Genome-Enabled Phylogeographic Investigation of the Quarantine Pathogen *Ralstonia solanacearum* Race 3 Biovar 2 and Screening for Sources of Resistance Against Its Core Effectors

Christopher R. Clarke, David J. Studholme, Byron Hayes, Brendan Runde, Alexandra Weisberg, Rongman Cai, Tadeusz Wroblewski, Marie-Christine Daunay, Emmanuel Wicker, Jose A. Castillo, and Boris A. Vinatzer

First, third, fourth, fifth, sixth, and eleventh authors: Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Latham Hall, Ag Quad Lane, Blacksburg, VA; second author: Biosciences, University of Exeter, Exeter, Devon, UK; seventh author: Genome Center and Department of Plant Sciences, University of California, Davis, CA 95616; eighth author: Unité de Genetique et Amelioration des Fruits et Legumes, INRA, Centre d'Avignon, Montfavet, France; ninth author: CIRAD, UMR Peuplements Vegetaux et Bioagresseurs en Milieu Tropical (PVBMT), Saint Pierre, La Reunion, France; and tenth author: PROINPA Foundation, Cochabamba, Bolivia.
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ABSTRACT

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Phylogeographic studies inform about routes of pathogen dissemination and are instrumental for improving import/export controls. Genomes of 17 isolates of the bacterial wilt and potato brown rot pathogen *Ralstonia solanacearum* race 3 biovar 2 (R3bv2), a Select Agent in the United States, were thus analyzed to get insight into the phylogeography of this pathogen. Thirteen of fourteen isolates from Europe, Africa, and

Asia were found to belong to a single clonal lineage while isolates from South America were genetically diverse and tended to carry ancestral alleles at the analyzed genomic loci consistent with a South American origin of R3bv2. The R3bv2 isolates share a core repertoire of 31 type III-secreted effector genes representing excellent candidates to be targeted with resistance genes in breeding programs to develop durable disease resistance. Toward this goal, 27 R3bv2 effectors were tested in eggplant, tomato, pepper, tobacco, and lettuce for induction of a hypersensitive-like response indicative of recognition by cognate resistance receptors. Fifteen effectors, eight of them core effectors, triggered a response in one or more plant species. These genotypes may harbor resistance genes that could be identified and mapped, cloned, and expressed in tomato or potato, for which sources of genetic resistance to R3bv2 are extremely limited.

There is no better disease control than disease prevention. Therefore, keeping pathogens out of an unaffected geographic area or eradicating recently introduced pathogens is paramount. To do this efficiently, it is necessary to understand how pathogens are disseminated. Phylogeography, i.e., inferring past migration events by combining phylogenetic reconstruction with geographic information, is instrumental to gain this understanding. Once a pathogen is introduced into a geographic area, disease control becomes necessary. Unfortunately, many crops are affected by pathogens against which no high-yielding, high-quality cultivars are resistant. In addition, complex genetics and heterozygous status of clonally propagated crop species, such as potato, make it extremely difficult to introduce resistance genes by classical breeding from wild resistant relatives (Jansky 2006). Yet, utilization of resistance genes provides a very effective strategy for crop protection, particularly against bacterial plant pathogens for which chemical control and agricultural practices are often only temporary and ineffective solutions (Strange and Scott 2005).

Today, genome sequencing in combination with biotechnology has the potential to greatly improve diagnostics and global tracking

of high-risk pathogens and to accelerate the creation of durable disease resistant cultivars. Here we show progress using such an approach for *Ralstonia solanacearum*, a beta-proteobacterium that enters plant roots from the soil through natural openings and wounds, enters and colonizes the xylem where it blocks flow of water and nutrients, and finally causes wilting and eventual death of the host (Denny 2006). *R. solanacearum* pathogenicity is based on a large repertoire of virulence genes encoding type II-secreted extracellular enzymes and type III-secreted effector (T3E) proteins (Genin and Denny 2012), which in some plant genotypes trigger immunity and thus contribute to host range (Poueymiro et al. 2009). *R. solanacearum* overall has a very large host range, encompassing more than 54 plant families (Hayward 1991). *R. solanacearum* thus constitutes a major biotic constraint in tropical and subtropical areas throughout the world, both on subsistence and cash crops (Elphinstone 2005).

