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Gene-for-Gene Tolerance to Bacterial Wilt in *Arabidopsis*

Liesl Van der Linden,[1] Jane Bredenkamp,[1] Sanushka Naidoo,[2] Joanne Fouché-Weich,[1] Katherine J. Denby,[3] Stephane Genin,[4] Yves Marco,[4] and Dave K. Berger[1]

[1]Department of Plant Science and [2]Department of Genetics, Forestry and Agricultural Research Institute (FABI), University of Pretoria, 0028, South Africa; [3]School of Life Sciences and Warwick Systems Biology Centre, University of Warwick, U.K.; [4]Laboratoire des Interactions Plantes-Microorganismes, UMR CNRS/INRA 2594/441, 31320 Castanet-Tolosan, France

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Bacterial wilt caused by *Ralstonia solanacearum* is a disease of widespread economic importance that affects numerous plant species, including *Arabidopsis thaliana*. We describe a pathosystem between *A. thaliana* and biovar 3 phylotype I strain BCCF402 of *R. solanacearum* isolated from *Eucalyptus* trees. *A. thaliana* accession Be-0 was susceptible and accession Kil-0 was tolerant. Kil-0 exhibited no wilting symptoms and no significant reduction in fitness (biomass, seed yield, and germination efficiency) after inoculation with *R. solanacearum* BCCF402, despite high bacterial numbers in planta. This was in contrast to the well-characterized resistance response in the accession Nd-1, which limits bacterial multiplication at early stages of infection and does not wilt. *R. solanacearum* BCCF402 was highly virulent because the susceptible accession Be-0 was completely wilted after inoculation. Genetic analyses, allelism studies with Nd-1, and *RRS1* cleaved amplified polymorphic sequence marker analysis showed that the tolerance phenotype in Kil-0 was dependent upon the resistance gene *RRS1*. Knockout and complementation studies of the *R. solanacearum* BCCF402 effector PopP2 confirmed that the tolerance response in Kil-0 was dependent upon the RRS1–PopP2 interaction. Our data indicate that the gene-for-gene interaction between RRS1 and PopP2 can contribute to tolerance, as well as resistance, which makes it a useful model system for evolutionary studies of the arms race between plants and bacterial pathogens. In addition, the results alert biotechnologists to the risk that deployment of RRS1 in transgenic crops may result in persistence of the pathogen in the field.

Ralstonia solanacearum, the causal agent of bacterial wilt disease affects several economically important plants worldwide (Hayward 1991). The pathogen infects Solanaceous crops such as tobacco, tomato, potato, and eggplant (Lebeau et al. 2010) and leguminous plants such as groundnut, French bean, and *Medicago truncatula* (Genin and Denny 2012; Vailleau et al. 2007). This pathogen is also pathogenic on several shrub and tree species such as cashew, mulberry, olive (Xu et al. 2009), and members of genus *Eucalyptus*. *Eucalyptus* spp. are hosts in Brazil, China, Australia, and Africa (Coutinho et al. 2000; Hayward 1991; Ran et al. 2005; Roux et al. 2000).

Corresponding author: D. K. Berger; Telephone:+27-12-4204634; Fax: +27-12-4206668; E-mail: dave.berger@fabi.up.ac.za

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R. solanacearum is a soilborne bacterium that enters hosts via root wounds or sites of secondary root emergence (Genin 2010). The bacteria spread toward the xylem vessels and colonize the root cortex and vascular parenchyma (Turner et al. 2009). *R. solanacearum* secretes copious amounts of extracellular products, resulting in cell damage. The accumulation of exopolysaccharide produced by the bacteria in the vascular bundle, pith, and cortex blocks the plant's vascular system (Monteiro et al. 2012). Water uptake is then compromised and the plant eventually wilts and dies (Genin and Denny 2012; Hayward 1991; Jacobs et al. 2012).

Sequence analyses of several *R. solanacearum* genomes have revealed that the pathogen has an arsenal of virulence factors and effectors purported to overcome host defenses (Poueymiro and Genin 2009). Host-plant resistance against *R. solanacearum* has been identified in several Solanaceous plants, such as tomato, eggplant, and pepper (Lebeau et al. 2010). In tomato, resistance against *R. solanacearum* is mainly polygenic and demonstrated to be strain specific (Thoquet et al. 1996; Wang et al. 2000). Quantitative trait loci (QTL) for resistance to *R. solanacearum* GMI1000 in the legume *M. truncatula* were identified, although no gene-for-gene interaction with any of 48 tested effectors could be detected (Vailleau et al. 2007). In contrast, PopP1 and PopP2 are effectors from GMI1000 whose recognition leads to resistance in petunia and *A. thaliana*, respectively (Deslandes et al. 2003; Lavie et al. 2002).

Several types of resistance have been described in *A. thaliana,* a host for *R. solanacearum*. Resistance in accession S96 to strain Ps95 was due to a single dominant locus (Ho and Yang 1999) whereas resistance against *R. solanacearum* 14.25 was controlled by three QTL in Col-0 (Godiard et al. 2003). Single-gene recessive resistance against *R. solanacearum* GMI1000 was identified in accession Nd-1 (Deslandes et al. 1998). Resistance was conferred by the *RRS1-R* allele of Nd-1 (Deslandes et al. 2002). RRS1-R confers resistance to strain GMI1000 by recognizing the matching PopP2 effector secreted from the pathogen (Deslandes et al. 2003). Both a physical interaction between PopP2 and RRS1-R in the nucleus and PopP2 auto-acetyltransferase activity are required for resistance (Tasset et al. 2010).

The outcomes of plant–pathogen interactions are susceptibility, tolerance, or resistance (Kover and Schaal 2002). Resistance is defined as the ability of the host plant to limit pathogen multiplication and spread in planta, whereas tolerance occurs without a significant reduction in fitness despite high pathogen numbers in planta (Kover and Schaal 2002). The relative importance of tolerance versus resistance in driving the arms race between plants and pathogens is currently of great interest to evolutionary biologists (Gao et al. 2009). Tolerance in insect–plant interactions has been well documented

(Leimu and Koricheva 2006; Mauricio et al. 1997; Schmidt and Baldwin 2009; Strauss and Agrawal 1999). However, until recently, the relationship between tolerance and resistance in bacteria–plant interactions has received little attention (Gao et al. 2009; Kover and Schaal 2002). A study of natural variation in susceptibility of 19 *A. thaliana* accessions to *Pseudomonas syringae* led the authors to conclude that tolerance appears to be an important driver in *Arabidopsis* variation in response to this bacterial pathogen (Kover and Schaal 2002).

