis following
139 infection with Emco5 (Rentel et al., 2004). Furthermore OXI1 is required for
140 the partial activation of MPK3 and MPK6 in response to treatment with H_2O_2
141 and cellulase, mimicking pathogen attack (Rentel et al., 2004). Both MPK3
142 and MPK6 are involved in the mitogen-activated protein kinase cascade
143 activated following recognition of bacterial flagellin by the receptor-like
144 kinase FLS2 (Asai et al., 2002) which initialises the induction of defence
145 genes such as *WRKY22/29* and *GST* and is effective in defence responses
146 against both bacterial and fungal pathogens (Gomez-Gomez et al., 2001;
147 Asai et al., 2002; Chinchilla et al., 2006). In this report a role for *OXI1* in
148 Arabidopsis is further extended to plant immunity against the bacterial
149 pathogen *P. syringae* and NADPH-produced ROS is shown to drive
150 expression of *OXI1* during this plant-pathogen interaction. Interestingly,
151 regulation of *OXI1* expression levels appears important in mediating an
152 appropriate defence response since both down regulation and
153 overexpression of *OXI1* results in enhanced susceptibility to biotrophic
154 pathogens.
155
156 **Materials and Methods**
157 Plant growth conditions

158 *Arabidopsis thaliana* plants were grown on a 1:1 soil mix composed of peat
159 (Jiffy Products, International AS, Norway) and vermiculite in a controlled
160 environment under a 16hr light:8 hr dark cycle at 21°C, 55% relative humidity
161 and fluorescent light of 80 to 100 μmol photon $s^{-1}m^{-2}$.
162
163 Plant lines
164 Wild type Arabidopsis seeds were acquired from Lehle Seeds (Lehle, Texas,
165 USA). The *oxi1* null mutant, *OXI1* complemented and *OXI1::GUS* transgenic
166 lines were the same as those used in Rentel et. al. (2004). The *atrbohD* T-
167 DNA mutant line used was that described in Torres et. al. (2002).
168
169 Generation of *35S::OXI1* and *35S::OXI1-YFP* constructs
170 A 1.4 kb DNA fragment of *OXI1* including the entire coding region and its
171 intron was PCR amplified from genomic DNA from the Ws-2 ecotype with the
172 primers 5′-GCGCCTGCAGGTCGACATTATGCTAGAGGG-3′ and
173 5′-GCGCGGATCCGTACACCA TAGTCCATAGAC-3′. The 2.5 kb OXI1-YFP
174 protein fusion comprising of 1.4 kb *OXI1* DNA fragment, 1.1 kb *YFP* coding
175 region and a *c-myc* epitope tag, was PCR amplified from the pBluescript SK$^-$
176 plasmid harbouring the *OXI1-YFP-cmyc* construct (Rentel, 2002) with the
177 primers 5′-GCGCGGATCCGTCGACATTATGCTAGAGGG-3′ and
178 5′-GCGCCCCGG GCAAGACCGGCAACAGGATTC-3′. Both PCR products
179 were cloned into the pUC2X35S plasmid containing two *35S CaMV*
180 promoters with the restriction enzymes *Pst*I and *BamH*I for *OXI1* and *BamH*I
181 and *Xma*I for *OXI1-YFP-cmyc* respectively, followed by subcloning into the
182 pBINPLUS binary vector through the unique restriction sites *Asc*I and *Pac*I.