R. solanacearum race 3 biovar 2 (R3bv2) is a subgroup of *R. solanacearum* that causes bacterial wilt on numerous solanaceous species. Because R3bv2 causes potato tubers to rot, the disease is also called “brown rot.” Based on phylogeny of the *egl* gene (Fegan and Prior 2005), most R3bv2 fall within phylotype IIB sequevar 1 (IIB-1) although a few isolates belong to other phylotype II sequevars (Cellier et al. 2012). Contrary to most *R. solanacearum* strains, which are adapted to hot humid climates, R3bv2 strains are able to cause disease at relatively low temperatures of temperate climate zones (Milling et al. 2009). Consequently, this pathogen constitutes a major problem for subsistence potato farmers in many mountainous regions of Africa, South America, and Asia. In the Mediterranean region, R3bv2 was first identified in the 1920s, in Egypt, Italy, and Spain. From there it probably spread with

First and second authors contributed equally.

Corresponding author: B. A. Vinatzer; E-mail address: vinatzer@vt.edu

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contaminated early ware potatoes to northern Europe where it started to cause frequent outbreaks in the 1990s after being disseminated via contaminated seed potatoes (Janse 1996; Grousset et al. 1998). It is now endemic in many waterways and on the bittersweet nightshade (*Solanum dulcamara*) in the Netherlands (Stevens and van Elsas 2010), France (Trigalet et al. 2002), the UK (Parkinson et al. 2013), Spain (Caruso et al. 2005; Lambert 2002), and Portugal (Cruz et al. 2012). R3bv2 is currently not present in North America although it was accidentally imported at least twice on geranium cuttings from Kenya and Guatemala, but in both cases it was possible to eradicate it before it became endemic (Williamson et al. 2002; Floyd and Michalak 2008). Because of its potentially devastating effect on the U.S. potato industry, R3bv2 is on the USDA-APHIS list of Select Agents (CDC and APHIS; Lambert 2002) and as such under stringent quarantine regulation.

The limited genetic diversity of R3bv2 strains identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), 16S rRNA, and 16S-23S suggests a recent evolutionary origin of R3bv2 (Poussier et al. 2000; Timms-Wilson et al. 2001). However, pulsed field gel electrophoresis and rep-PCR (Smith et al. 1995), microarrays (Cellier et al. 2012), and multilocus variable number tandem repeat analysis (MLVA) (N'Guessan et al. 2013) provided enough resolution to distinguish R3bv2 isolates into separate populations, some of which were found to be associated with different geographic regions. Single nucleotide polymorphisms (SNPs) have been successful in reconstructing detailed phylogeographies of several human pathogens, for example of the plague pathogen *Yersinia pestis* (Morelli et al. 2010b), because they allow determination of ancestral versus derived relationships. SNPs have also been used with some success in resolving the phylogeography of plant pathogenic bacteria, for example of *Pseudomonas syringae* pv. *actinidiae* (Mazzaglia et al. 2012), but they have not yet been used for phylogeographic studies of *R. solanacearum*.

Unfortunately, there is not a single potato cultivar resistant to any *R. solanacearum* strain (Fock et al. 2005). While some wild potato relatives are resistant, for example *S. commersonii* (Siri et al. 2009), resistance is difficult to introduce into cultivated potato because of different ploidy levels (Jansky 2006) and mismatches in the endosperm balance number (Ortiz and Ehlenfeldt 1992). Moreover, R3bv2 has been found virulent and very aggressive on most bacterial wilt-resistant tomato genotypes that contain genes for quantitative resistance (Carmeille et al. 2006; Wang et al. 2000) (Lebeau et al. 2011; Wang et al. 2013). The best-characterized gene that provides resistance to *R. solanacearum* is the *Arabidopsis thaliana* *RRS1-R* gene, which recognizes the T3E PopP2 (Deslandes et al. 2002; Deslandes et al. 2003), recently renamed RipP2 (Peeters et al. 2013). In crop species, the only dominant genes for resistance to *R. solanacearum* have so far been mapped in eggplant (Lebeau et al. 2013; Xi'ou et al. 2014), and indication for a resistance gene to an *R. solanacearum* effector has also been found in the wild eggplant relative *Solanum torvum* (Nahar et al. 2014). Moreover, Lebeau et al. (2011) found eggplant germplasm to encompass some remarkable genotypes with resistance to multiple *R. solanacearum* strains, including R3bv2. Because tomato, potato, and eggplant all belong to the same botanical family, expression of disease resistance genes from eggplant in potato or tomato will likely provide resistance, due to compatibility of signal transduction components (Hulbert et al. 2001; Tai et al. 1999). Therefore, identifying the underlying resistance genes in these eggplant genotypes is paramount.