Current understanding has led to the model that resistance to bacterial pathogens is conferred by resistance (*R*) genes in a gene-for-gene relationship, whereas tolerance is a polygenic quantitative trait (Barrett et al. 2009). However, a recent study that compared several natural accessions and transgenic acces-

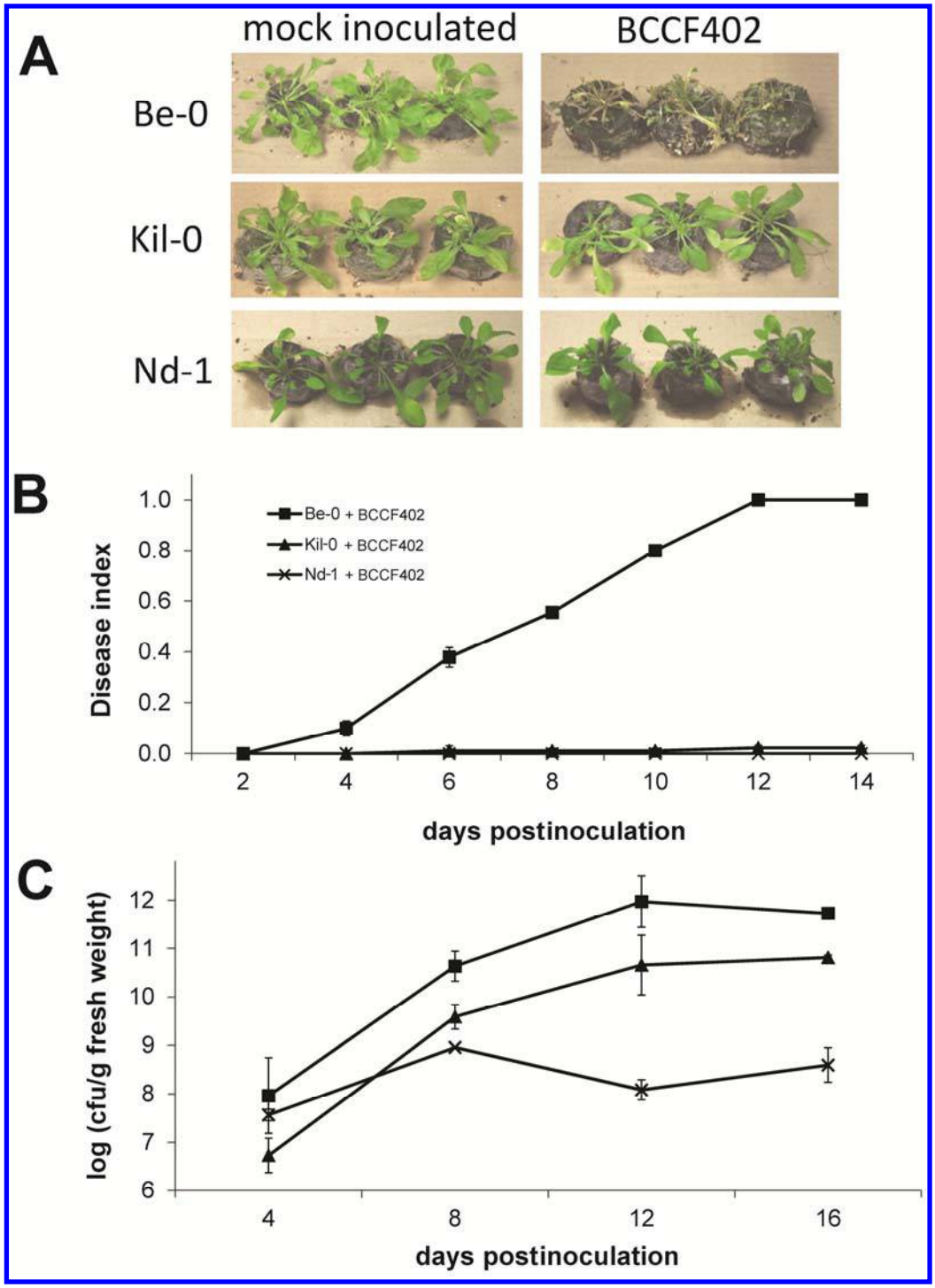

Fig. 1. Disease responses of *Arabidopsis thaliana* accessions Be-0, Kil-0, and Nd-1 to *Ralstonia solanacearum* isolate BCCF402. **A,** Symptoms at 14 days postinoculation of Be-0, Kil-0, and Nd-1 plants inoculated with *R. solanacearum* BCCF402 at 1×10^8 CFU/ml using a root-inoculation method. Three replicate representative plants for treatment are shown. **B,** Disease index for *A. thaliana* accessions Be-0 (■), Kil-0 (▲), and Nd-1 (X) inoculated with *R solanacearum* BCCF402. The disease index for each treatment was calculated based on data from 30 plants (three replicates of 10 plants each) over 12 days. Error bars illustrate standard deviations. **C,** Bacterial numbers expressed as log(CFU per gram of fresh weight) in *Arabidopsis* Be-0 (■), Kil-0 (▲), and Nd-1 (X) accessions inoculated with *R. solanacearum* BCCF402. Bacterial counts were calculated on nine plants per treatment per time point. Error bars illustrate standard deviations. Replicate inoculation experiments yielded similar results.

sions that differed for the presence of the *Rpm1* resistance gene provided evidence that the *R* gene itself could contribute to both resistance and tolerance (Roux et al. 2010).

We previously reported that *R. solanacearum* BCCF401, collected from *Eucalyptus grandis* × *E. camaldulensis* plantations in South Africa (Fouché-Weich et al. 2006), was pathogenic on *Arabidopsis* accession Col-0 (Naidoo et al. 2011). In the current study, we characterized the response of *Arabidopsis* accessions to another *Eucalyptus* strain of *R. solanacearum* called BCCF402. The strain caused wilting symptoms on accession Be-0 and no apparent disease symptoms on accessions Kil-0 or Nd-1. However, Kil-0 harbored high bacterial populations, indicative of a tolerant response. Plant fitness in Kil-0 was not compromised by infection with *R. solanacearum* BCCF402, and tolerance in Kil-0 was dependent on both PopP2 and RRS1. This work demonstrates that an *R* gene can confer either resistance or tolerance, and highlights the fact that plant responses to bacterial pathogens fall within a continuum from susceptible to highly tolerant (resistant).