183 Both vectors were a gift from Malcolm Campbell (Department of Botany,
184 University of Toronto, Canada). The resulting plasmids were transformed
185 into the C58C1 strain of *Agrobacterium tumefaciens* and transformed into
186 Arabidopsis plants of the Ws-2 ecotype by the floral dip method (Clough and
187 Bent, 1998). 25 µg mL^{-1} kanamycin was used for selection of homozygous
188 lines.
189
190 Pathogen infections
191 Inoculations with virulent *Pseudomonas syringae* pv. *tomato* DC3000 and
192 avirulent *P. syringae* harbouring the *avrB* gene were performed as
193 described in Murray et. al. (2002). The avirulent strain was maintained and
194 grown on King's broth media (King et al., 1954) supplemented with 50 µg
195 mL^{-1} rifampicin and 50 µg mL^{-1} kanamycin. Inoculation and assessment of
196 *Hyaloperonospora parasitica* sporulation was determined as described in
197 Rentel et. al. (2004). All pathogen infection experiments were repeated at
198 least 3 times.
199
200 *In vivo* histochemical GUS and DAB staining
201 GUS staining of Arabidopsis leaves was performed as previously described
202 (Rentel et al., 2004). The presence of H_2O_2 was detected by gently shaking
203 leaves submerged in a 1 mg mL^{-1} 3,3'-diaminobenzidine (DAB) solution for
204 2 to 3 h at room temperature until a reddish-brown precipitate was observed.
205 Images for both GUS and DAB staining were obtained by scanning the
206 leaves with a Canonscan 8400F Scanner.
207

208 Northern blot analysis

209 Total RNA was extracted using either the RNeasy Plant Total RNA kit
210 (Qiagen, UK) as per manufacture's instructions or a guanidinium
211 thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi,
212 1987). Electrophoresis and transfer of RNA onto nylon membrane was
213 performed as previously described (Murray et al., 2007). DNA probes were
214 labelled with ^{32}P using a Megaprime DNA labelling kit (Amersham, UK) and
215 hybridised to total RNA in hybridisation buffer composed of 5 x SSC, 50%
216 (v/v) formamide, 0.5% (v/v) SDS, 5 x Denhardt's solution and 100 μg mL^{-1}
217 denatured salmon sperm DNA. A full length 1.4 kb DNA probe of *OXI1*
218 (At3g25250) was obtained through restriction digest of *OXI1* cloned into the
219 pUC2X35S plasmid with the enzymes *Pst*I and *BamH*I. The *VSP1*
220 (At5g24780) template of approximately 300 bp was amplified by PCR of
221 genomic DNA with the primers 5′-CGGCATCCGTTCCAGCCGTC-3′ and
222 5′-CTAGAGAGGAGAGTGTCGTC-3′. The *PR-1* (At2g14610) probe was
223 amplified from genomic DNA using primers previously described (Denby et
224 al., 2005).

225

226 **Results**

227 *OXI1 is necessary for full resistance to P. syringae*

228 Given the requirement for OXI1 in the basal defence response to virulent
229 *H. parasitica* (Rentel et al., 2004) we investigated whether OXI1 is required
230 for defence against other virulent plant pathogens. The *oxi1* null mutant, wild
231 type (Ws-2) and the *oxi1* mutant complemented with the wild type *OXI1* gene
232 (*oxi1* + *OXI1*) transgenic line were challenged with virulent *P. syringae* pv.

233 *tomato* DC3000 (*Pst*DC3000). The *oxi1* mutant exhibited increased
234 susceptibility at 2 and 3 days post inoculation compared to wild-type (Fig. 1A
235 and Fig. S1A). Importantly, the complemented line exhibited wild-type
236 bacterial titres demonstrating that the increased susceptibility phenotype of
237 the *oxi1* mutation was due to the lack of *OXI1* expression (Fig. 1A and Fig.
238 S1B). OXI1 is therefore required for basal resistance against both an
239 oomycete (*H. parasitica*) and bacterial (*Pst*DC3000) biotrophic pathogen.
240 Despite strong induction after infection with *Botrytis cinerea* (Fig. S2), o*xi1*
241 mutants did not show altered susceptibility to this necrotrophic pathogen
242 (data not shown).
243
244 We also found that OXI1 is necessary for full resistance against an avirulent
245 isolate of *P. syringae* which carries the *avrB* gene (*Pst*DC3000 *avrB*) (Fig.
246 1B and Fig. S1C). Again the complemented line contained bacterial titres
247 similar to wildtype. The requirement for *OXI1* for full resistance was
248 confirmed using an additional avirulent isolate of *P. syringae* (*Pst*DC3000
249 carrying *avrRpt2*) (Fig. S3). Hence, although defence against avirulent *H.*
250 *parasitica* isolates is OXI1-independent (Rentel, 2002), *OXI1* is required for
251 full resistance against both virulent and avirulent *P. syringae*.
252
253 *Overexpression of OXI1 results in increased susceptibility to biotrophic*
254 *pathogens*
255 Having demonstrated that *oxi1* mutants are more susceptible to *P. syringae*,
256 we tested whether increased expression of *OXI1* could lead to enhanced
257 resistance. Two independent overexpressor lines were generated; both drive