We have shown in the past that using *Agrobacterium*-mediated transient assays to express individual T3Es in plants and screening for a hypersensitive response (HR)-like plant reaction is an efficient way to detect the presence of potential resistance genes (Vinatzer et al. 2006; Wroblewski et al. 2009). These genes could then be genetically mapped, cloned and expressed in crop cultivars. The problem is that pathogens can overcome a single resistance gene relatively easily by losing or mutating the effector that is being

recognized (Gassmann et al. 2000; Kunkeaw et al. 2010). However, Bart and colleagues used the example of *Xanthomonas axonopodis* pv. *manihotis* to show that by sequencing a large number of strains it is possible to identify the core set of T3Es of a pathogen (Bart et al. 2012). The assumption is that effectors present in most strains of a pathogen play an essential role in pathogen virulence and thus cannot be easily lost by the pathogen after a new resistance gene is employed in the host. Resistance genes that recognize such conserved effectors are thus expected to be more durable than resistance genes that recognize dispensable effectors.

Here, we analyzed the genomes of seventeen R3bv2 isolates collected over 58 years on different continents to get insight into R3bv2 phylogeography and to identify the R3bv2 core effector repertoire to target in crop breeding. We previously tested putative R3bv2 effectors in transient assays on lettuce, tomato, pepper, and tobacco genotypes to survey them for potential resistance genes (Wroblewski et al. 2009). Here we integrate these results with new results obtained with two eggplant genotypes, one of which is resistant to R3bv2 (Lebeau et al. 2013), to prioritize effectors to target in breeding for bacterial wilt/brown rot resistance

MATERIALS AND METHODS

Bacterial growth and DNA extraction. Bacterial strains were stored in Cryobank beads at -80°C . They were grown first on nutrient broth overnight at 28°C , and then streaked (50 μl) on Kelman's triphenyl tetrazolium chloride (TZC) agar medium (Kelman 1954) supplemented with 1% yeast extract, and subcultured 2 days at 28°C . DNA was extracted from fresh cultures (~ 1.0 to 2.0×10^9 cells) using the DNeasy Blood and Tissue Kit (Qiagen), and checked for titer and quality using a Nanodrop device.

Genome sequencing. Paired-end Illumina sequencing was performed for 76 cycles on an Illumina GAIIx with PE module at the Center for the Analysis of Genome Evolution and Function, University of Toronto, Toronto, Ontario, Canada as previously described (Mazzaglia et al. 2012). 454 sequencing was performed on a GS FLX sequencer with Titanium chemistry and sequencing reads were assembled with Newbler v. 2.5 at the Virginia Bioinformatics Institute, Blacksburg, Virginia. Sequencing details are listed in Table 1.

SNP identification. To facilitate identification of SNPs, we aligned the sequence reads (454 or Illumina; Table 1) against the reference genome sequence of *R. solanacearum* strain Po82 (Xu et al. 2011) using BWA-mem version 0.7.5a-r405 (Li and Durbin 2009) with default parameter values and excluding any reads that did not map uniquely to a single site on the reference genome. From the resulting alignments, we generated pileup files using SAMtools version 0.1.19-96b5f2294a (Li et al. 2009). We then parsed the pileup-formatted alignments to examine the polymorphism status of each single-nucleotide site in the entire 5,430,263-bp reference genome. For each single-nucleotide site we categorized it as either ambiguous or unambiguous. A site was considered to be unambiguous only if there was at least 5 $\times$ coverage by genomic sequence reads from each and every bacterial isolate and only if for each and every bacterial isolate, at least 95% of the aligned reads were in agreement. Any sites that did not satisfy these criteria were considered to be ambiguous and excluded from further analysis. Over the remaining unambiguous sites, we could be very confident in the genotype for all the sequenced isolates.

The sequence reads for several genomes that had been previously published by other laboratories were not publicly available at the time of this study. Therefore, we generated 50 $\times$ coverage of simulated 'pseudo-reads' and aligned these to the reference genome by the same method. The genomes for which we had to resort to using pseudo-reads were the genomes of the R3bv2 isolates UW551 and IPO1609 and the following non-R3bv2 genomes: CMR15, FJAT-1458, FJAT-91, FQY_4, GMI1000, MoIK2, NCPPB909, P673, Po82, PSI07, R229, R24, Rs-09-161, Rs-10-244, SD54, and

Y45. The pseudo-reads comprised randomly selected 50-bp substrings from the genome sequence; substitution errors were introduced randomly into the pseudo-reads at a rate of 0.1% per base pair. Clearly, there is a significantly higher risk of false-positive variants being called based on these pseudo-reads since the use of pseudo-reads derived from a single consensus genome assembly does not benefit from the alignment of multiple independent sequence reads as is the case for the Illumina and 454 reads. The accuracy of these previously published genome assemblies (and hence the pseudo-reads derived from them) is limited by the depth and reliability of the Sanger sequence reads used to assemble them and by the accuracy of the assembly process.