RESULTS

Differential disease responses of *A. thaliana* accessions Be-0, Kil-0, and Nd-1 to *R. solanacearum* BCCF402.

R. solanacearum BCCF402 is a biovar 3 phylotype I strain from *Eucalyptus* trees (Fouché-Weich et al. 2006). Root inoculations were performed on *A. thaliana* accessions Be-0, Kil-0, and Nd-1, and plants were monitored for disease development. *A. thaliana* accession Be-0 developed severe wilt symptoms a week after inoculation and disease index (DI) scores plateaued at 12 days postinoculation (dpi), after which the plants died (Fig. 1A and B). In contrast, accessions Kil-0 and Nd-1 remained symptomless 14 dpi with *R. solanacearum* BCCF402 (Fig. 1A and 1B). Bacterial numeration experiments showed an increase over time by several orders of magnitude of *R. solanacearum* BCCF402 in aerial parts of accession Be-0 (Fig. 1C). This was *hrp*-dependent because bacterial numbers did not increase in a Δ*hrp* mutant (Supplementary Fig. S1). Despite the absence of symptoms in Kil-0 (Fig. 1A and B), BCCF402 bacterial numbers increased in these plants and were only one order of magnitude lower than the susceptible accession Be-0 (Fig. 1C). This result was consistent in replicate independent experiments, although a few Kil-0 plants exhibited minor wilting symptoms (DI < 0.2) after 12 dpi in some experiments, which was most likely due to nonreproducible physiological differences of individual plants (data not shown). Bacterial multiplication in Kil-0 was *hrp* dependent because bacterial numbers did not increase when inoculated with a Δ*hrp* mutant. Accession Nd-1 exhibited the same resistant response to *R. solanacearum* BCCF402 as previously reported for *R. solanacearum* GMI1000 (Deslandes et al. 1998), in that it was symptomless (Fig. 1A and B) and bacterial numbers did not change by more than one order of magnitude (Fig. 1C).

A. thaliana accession Kil-0 exhibits tolerance to *R. solanacearum* BCCF402.

We hypothesized that Kil-0 is tolerant to *R. solanacearum* BCCF402 because it harbors high bacterial numbers without exhibiting disease symptoms (Fig. 1). Our working definition of tolerance is that the infected plant does not show a significant reduction in fitness despite high pathogen numbers in planta (Kover and Schaal 2002). Resistant plants severely restrict pathogen growth after infection and show no reduction in fitness (Kover and Schaal 2002). Dry weight of accession Kil-0 was not significantly reduced by inoculation with *R. solanacearum* BCCF402 (Table 1), whereas dry weight of the susceptible accession Be-0 was reduced more than twofold ($P < 0.01$) (Table 1). Accessions Be-0 and Kil-0 produced similar numbers of seed per plant, with a >87% germination rate under control conditions. Susceptible Be-0 plants died and, therefore, did not produce any seed when inoculated with the pathogen (Table 1). In contrast, there was no significant difference in the amount of seed produced by accession Kil-0 between mock-inoculated and *R. solanacearum* BCCF402-inoculated plants (Table 1; *t* test, $P < 0.05$). Furthermore, the germination rate of Kil-0 seed remained high (>93%) after inoculation with *R. solanacearum* BCCF402 (Table 1). Interestingly, Kil-0 produced a twofold greater average amount of seed per plant when inoculated compared with the control, although the difference was not significant (Table 1; *t* test, $P > 0.05$). Inoculation of accession Nd-1 with *R. solanacearum* BCCF402 did not result in a reduction in yield, consistent with its resistant phenotype (data not shown). Taken together, these results showed that *A. thaliana* accession Kil-0 is tolerant, Nd-1 is resistant, and Be-0 is susceptible to *R. solanacearum* BCCF402.

Table 1. *Arabidopsis thaliana* accession Kil-0 shows no reduction in yield or fecundity in response to inoculation with *Ralstonia solanacearum* BCCF402, in contrast to *A. thaliana* accession Be-0

	Kil-0			Be-0		
Yield, fecundity	**Mock-inoculated**	**Inoculated**	**$P < 0.05$[a]**	**Mock-inoculated**	**Inoculated**	**$P < 0.05$[b]**
Dry weight (g)[c]	0.28 ± 0.02	0.26 ± 0.092	No ($P = 0.656$)	0.29 ± 0.035	0.117 ± 0.061	Yes ($P = 0.0129$)
Number of seeds/plant[d]	426.00 ± 209	839.00 ± 322	No ($P = 0.136$)	639.00 ± 241	0	Yes (n/a)
Germination (%)[e]	96.00 ± 4.10	93.00 ± 5.5	No ($P = 0.496$)	86.60 ± 6.35	0	Yes (n/a)

[a] Significant difference between Kil-0 mock-inoculated compared with Kil-0 inoculated based on a Student's *t* test, $P < 0.05$.
[b] Significant difference between Be-0 mock-inoculated compared with Be-0 inoculated based on a Student's *t* test, $P < 0.05$.
[c] Dry weight was measured at 16 days postinoculation from 30 plants per treatment (three biological replicates of 10 plants each), values ± standard deviations.
[d] Seed yield was calculated from nine plants per treatment (three biological replicates of three plants each), values ± standard deviations.
[e] Germination was measured from seed collected from nine plants per treatment, values ± standard deviations.

Table 2. Segregation analysis of response to *Ralstonia solanacearum* BCCF402 in the F_2 progeny from a cross between *Arabidopsis thaliana* accessions Kil-0 and Be-0[a]

	Number of plants			Ratio (T/S)			
Trial	**T**	**S**	**Total**	**Expected**	**Observed**	**χ^2**	***P***
1	74	215	289	1:3	1:2.9	0.06	$0.9 > P > 0.7$
2	92	295	387	1:3	1:3.2	0.31	$0.7 > P > 0.5$

[a] T = tolerant and S = susceptible; χ^2 values were calculated for an expected segregation ratio of 1:3 (T/S) plants.

Tolerance in *A. thaliana* accession Kil-0 against *R. solanacearum* BCCF402 is due to a single recessive gene.