258 *OXI1* expression from the *35S* CaMV promoter but one contains OXI1 fused
259 to the reporter gene YFP. Both lines show increased *OXI1* expression at the
260 mRNA level (Fig. 2). Surprisingly, these overexpressor lines displayed
261 enhanced susceptibility to both virulent and avirulent isolates of *P. syringae*
262 (Fig. 3A and B). Since both overexpressor lines showed the same
263 phenotype, the increased susceptibility was not due to the position of the
264 transgene or as a consequence of the YFP fusion. Due to this unexpected
265 result, and as *oxi1* mutants show increased susceptibility to virulent *H.*
266 *parasitica* (Rentel et al., 2004), we tested the susceptibility of these
267 overexpressing lines to the virulent *H. parasitica* isolate Emco5 (Fig. 3C).
268 Again, the *35S::OXI1* overexpressor showed enhanced susceptibility (as
269 seen by increased sporulation) compared to wildtype. Sporulation in the
270 *35S::OXI1-YFP* line was highly variable hence, although the average
271 susceptiblity was increased, the result was not statistically significant. From
272 these results we conclude that modulation of *OXI1* expression levels (either
273 increased or knocked out) causes increased susceptibility to virulent and
274 avirulent *P. syringae* as well as virulent *H. parasitica* isolates.
275
276 *Expression of two defence marker genes is uncompromised in the oxi1 null*
277 *mutant*
278 As OXI1 is required for resistance against at least two biotrophic pathogens,
279 we sought to establish a functional basis for this requirement. However,
280 expression of the classic defence gene *PR-1* was not compromised in the
281 *oxi1* mutant following infection with avirulent *P. syringae* (Fig. 4A and Fig.
282 S4A). Given that OXI1 is required for full activation of MPK3 amd MPK6 in

283 response to ROS and cellulase treatment (Rentel et al., 2004), we
284 investigated whether OXI1 regulates the expression of MPK6-dependent
285 *Vegetative Storage Protein1* (*VSP1*) in response to pathogen challenge.
286 *VSP1* was induced only 48 h after infection with virulent *P. syringae,* again
287 this induction was not affected in the *oxi1* mutant (Fig. 4B and Fig. S4B). As
288 expected, *VSP1* was not induced in response to challenge with avirulent *P.*
289 *syringae* in either wild-type or *oxi1* mutant plants (data not shown).
290
291 *The oxidative burst mediates induction of OXI1 expression*
292 Expression of *OXI1* is known to be induced in response to ROS (H_2O_2) and
293 in cells adjacent to *H. parasitica* hyphae (Rentel et al., 2004). As ROS
294 production is one of the earliest plant responses to pathogen infection (Lamb
295 and Dixon, 1997), we investigated whether ROS accumulation was
296 responsible for induction of *OXI1* gene expression after *P. syringae* infection.
297 After infection with either virulent or avirulent *P. syringae*, GUS expression
298 driven by the *OXI1* promoter increased and was confined to the regions of
299 ROS accumulation in the leaf (Fig. 5A). We used two methods to reduce the
300 rapid oxidative burst which occurs after avirulent *P. syringae* infection and
301 determined the effect on *OXI1* expression. As expected, an *atrbohD* mutant
302 failed to accumulate H_2O_2 during infection with avirulent *Pst*DC3000 *avrB*
303 (Fig. 5B). *OXI1* expression in this mutant background was reduced
304 compared to the wild-type control (Fig. 5C), suggesting that ROS generated
305 through NADPH oxidase is at least partly responsible for the induction of
306 *OXI1* during ETI. This conclusion was strengthened by reduced GUS activity
307 in leaves of *OXI1::GUS* plants co-infiltrated with *Pst*DC300*0 avrB* and 10 μM