Construction of phylogenetic trees. We used the results of the genome-wide SNP-calling to investigate the phylogenetic relationships among the sequenced bacterial isolates. The SNP sites were concatenated together to generate a multiple sequence alignment, on which a maximum likelihood (ML) phylogenetic tree was built using RAxML (Stamatakis 2014). Twenty ML searches were performed for each tree along with 1,200 nonparametric bootstrap replicates using the ASC_GTRGAMMA model. Bootstrap values as a percentage of 1,200 replicates were mapped to branches on the best ML tree, and branches with less than 50% bootstrap support were collapsed into polytomies using TreeCollapseCL4 (Hodcroft).

Identification of effector repertoires. We checked for presence of each of the 60 predicted effector genes previously cataloged (Peeters et al. 2013) in *R. solanacearum* UW551 (Gabriel et al. 2006) by aligning sequence reads against the gene sequences using BWA-mem version 0.7.5a-r405 (Li and Durbin 2009) with default parameter values (allowing nonuniquely mapping reads to match multiple genomic sites). From the resulting alignments, we generated pileup files using SAMtools version 0.1.19-96b5f2294a (Li et al. 2009) and parsed these pileup files to determine the proportion of the gene that was covered by aligned sequence reads (that is, the breadth of coverage for each gene). To check for frame-shifts and premature stop-codons, we generated consensus sequences for each gene for each sequenced isolate (based on the pileup files). We then checked the integrity of each consensus effector gene sequence by translating from nucleotide into amino acid sequence using BioPerl (Stajich et al. 2002).

Agrobacterium-mediated transient expression of effectors. Eggplant seeds were sowed in Sunshine Mix 1 Soil (Sun Gro Horticulture, Agawam, MA) and grown at 22°C on a 16 h day cycle in Conviron (Henderson, NC) PGC20 chamber. Other plant species and cultivars tested were grown in the greenhouse condition as described previously (Wroblewski et al. 2009). Five- to seven-week-old eggplants, 2- to 3-week-old lettuce plants, and 4- to

6-week-old tomato, pepper, and *N. benthamiana* plants were used for transient assays.

Thirty-two effectors from *R. solanacearum* strain UW551 that were previously cloned into the vector pBAV150 (Vinatzer et al. 2006) under control of a dexamethasone-inducible promoter were transformed via conjugation into *A. tumefaciens* strain C58 (Hamilton and Fall 1971). *Agrobacterium*-mediated transient expression was performed similar to previously described protocols (Wroblewski et al. 2009). Briefly, *Agrobacterium* was inoculated into liquid Luria-Bertani broth with antibiotics and 200 µM acetosyringone and incubated at 28°C at 200 rpm for 18 h in 50 ml conical tubes. Cells were harvested via centrifugation at 4,000 rpm. Cells were resuspended in sterile MgSO₄ and centrifuged to pellet a second time. Cells were resuspended in sterile MgSO₄ again and adjusted to an approximate optical density at 600 nm (OD₆₀₀) of 0.5 and acetosyringone was added to a final concentration of 200 µM and incubated at 28°C at 200 rpm for an additional 4 to 6 h. The OD₆₀₀ was adjusted to 0.3 and the adaxial side of leaves were infiltrated with a 1 ml blunt-end syringe. Two days after infiltration, infiltrated leaves were sprayed with 30 µM dexamethasone. Five days after infiltration, the severity of induced HR-like symptoms was scored. At least two independent experiments with six leaves per experiment were performed for each effector on the two eggplant genotypes. Any effector that elicited an HR-like response was tested in at least three independent experiments. For the other plant species, the number of repetitions are reported for each individual effector on each genotype in the CHARGE database at http://charge.ucdavis.edu/charge_db/chargedb_index.php.

RESULTS

A genome-based phylogeny and estimation of the time since the most recent common ancestor for R3bv2. To investigate phylogeny and phylogeography of R3bv2, we chose to sequence the genomes of 14 R3bv2 isolates (Table 2). The isolates were chosen to maximize the time span of isolation (from 1950 to 2008), geographic location (South America, Africa, Europe, and Asia), and genetic diversity based on previously obtained microarray analysis (Cellier et al. 2012). The publicly available genome sequences of the R3bv2 isolates UW551 (Gabriel et al. 2006), IPO1609 (Guidot et al. 2009), and NCPPB909 (GenBank: JNGD000000000.1, unpublished) were also included in the analysis. Details of the employed sequencing technology and the obtained genome coverage for each genome are listed in Table 1.