In order to determine the genetic basis of tolerance in accession Kil-0 to *R. solanacearum* BCCF402, a cross was performed between Kil-0 and Be-0 plants. Heterozygous F_1 plants were challenged with BCCF402 by root inoculation and were completely wilted by 17 dpi (data not shown), indicating that tolerance in Kil-0 is a recessive trait. F_1 plants were selfed to produce an F_2 segregating population, which was challenged with *R. solanacearum* BCCF402 by root inoculation in two independent trials. F_2 plants that showed the same symptomless phenotype as Kil-0 at 14 dpi were scored as tolerant, whereas plants that were completely wilted (as seen for Be-0 at 14 dpi) were scored as susceptible (Table 2). Both trials produced tolerant/susceptible ratios of approximately 1:3 (Table 2). The χ^2 tests of both trials demonstrated that the observed ratio was not significantly different from an expected ratio of 1:3 (Table 2). These results indicated that the tolerance phenotype in accession Kil-0 was conferred by a single recessive gene. This was further verified by analysis of F_3 progeny from six tolerant F_2 lines that were selfed. Consistent with tolerance conferred by a single gene, all F_3 progeny showed the same tolerant phenotype as Kil-0 after inoculation with *R. solanacearum* BCCF402 (data not shown).

Tolerance in Kil-0 co-segregates with the RRS1 allele.

Resistance and not tolerance had previously been documented in another *Arabidopsis*–bacterial wilt pathosystem (Deslandes et al. 1998). In a cross between *A. thaliana* accessions Col-5 and Nd-1, the resistance segregated as a simply inherited recessive trait, and *RRS1-R* was identified as the major determinant of resistance against this strain (Deslandes et al. 1998, 2002). To determine whether tolerance in Kil-0 was linked to the *RRS1* allele, 47 F_2 plants resulting from a cross between Kil-0 and Be-0 were screened with a cleaved amplified polymorphic sequence (CAPS) marker developed to distinguish between the alleles of *RRS1* in Kil-0 and Be-0. In total, 14 plants scored as tolerant were homozygous for the Kil-0 allele of *RRS1* (Fig. 2A, representative CAPS marker profiles of 9 tolerant F_2 plants). Susceptible plants were either homozygous for the Be-0 allele (16 plants) or heterozygous for the Kil-0 and Be-0 alleles of *RRS1* (17 plants) (Fig. 2B, representative CAPS marker profiles of 9 susceptible F_2 plants). These results suggested that tolerance co-segregated with the *RRS1* allele of Kil-0.