308 diphenylene iodinium (DPI), a chemical inhibitor of NADPH oxidase,
309 compared to leaves infiltrated with *Pst*DC3000 *avrB* alone (Fig. 5D and Fig.
310 S5).
311
312 **Discussion**
313 Pathogen-induced *OXI1* expression is the result of ROS accumulation,
314 produced at least in part, via the AtrbohD NADPH-oxidase mechanism (Fig.
315 5) and OXI1 clearly contributes to both basal and effector-triggered
316 resistance to the bacterial pathogen *P. syringae* (Fig. 1). It is thought that the
317 regulation of PTI and ETI resistance responses overlaps considerably, with
318 ETI being an accelerated and amplified PTI response (Jones and Dangl,
319 2006). Large-scale expression profiling provided evidence that ETI is
320 qualitatively similar to PTI as the expression profiles, as well as the level of
321 induction of genes, during the early stages of infection with *P. syringae* pv.
322 *maculicola* (*Psm*) harbouring the avirulence *avrRpt2* gene were similar to
323 those produced during the late stages of infection with virulent *Psm* (Tao et
324 al., 2003). Furthermore, Arabidopsis mutant analysis has identified many
325 molecular components in the defence signalling network involved in both PTI
326 and ETI. For example, a mutation in the *ENHANCED DISEASE*
327 *SUSCEPTIBILITY 1* (*EDS1*) gene results in *eds1* mutants being more
328 susceptible to both virulent and avirulent isolates of *H. parasitica* (Parker et
329 al., 1996). Similarly, the Arabidopsis mutants *eds5, npr1*, *sid2* and *pad4* are
330 compromised in their resistance responses to both virulent and avirulent
331 isolates of *H. parasitica* and/or *P. syringae* (Cao et al., 1994; Zhou et al.,
332 1998; Nawrath and Métraux, 1999). Silencing of MPK6, a component of the

333 Arabidopsis MAPK cascade induced by flagellin during PTI, also leads to
334 enhanced susceptibility to virulent and avirulent isolates of *P. syringae*
335 (Menke et al., 2004). Our data indicate that OXI1 represents another shared
336 component between PTI and ETI since the *oxi1* mutant exhibits enhanced
337 bacterial titres compared to wildtype following infection with both virulent and
338 avirulent *P. syringae.* OXI1 is likely to trigger phosphorylation events that
339 result in activation of defence responses that serve to restrict or slow the
340 process of pathogen growth.

341

342 In contrast to infection by *P. syringae*, a role for OXI1 could only be
343 discerned in defence against a virulent *H. parasitica* isolate and not the
344 avirulent isolate Emoy2 (Rentel et al., 2004). We have only tested one
345 avirulent isolate of *H. parasitica* and it is possible that OXI1 may play a role
346 in ETI against isolates with different effector complements. Given that
347 effectors themselves target different components of the defence system
348 (which are then unavailable for signaling), it is unlikely that ETI signaling will
349 be identical in response to all effectors.