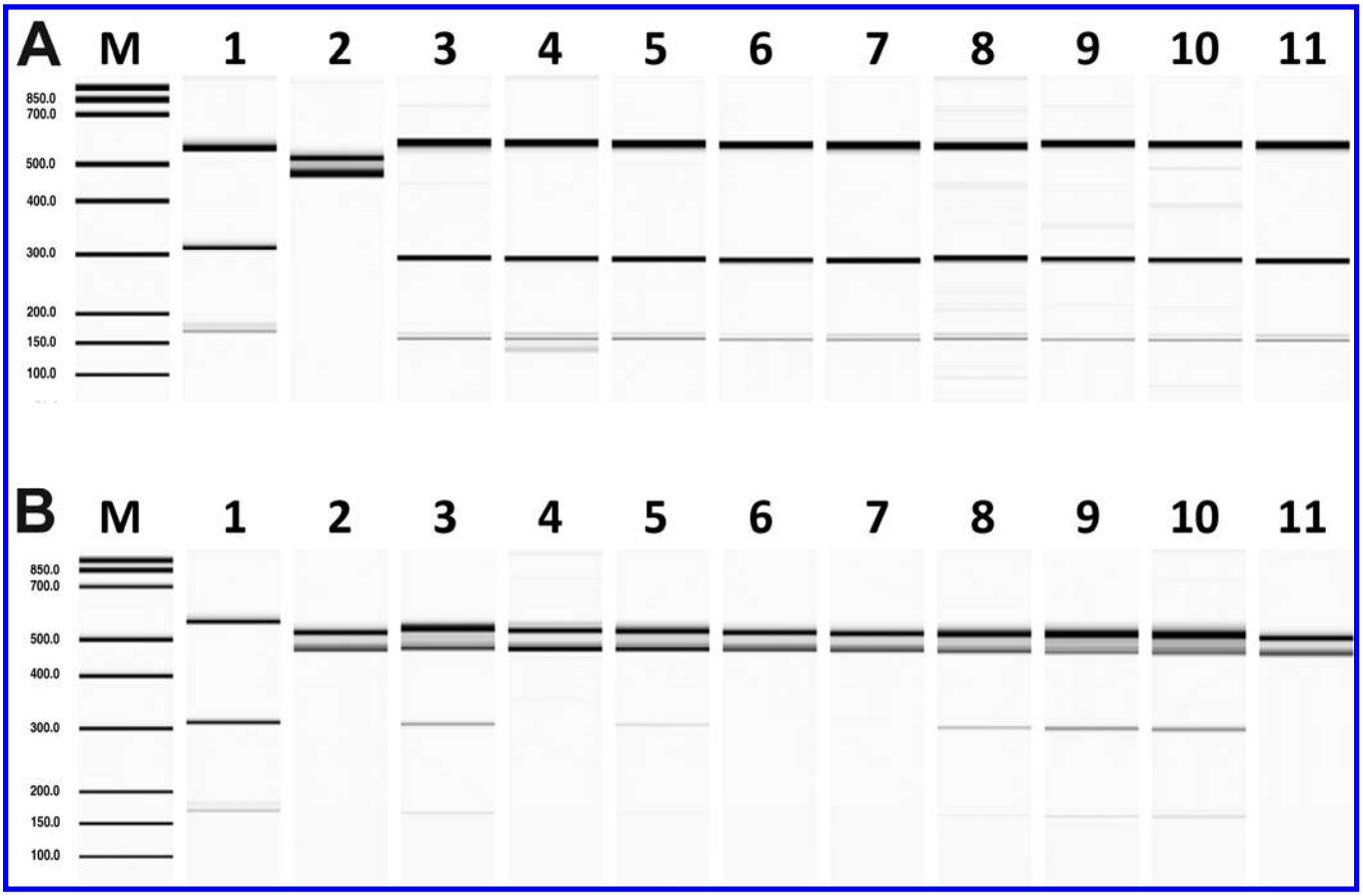

Fig. 2. Cleaved amplified polymorphic sequence (CAPS) marker analysis shows that tolerance segregates with the Kil-0 allele of *RRS1*. **A,** CAPS marker products of *RRS1* digested with *Lwe*I from *Arabidopsis thaliana* accession Kil-0 (lane 1), Be-0 (lane 2), and nine representative F_2 progeny from the Kil-0 X Be-0 cross that were scored as tolerant after inoculation with *Ralstonia solanacearum* BCCF402 (lanes 3 to 11). CAPS products of 516, 313, bp and 164 bp were obtained from accession Kil-0 (lane 1), and products of 504 and 479 bp from accession Be-0 (lane 2). Tolerant F_2 plants were homozygous for the Kil-0 allele of *RRS1* (lanes 3–11). Lane M: 1-kb DNA marker (Bio-Rad Laboratories Inc.). Additional faint bands such as the 150-bp product in lane 4 and several faint bands in lanes 8–10 represent nonspecific products that are not consistent between samples that were detected due to the high sensitivity of the Experion electrophoresis system. **B,** CAPS marker products of *RRS1* digested with *Lwe*I from *A. thaliana* accession Kil-0 (lane 1), Be-0 (lane 2), and nine representative F_2 progeny from the Kil-0 X Be-0 cross that were scored as susceptible after inoculation with *R. solanacearum* BCCF402 (lanes 3 to 11). Susceptible F_2 plants were either homozygous for the Be-0 allele of *RRS1* (products of 504 and 479 bp; lanes 4, 6, 7, and 11) or heterozygous (lanes 3, 5, 8, 9, and 10). Lane M: 1-kb DNA marker (Bio-Rad Laboratories Inc.). CAPS marker analysis was carried out by polymerase chain reaction amplification with the RRS1F-925 and RT3 primers of a C-terminal genomic DNA fragment of the *RRS1* gene from each plant, which was digested with *Lwe*I restriction enzyme. Products were then resolved on a DNA 1-kb chip on the Experion automated electrophoresis system (Bio-Rad Laboratories Inc.).

Tolerance in Kil-0 is allelic to resistance in Nd-1.

The F_1 progeny from a cross between Kil-0 and Nd-1 was inoculated with *R. solanacearum* BCCF402. CAPS marker analysis confirmed the heterozygosity of the progeny (data not shown). Susceptible Be-0 plants were severely wilted by 14 dpi (DI = 0.9 ± 0.1), whereas Nd-1 and Kil-0 plants were not (DI < 0.1). The F_1 progeny (15 plants) from the cross between Kil-0 and Nd-1 did not exhibit wilt symptoms at 14 dpi after inoculation with *R. solanacearum* BCCF402 (DI < 0.1) (Supplementary Fig. S2). These results indicated that it is unlikely that

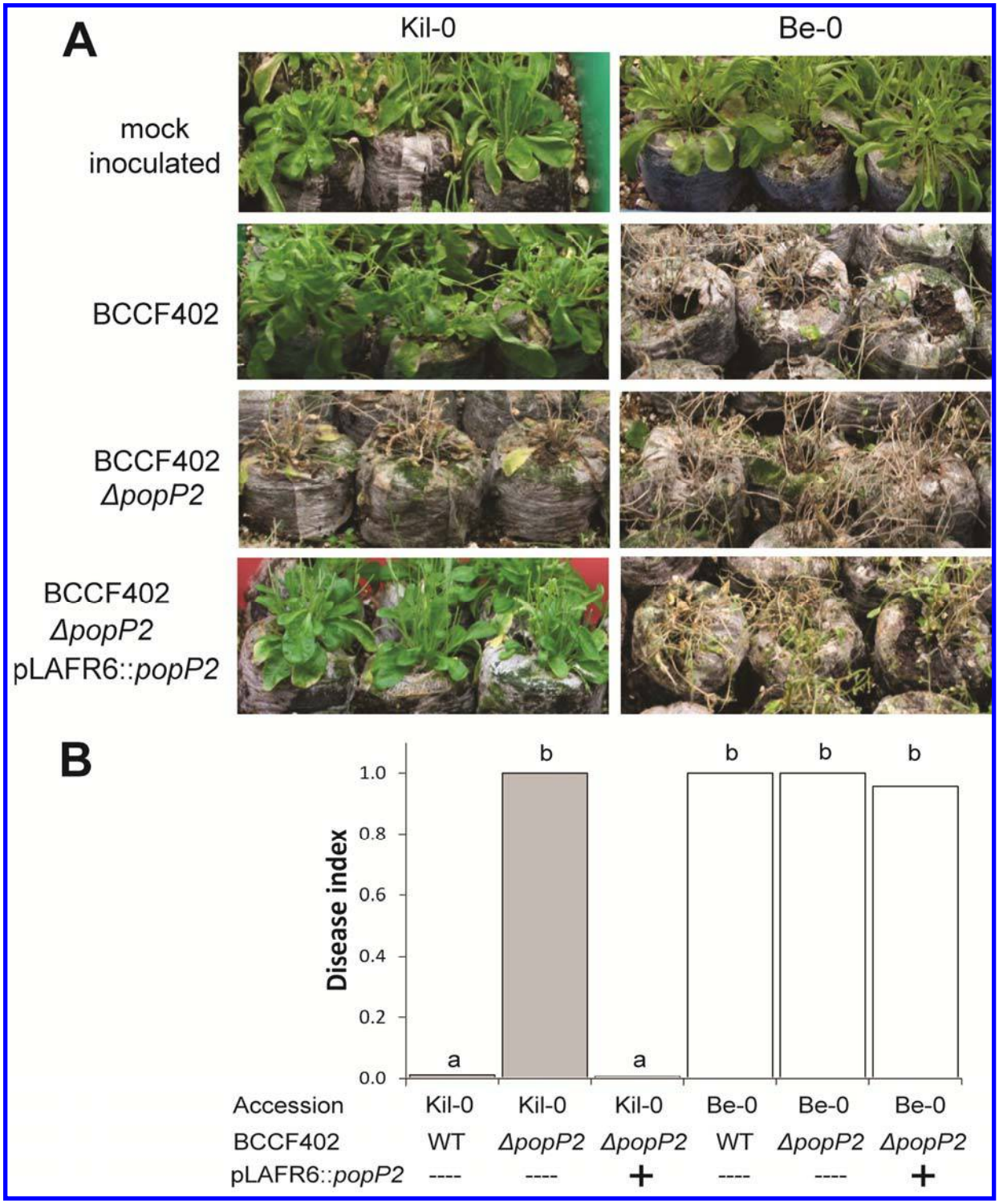

Fig. 3. Tolerance in *Arabidopsis thaliana* accession Kil-0 to *Ralstonia solanacearum* isolate BCCF402 requires *popP2*. **A,** Photographs of representative plants of *A. thaliana* accessions Kil-0 (left hand panel) and Be-0 (right hand panel) are shown at 19 days postinoculation (dpi) after mock inoculation or inoculation with *R. solanacearum* BCCF 402, BCCF 402 (*ΔpopP2*), or BCCF 402 (*ΔpopP2*) + (pLAFR6::popP2). Accession Kil-0 does not display symptoms after inoculation with wild-type (WT) *R. solanacearum* BCCF 402 or the complemented *popP2* mutant; however, it is susceptible to the *popP2* mutant strain of *R. solanacearum* BCCF 402. **B,** Disease index values for *A. thaliana* accessions Kil-0 (gray columns) and Be-0 (white columns) root-inoculated with either *R. solanacearum* BCCF402, the BCCF402 *ΔpopP2* mutant strain, or the mutant strain complemented with *popP2* at 1×10^8 CFU/ml. Means and standard deviations were calculated for disease index values of a total of 40 plants per accession (four replicates of 10 plants each) at 19 dpi. Treatments with the same letters were not significantly different (Wilcoxon Rank Sum Test, $P < 0.05$).

tolerance in Kil-0 and resistance in Nd-1 are conferred by different genetic loci, and supported the role of the recessive alleles linked to the *RRS1* locus for conferring the phenotype of each accession. Interestingly, bacterial counts at 8 and 14 dpi indicated that the F_1 progeny exhibited the tolerance phenotype, because they had high bacterial numbers similar to Kil-0 but significantly different from Nd-1, which had low bacterial numbers.

The RRS1 alleles of Kil-0 and Nd-1 show high amino acid similarity.

The full-length cDNAs of *RRS1* from Kil-0 and Be-0 were cloned and sequenced. The predicted amino acid sequences were compared with those of Nd-1 and Col-0 (Deslandes et al. 2002) (Supplementary Fig. S3). RRS1 from Nd-1 is predicted to be 1,378 amino acids (aa) in size. All four RRS1 proteins contained an identical WRKY domain near the C-terminus. However, the Kil-0, Be-0, and Col-0 proteins were 5, 23, and 90 aa shorter, respectively, than Nd-1. RRS1 from Kil-0 had the highest amino acid identity to RRS1 from Nd-1 (99%), with only eight different amino acids, whereas Be-0 and Col-0 were 97 and 92% identical, with different amino acids at 11 and 18 positions, respectively.

PopP2 is the tolerance determinant in *R. solanacearum* BCCF402 recognized by RRS1 in Kil-0.

The *RRS1* gene in accession Nd-1 confers resistance against *R. solanacearum* GMI1000 by recognizing the matching PopP2 effector in the tomato strain GMI1000 (Deslandes et al. 2003). To confirm the hypothesis that *RRS1* in Kil-0 is the locus conferring tolerance against *R. solanacearum* BCCF402, the tolerant Kil-0 plants were challenged with a BCCF402 *ΔpopP2* mutant strain. The disruption of *popP2* in *R. solanacearum* BCCF402 abolished tolerance in accession Kil-0 and the plants were completely wilted (Fig. 3A and B). Kil-0 plants were also challenged with the *R. solanacearum* BCCF402 *ΔpopP2* mutant strain complemented with a functional *popP2* gene (pLAFR6::*popP2*), which restored the tolerant phenotype (Fig. 3A and B). As expected, Be-0 plants were susceptible to the wild-type, *ΔpopP2* mutant, and complemented *ΔpopP2* mutant strains of *R. solanacearum* BCCF402 (Fig. 3A and B). These results supported the hypothesis that PopP2 is the effector recognized by RRS1 in Kil-0 to confer tolerance in this accession to *R. solanacearum* BCCF402.

DISCUSSION

Bacterial wilt is a disease of many plant species caused by the *R. solanacearum* species complex. Progress has been made in understanding susceptibility and resistance to bacterial wilt (Deslandes et al. 2002; Narusaka et al. 2009; Turner et al. 2009); however, very little is known about mechanisms conferring tolerance at the molecular level. Our study reports on a case of tolerance in the *A thaliana* accession Kil-0 to *R. solanacearum* BCCF402, a phylotype I strain from *Eucalyptus* trees. Several observations suggest that Kil-0 is tolerant to BCCF402 (Fig. 1; Table 1), namely i) Kil-0 plants exhibited a "resistant" phenotype in terms of lack of wilting symptoms but had high bacterial multiplication in planta; ii) plant biomass yield, seed number, and seed germination of Kil-0 plants were not significantly affected by inoculation with BCCF402, in contrast to the susceptible accession Be-0; and iii) the response of Kil-0 plants was distinct from that of resistant Nd-1 plants that were symptomless but did not support high bacterial multiplication when inoculated with the same bacterial strain BCCF402.

Tolerance in plant–pathogen interactions is believed to be a polygenic trait conferred by multiple genes with small effects (Barrett et al. 2009). Therefore, we expected that Kil-0 tolerance to *R. solanacearum* BCCF402 would be polygenic. However, genetic analysis indicated that the tolerance phenotype in Kil-0 was conferred by a single recessive locus (Table 2). RRS1-R, an atypical R protein, is the determinant of recessive resistance in *A. thaliana* accession Nd-1 to *R. solanacearum* GMI1000 (Deslandes et al. 2002). The use of a CAPS marker that distinguished between the *RRS1* alleles of Kil-0 and Be-0 showed that the tolerant response to BCCF402 co-segregated with the *RRS1* allele in Kil-0 (Fig. 2). This provided strong evidence that tolerance is conferred by *RRS1* or a closely linked gene, which was further confirmed by allelism studies.