350

351 While infection with the necrotrophic pathogen *B. cinerea* resulted in
352 increased expression of *OXI1* in Arabidopsis (Fig. S1), lack of OXI1 did not
353 increase susceptiblity compared to wildtype (data not shown). Induction of
354 *OXI1* in response to *B. cinerea* appears to be a consequence of ROS
355 accumulation during this interaction, without an active role in defence. Hence
356 our results currently limit the role of OXI1 to disease resistance against
357 biotrophic pathogens.

358

359 A surprising feature of OXI1 is that reduced expression and overexpression
360 of *OXI1* both led to enhanced susceptibility to biotrophic pathogens (Fig. 3).
361 It is unlikely that a single kinase plays both a positive and a negative role
362 during the same defence response. Hence we propose that the level of OXI1
363 protein is crucial for appropriate signalling, and modulation of these levels
364 (either higher or lower) disrupts OXI1 function. As transcript levels do not
365 necessary correlate to protein levels, it is possible that the OXI1
366 overexpression lines either have lower or higher OXI1 protein levels in
367 comparison to that of wild-type since these lines mirror the loss of function
368 mutant. In the first instance, if the protein levels are actually reduced in the
369 overexpression lines relative to wild-type, it could be that prolonged
370 expression of OXI1 protein from a constitutive promoter (35S) might lead to
371 enhanced activation of pathways naturally present to negatively regulate the
372 OXI1 protein i.e. by protein degradation. Alternatively, it would be more
373 orthodox and parsimonious to assume that protein levels are higher in the
374 overexpression lines than in wild-type. It is not uncommon for plants
375 overexpressing proteins to show the same phenotypes as loss of function
376 mutants, for example, FIP1 and EBS, also in Arabidopsis (Chen *et al.*, 2007;
377 Pineiro *et al.*, 2003). In these cases the perceived wisdom is that these
378 proteins operate in complexes in which the stoichiometry is crucial and
379 regulated by the levels of protein expression. Therefore both under- or over-
380 expression would lead to suboptimal complex formation, leading to reduced
381 function. Therefore the absolute level of abundance of such components is
382 crucial and OXI1 may be one such component. Furthermore, OXI1 levels

383 appear to be tightly controlled *in planta*; *35S::OXI1-YFP* lines show low
384 levels of YFP protein compared to lines expressing YFP-aequorin protein in
385 single root cell types, and treatment of seedlings with the proteasome
386 inhibitor MG132 for 1 h results in dramatically increased protein levels
387 indicating a short half-life (data not shown). This rapid turnover of OXI1
388 protein together with the importance of timing in pathogen responses
389 presents another scenario whereby the key factor could also be one of
390 appropriate timing. For example, having either OXI1 protein or transcript
391 already present when plants are challenged with pathogen may be
392 detrimental to establishing an optimal defence response.
393
394 Despite the demonstration of MPK6 as a downstream component of OXI1 in
395 response to H_2O_2 and cellulase treatment (Rentel et al., 2004) and the fact
396 that the defence phenotype of *mpk6* silenced Arabidopsis mutant resembles
397 that of *oxi1* (Menke et al., 2004), MPK6-dependent expression of *VSP1*
398 during PTI appears to be independent of OXI1 (Fig. 4B). Similarly, despite
399 the importance of SA signalling to disease resistance against biotrophic
400 pathogens, OXI1 was not required for expression of the SA marker gene
401 *PR1* (Fig. 4A) or development of systemic acquired resistance (Fig. S6).
402 OXI1 interacts directly with, and can phosphorylate, the Ser/Thr protein
403 kinase PTI1-2 (Anthony *et al.*, 2006). PTI1-2 is activated in response to
404 various stress treatments and is dependent on OXI1 for its activation in
405 response to flagellin and H_2O_2 (Anthony *et al.*, 2006). Given the homology
406 between PTI1-2 and the tomato Pto kinase, which confers resistance to
407 avirulent *P. syringe* carrying the *avrPto* gene (Zhou *et al.*, 1995), it is