Recognition of the effector PopP2 by RRS1-R and physical interaction between RRS1-R and PopP2 in the nucleus are key steps of resistance in accession Nd-1 (Deslandes et al. 2003). Several lines of evidence indicated that RRS1 was the major determinant of Kil-0 tolerance, namely i) inactivation of *popP2* in strain BCCF402 resulted in loss of Kil-0 tolerance, leading to appearance of disease symptoms; ii) loss of tolerance was reversed by complementation of the *popP2* mutant with a low-copy-number plasmid expressing *popP2*; and iii) genetic studies showed that the RRS1 allele segregated with the tolerance trait, and that tolerance in Kil-0 was allelic to resistance in Nd-1. In *A. thaliana* accession Ws-0, both *RRS1* and *RPS4,* an adjacent *R* gene, are required for resistance to *R. solanacearum* isolate 1002 (Narusaka et al. 2009). The dependence upon *popP2* for the tolerance phenotype in Kil-0 makes it more likely that RRS1 and not RPS4 is the major determinant of this phenotype. However, the possibility that RPS4 is implicated in Kil-0 tolerance cannot be excluded, and experiments are underway to test this.

Our results indicated that the RRS1–PopP2 interaction can result in either resistance in Nd-1 or tolerance in Kil-0 to *R. solanacearum* BCCF402. It was unexpected that a major R gene (*RRS1*) could confer tolerance as well as resistance. However, it was recently shown that another *R* gene, *Rpm1*, exhibits similar features (Roux et al. 2010). Responses of *Arabidopsis* accessions carrying the resistance gene *Rpm1* were compared with those without the *R* gene after challenge with different inoculum levels of *P. syringae* DC3000::AvrRpm1. As expected, Rpm1-mediated resistance was observed at intermediate inoculum levels. However, at inoculum levels where no significant difference in symptoms was observed, there was a clear fitness benefit (46 to 77%) associated with the *Rpm1* gene, providing evidence that *Rpm1* contributes to tolerance (Roux et al. 2010). This is in contrast to *Rps5*, another *R* gene, which was not associated with tolerance in a different *Arabidopsis-Pseudomonas* pathosystem (Gao et al. 2009).

In our study, we demonstrated that *popP2* and *RRS1* were required for the tolerance phenotype seen in *A. thaliana* accession Kil-0 to *R. solanacearum* BCCF402. A point to consider is whether the RRS1 or PopP2 proteins may explain the differences in tolerant and resistant phenotypes. Sequence comparison of RRS1 from Kil-0 and Nd-1 showed that the proteins share 99% identity, with only eight amino acid differences, and Kil-0 is five amino acids shorter at the 3′ end. This is in contrast to RRS1-S from accession Col-0, which lacks 83 C-terminal amino acids, a feature that is hypothesized to explain why RRS1-S is unable to detect PopP2 activity and trigger a defense response (Tasset et al. 2010). In addition, the predicted PopP2 sequence from BCCF402 differed by only four amino acids with PopP2 from GMI1000, and the functionally important residues of the catalytic triad and the auto-acetylated lysine were conserved (Supplementary Fig. S4) (Tasset et al. 2010). GMI1000 is also classified as a biovar 3 phylotype I strain of *R. solanacearum* (Fegan and Prior 2005). However, despite the high degree of similarities between the RRS1 and

PopP2 proteins from different accessions, we cannot exclude the possibility that subtle differences may explain our data.

It is more likely that differences in the downstream responses explain the tolerant and resistant responses of Kil-0 and Nd-1 plants, respectively. Interestingly, the *A. thaliana ein2.1* mutant, which is defective in ethylene signaling, exhibits a phenotype reminiscent of tolerance because, despite a delayed symptom development, it supports high bacterial multiplication (Hirsch et al. 2002). Microarray expression profiling of susceptible responses of *A. thaliana* accessions to *R. solanacearum* strains has been reported (Hu et al. 2008; Naidoo et al. 2011). It would be interesting to compare expression profiles from tolerant and susceptible plants that both support high bacterial numbers but differ in symptom development.

Our observation that the F_1 progeny of Kil-0 and Nd-1 were tolerant indicated that the tolerance phenotype appears to be dominant over the resistant phenotype. Further biochemical characterization of RRS1 protein interactions or downstream responses could shed light on the reasons for this phenomenon. In conclusion, we have shown that the bacterial wilt pathogen *R. solanacearum* BCCF402 was virulent on *A. thaliana* accession Be-0 plants, which were fully susceptible. The responses of *A. thaliana* accessions Kil-0 and Nd-1 were distinct and could be defined as tolerant and highly tolerant (resistant), respectively, and were both most likely mediated through RRS1. Therefore, tolerance is not always a polygenic trait. Gene-for-gene interactions between plants and pathogens appear to lead to a continuum of responses between susceptibility and high levels of tolerance (resistance).

Finally, in the context of disease management of crops, including *Eucalyptus* trees from which *R. solanacearum* BCCF402 was isolated, our work highlights the possibility that deployment of the *RRS1* gene in transgenic trees may result in tolerance associated with high bacterial numbers in planta, with a greater risk for persistence of the pathogen in the plantation environment over the long term.

MATERIALS AND METHODS

R. solanacearum strains.

R. solanacearum biovar 3 phylotype I strain BCCF402 (Fouché-Weich et al. 2006; Roux et al. 2000) was grown on solidified bacto-agar glucose triphenyltetrazolium chloride (BGT) medium at 28°C for 48 h and mucoid colonies were transferred to liquid B medium (Deslandes et al. 1998). Rifampicin mutants were prepared as described previously (Naidoo et al. 2011).

Preparation of the BCCF402 Δ*hrp* mutant was conducted as described for BCCF401 (Naidoo et al. 2011) using genomic DNA from Δ*hrp R. solanacearum* GMI1402, a derivative of GMI1000 carrying a disruption in the *hrc*S (*hrp*-conserved) gene (Arlat et al. 1992). BCCF402 Δ*hrp* mutants that were unable to induce a hypersensitive response on tobacco were selected (data not shown).

The BCCF402 *ΔpopP2* mutant strain was generated by introducing a PCZ367 integrative plasmid containing a disrupted *popP2* gene into BCCF402, as described previously (Deslandes et al. 2003). Strains were screened using polymerase chain reaction (PCR) to verify that the *popP2* gene was disrupted. The *popP2* mutant strain was termed BCCF402 *ΔpopP2*. The *R. solanacearum* BCCF402 *popP2* gene, together with its promoter (GenBank accession number JX135562), was amplified from genomic DNA using the POPP2FLF (5′GAGGGTGG TCGTAATGGTTG3′) and POPP2FLR (5′CTGGTTTGTG AGTTGTTGTC3′) primers. The product was cloned into the PTZ57R/T vector (InsTAclone PCR cloning kits; Fermentas Inc., Vilnius, Lithuania). The full-length *popP2* gene and its promoter were cloned into the linearized pLARF6 cosmid (Deslandes et al. 2003) using T4 DNA ligase (Fermentas, Inc.), according to the manufacturer's instructions. The pLAFR6:: popP2 construct was transformed into the *R. solanacearum* BCCF402 *ΔpopP2* strain by electroporation (Deslandes et al. 2003). The transformed cells were selected on BGT plates containing gentamycin at 10 μg/ml and tetracycline at 10 μg/ml and designated BCCF402 *ΔpopP2* + (pLAFR6::popP2).

Arabidopsis accessions and bacterial inoculations.

Arabidopsis accessions Be-0, Kil-0 and Nd-1, obtained from the Nottingham *Arabidopsis* Stock Centre, were grown on Jiffy pots (Jiffy France, Lyon, France), as described previously (Naidoo et al. 2011). The plantlets were maintained in a growth chamber at a temperature of 22°C with 16 h of light, 25 to 30% relative humidity, and 300 to 350 lum/ft^2. Plantlets were watered four times (once per week) with a solution of Multifeed (Plaaskem [Pty.] Ltd., Johannesburg, South Africa) at 2.5g/liter.

Crosses between Kil-0 and Be-0 and between Kil-0 and Nd-1 were performed according to Weigel and Glazebrook (2002). The F_2 and F_3 progeny were obtained by selfing F_1 and F_2 progeny, respectively. All *R. solanacearum* strains used in this study were grown overnight in B medium (Deslandes et al. 1998) using respective antibiotics. Inoculations were performed according to Deslandes and associates (1998) using an inoculum of 1×10^8 CFU/ml. Symptom development was recorded and rated on a scale from 0 (no disease) to 4 (100% wilted or dead plants) according to the method of Deslandes and associates (1998). The DI was calculated using the following formula: $DI = [\Sigma(n_i \times v_i)/(V \times N)]$, where n_i = number of plants with respective disease rating, v_i = disease rating (0, 1, 2, 3, or 4), V = the highest disease rating (= 4), and N = the number of plants observed (Naidoo et al. 2011). Bacterial enumerations were carried out as described by Deslandes and associates (1998), with selection for *R. solanacearum* BCCF402 on rifampicin at 50 μg/ml and for the BCCF402 *Δhrp* mutant on kanamycin at 50 μg/ml.

DNA extractions.

Arabidopsis and *R. solanacearum* genomic DNA was isolated based on the cetyl-trimethyl-ammonium bromide extraction procedure described by Lukowitz and associates (2000). Plasmid DNA was isolated using the Invisorb Spin Plasmid MiniTwo Kit (Invitek, Berlin) according to the manufacturer's instructions.

RNA extractions and cDNA synthesis.

RNA was isolated from leaves of Be-0 and Kil-0 using the QIAzol lysis reagent (Qiagen, Valencia, CA, U.S.A.) according to manufacturer's instructions and subsequently purified using the Qiagen RNeasy Plant Mini Kit. cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, U.S.A.) and cleaned using the Qiagen RNeasy plant mini kit.

Biomass, seed yield, and seed viability measurements.

Whole-plant tissue was harvested 16 days after BCCF402 challenge to evaluate biomass yield changes. The plant material was weighed after drying in an oven at 36°C for 24 h to obtain the dry weight. Three biological replicates were included, consisting of 10 plants each. Seed were collected for 4 weeks after BCCF402 inoculation or until plant senescence, according to Weigel and Glazebrook (2002). In all, 1,000 seeds of each accession were counted and weighed, forming an estimate of the mass per 1,000 seeds (in milligrams). The total seed of each accession were weighed and the approximate number of

seed per accession was calculated. Three biological replicates of three plants each were included in this analysis. Seed were germinated to evaluate the seed viability of inoculated compared with uninoculated plants. In total, 100 seeds were germinated on plates of Murashige and Skoog medium according to Naidoo and associates (2011). Germinating plantlets, indicated by the formation of roots and shoots, were counted and the experiment was completed in triplicate.

Genotyping.

The *RRS1* CAPS marker was developed as follows. Primers RRS1F-925 (5′TCCATACCGGCTATAGACGA3′) and RT3 (5′AACTCCTCCATGTCCGTC3′) were designed from the C-terminal region of *RRS1-R* from *A. thaliana* ecotype Nd-1 (GenBank accession number AX103684). These primers amplified products of 983 and 993 bp of the C-terminal region of *RRS1* from genomic DNA of *A. thaliana* accessions Be-0 and Kil-0, respectively (data not shown). The DNA sequences of the products were compared and showed that Kil-0 contained an additional *Lwe*I restriction enzyme site (data not shown). Digestion of the Be-0 product with *Lwe*I was predicted to yield two products of 479 and 504 bp, whereas digestion of the Kil-0 product with *Lwe*I was predicted to yield three products of 516, 313, and 164 bp (data not shown). Genomic DNA from the *Arabidopsis* plants was screened with the *RRS1* CAPS marker by amplifying the C-terminal region of the *RRS1* gene with the RRS1F-925 and RT3 primer pair and digesting the PCR products overnight at 37°C using *Lwe*I (Fermentas, Inc.). Products were resolved by a DNA 1K kit on the Experion automated electrophoresis system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.).

PCR and sequencing.

The sequences of the full-length *RRS1* cDNA from *A. thaliana* accessions Kil-0 and Be-0 were determined after PCR amplification of three overlapping cDNA fragments using three sets of primer pairs: RRS1-C1F (ATTTCTCAAAATCTTAAA AACTT) and RRS1-C1R (GAAGGTTGTTTTAGGTTCTC CA), RRS1-C2F (CATGTTGAAATTGATGTCCTTG) and RRS1-C2R (CGTTGACCACTTGTTGAGATAG), and RRS1-C3MF (GACTACTGTGATGCTACAGATG) and RRS1-C3MR (GTCATTATCCACCTCCATATTG). Each PCR contained 1× *Taq* reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM each primer, and 1 U of *Taq* DNA polymerase (BIOTAQ DNA polymerase; Bioline Ltd., London). Standard cycling conditions were used with specific annealing temperatures per primer pair. Sequencing reactions were performed at Macrogen (Rockville, MD, U.S.A.) or at InqabaBiotech (Pretoria, South Africa). Be-0 and Kil-0 *RRS1* cDNA sequences were submitted to GenBank (accession numbers JX135560 and JX135561, respectively).

Data analysis.

Statistical analyses were performed using R and GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, U.S.A.).

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