408 tempting to speculate that OXI1 promotes defence against *P. syringae*
409 through the activation of PTI1-2. However, no targets of PTI1-2 have been
410 identified and, unlike Pto, OXI1 is likely to function downstream of ROS.
411 Hence, the identification of additional direct targets of OXI1 will be vital in
412 elucidating the function of this ROS-responsive kinase during *Arabidopsis-*
413 *Pseudomonas/Hyaloperonospora* interactions and in addressing how
414 specificity of ROS signaling is achieved.
415

416 **Supplementary material**

417 The *oxi1* null mutant is more susceptible to both virulent and avirulent *P.*
418 *syringae* (Fig. S1A and S1C) and this phenotype of the null mutant is
419 rescued in the complemented line which exhibits wild-type bacterial titres
420 when challenged with *P. syringae* (Fig. S1B and S1C). *OXI1* expression is
421 induced following infection of Arabidopsis leaves with the necrotrophic
422 pathogen *Botrytis cinerea* (Fig. S2). The *oxi1* null mutant is more
423 susceptible than wildtype to the avirulent strain *Pst*DC3000 *avrRpt2* (Fig.
424 S3). Expression of *PR-1* and *VSP1* is unaffected in the oxi1 null mutant in
425 response to pathogen challenge (Fig. S4). DPI treatment alone has no effect
426 on OXI1::GUS expression (Fig. S5). The *oxi1* mutation does not affect the
427 development of systemic acquired resistance (Fig. S6).
428

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436

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572 **Figure Legends**

573

574 **Figure 1.** The *oxi1* mutant exhibits increased susceptibility to both virulent

575 and avirulent strains of *P. syringae*

576 Leaves of 3 week old wild-type Ws-2, *oxi1* mutant and the *oxi1* mutant line

577 complemented with the wild type *OXI1* gene (*oxi1* + *OXI1*) were pressure

578 inoculated with either virulent *Pst* DC3000 (A) or avirulent *Pst* DC3000

579 harbouring the *avrB* (B) gene at $5x10^5$ cfu mL^{-1} and bacterial titre

580 determined. The bars represent the mean log bacterial titre expressed as cfu

581 cm^{-2} at 24, 48 and 72 h post infection ± SEM (n = 3 biological replicates,

582 each consisting of 3 leaf discs per replicate plant). * indicates a significant

583 increase in pathogen growth compared to wildtype (student's t-test, $P<0.05$).

584 Results shown are for one representative experiment of four.

585

586 **Figure 2.** Transgenic Arabidopsis lines overexpressing *OXI1* and *OXI1-YFP*

587 Northern analysis confirmed constitutive overexpression of *OXI1* (A) or

588 *OXI1-YFP* (B) in transgenic Arabidopsis lines. Blots were probed with a full

589 length *OXI1* DNA fragment that recognises wild-type *OXI1* and the larger

590 *OXI1-YFP* transcript. Ethidium bromide (A) or methylene blue (B) stained

591 rRNA was used as a loading control. Results shown are for one

592 representative experiment of two.

593

594 **Figure 3.** Overexpression of *OXI1* increases susceptibility to the biotrophic

595 pathogens *P. syringae* and *H. parasitica*

596 Analysis of bacterial titres in 3 week old leaves of Ws-2, *oxi1*, *35S::OXI1* and

597 *35S::OXI1-YFP* plants pressure inoculated with either $5x10^5$ cfu mL^{-1} virulent

598 *Pst* DC3000 (A) or avirulent *Pst* DC3000 *avrB* (B). The bars represent the
599 mean log bacterial titre expressed as cfu cm^{-2} ± SEM (n = 3 biological
600 replicates, each consisting of 3 leaf discs per replicate plant). (C) Seven day
601 old seedlings of genotypes Ws-2, *oxi1*, *35S::OXI1* and *35S::OXI1-YFP* were
602 sprayed with spores of the virulent *H. parasitica* strain Emco5 at a spore
603 suspension of $5x10^4$ spores mL^{-1}. Bars represent the average sporulation of
604 4 independent samples of pooled seedlings for each genotype, 7 days post
605 infection ± SEM. * indicates a significant increase in pathogen growth
606 compared to the wild type (student's t-test, P<0.05). Results shown are for
607 one representative experiment of four.
608
609 **Figure 4.** Expression of defence associated genes in the *oxi1* mutant
610 Three week old Ws-2 and the *oxi1* mutant were pressure inoculated with
611 either 10 mM $MgCl_2$ alone (-) or $5x10^5$ cfu mL^{-1} avirulent *Pst* DC3000 *avrB*
612 (*avrB*) or virulent *Pst* DC3000 (*Pst*) in a 10 mM $MgCl_2$ suspension. Leaves
613 were harvested before the start of the experiment (0) or at various time
614 points post infection as indicated. Expression of *PR-1* (A) and *VSP1* (B) was
615 assessed via Northern analysis. Ethidium bromide (A) and methylene blue
616 (B) stained RNA were used as loading controls. Results shown are for one
617 representative experiment of two.
618
619 **Figure 5.** *OXI1* expression correlates with the oxidative burst following
620 pathogen infection and is at least partially dependent on NADPH oxidase.
621 (A) A small area of leaf tissue from 4 week old *OXI1::GUS* transgenic plants
622 was pressure inoculated with either 10 mM $MgCl_2$, avirulent *Pst* DC3000

623 *avrB* (*avrB*) or virulent *Pst* DC3000 (*Pst*) suspension in 10 mM $MgCl_2$ at
624 $5x10^6$ cfu mL^{-1}. At 3, 8 and 24 h post-infection leaves were excised and
625 stained with DAB for the presence of an oxidative burst (forms a reddish-
626 brown precipitate with H_2O_2) or stained for GUS activity. Three leaves were
627 analysed per treatment per time point; one representative leaf of each
628 sample is shown. Both the oxidative burst and *OXI1* expression occurred
629 within the infected area.
630 (B) A small area of leaves from 4 week old *atrbohD* mutant and wild-type
631 Col-0 plants were pressure inoculated with either 10 mM $MgCl_2$ or avirulent
632 *Pst* DC3000 *avrB* (*avrB*) suspension in 10 mM $MgCl_2$ at $5x10^6$ cfu mL^{-1}. DAB
633 staining at 6 h post infection demonstrated lack of the oxidative burst in the
634 *atrbohD* mutant. A representative of 3 leaves per treatment of Col is shown
635 and the red circle indicates the infected area exhibiting an oxidative burst.
636 (C) Entire leaves of 4 week old *atrbohD* mutants and wild-type Col were
637 pressure inoculated with either 10 mM $MgCl_2$ or avirulent *Pst* DC3000 *avrB*
638 (*avrB*) suspension in 10 mM $MgCl_2$ at $5x10^6$ cfu mL^{-1}. A minimum of 4 leaves
639 were harvested at 8 h post inoculation for each sample. Duplicate samples
640 for each genotype are shown. Northern blots were probed with a full length
641 *OXI1* DNA fragment. Methylene blue stained RNA was used as a loading
642 and transfer control.
643 (D) A small area of leaves from 4 week old OXI1::GUS transgenic plants was
644 pressure inoculated with either 10 mM $MgCl_2$ or avirulent *Pst* DC3000 *avrB*
645 suspension in 10 mM $MgCl_2$ containing either 0.1% (v/v) DMSO (*avrB* +
646 DMSO) or 10 µM DPI (*avrB* + DPI) at $5x10^6$ cfu mL^{-1}. DPI is a chemical
647 inhibitor of NADPH oxidase and co-infiltration with *avrB* + DMSO was used

648 as a control for the DPI treatment. GUS activity was visualised 24 h post
649 infection and showed a reduction with DPI treatment.
650 In all cases, results are shown from one representative experiment of three.