To start investigating the phylogeny of R3bv2 strains, we first placed them into a larger phylogenetic context by building a phylogenetic tree including publicly available representatives of

TABLE 1. Genome sequencing data

Strain	SRA accession numbers	Average read length (bp)	Number of reads	Genome coverage depth
23-10BR ^a	SRX702422	433.59 ^b	271,396	20×
NCPPB282 ^a	SRX703654	431.73 ^b	290,941	22×
POPS2 ^a	SRX703652	422.94 ^b	230,546	16×
Rs2	SRX703653	427.76 ^b	277,564	23×
CFBP4808	SRX701225	2 × 70 ^c	5,065,895 ^d	102×
CFBP4578	SRX701307	2 × 70 ^c	755,467 ^d	13×
CFBP3927	SRX701324	2 × 70 ^c	1,511,314 ^d	24×
CFBP3884	SRX701786	2 × 70 ^c	2,711,618 ^d	45×
CFBP3873	SRX701787	2 × 70 ^c	4,035,125 ^d	84×
JT516	SRX701788	2 × 70 ^c	3,214,532 ^d	54×
CFBP3858	SRX701790	2 × 70 ^c	9,365,297 ^d	140×
LNVP28.23	SRX701791	2 × 70 ^c	6,619,254 ^d	108×
KZR-5	SRX701792	2 × 70 ^c	4,111,225 ^d	81×
PA1	SRX701793	2 × 70 ^c	4,145,998 ^d	85×

^a Genome assemblies have been deposited in GenBank under accession numbers JQOI000000000.1, JQSH000000000.1, and JQSI000000000.1.

^b Genomes of strains 23-10BR, NCPPB282, POPS2, and Rs2 were sequenced by 454.

^c Genomes of these strains were sequenced by Illumina HiSeq, generating paired reads.

^d These numbers refer to the numbers of read-pairs (rather than individual reads).

the entire *R. solanacearum* species complex. The genome of strain Po82 isolated from potato but also pathogenic to other Solanaceae and to banana (Xu et al. 2011) was used as reference since Po82 is the strain most closely related to R3bv2 whose genome sequence is completely assembled. A total of 1,108,724 bp of this genome were covered at least five times by sequencing reads with consistent base calls (or assembled genome sequences) from each of the sequenced genomes. Therefore, the tree (Fig. 1A) was built based on this 20% of the genome for which the genotype could be unambiguously determined for all sequenced isolates. As expected, all newly sequenced R3bv2 isolates cluster closely together in a clade shared with previously sequenced known members of phylotype IIB: the R3bv2 isolates UW551 (Gabriel et al. 2006), IPO1609 (Guidot et al. 2009), and NCPPB909, strain Molk2 (causative agent of banana Moko disease), strain P673 isolated from the ornamental plant *Epipremnum aureum* (pothos) and also able to infect tomato and, to a lesser degree, potato at 18°C (Bocsanczy et al. 2014) and the reference strain Po82 (Xu et al. 2011).

Next, we built a tree of only R3bv2 genomes. For this tree, 2,006,873 positions were unambiguous (37% of the total Po82 genome), providing greater phylogenetic resolution than the previous tree. Moreover, SNPs were distributed quite evenly over both the chromosome and the megaplasmid (17,852 SNPs within the main chromosome and 7,136 SNPs within the megaplasmid). Importantly, the tree built on all SNPs (Fig. 1B) and the trees built using SNPs from only the main chromosome had the same branching order and the tree built only on SNPs from the megaplasmid had a very similar topology (Supplementary Fig. 1A and B), which is in agreement with an earlier study that showed that the two replicons coevolve (Guidot et al. 2007).

The South American isolates 23-10BR, NCPPB282, and Rs2 (isolated in Brazil, Colombia, and Bolivia, respectively), and the Dutch isolate CFBP3858 are on relatively long branches and are distinct from all other R3bv2 isolates, which instead form a tight cluster of highly similar isolates (see Supplementary Table 1 for the exact number of pairwise SNPs for all R3bv2 genomes). Within this cluster of 13 isolates, 11 isolates collected in Europe, Africa, and Asia only differ at one to seven SNP loci from each other although they were collected over a time period of 50 years. We thus used the two most similar isolates collected the furthest apart in time (CFBP4808 isolated in 1954 and PA1 isolated in 2004) to infer a minimal yearly mutation rate by dividing the number of mutations that distinguished these two genomes by the difference in years of

isolation divided by two (since both strains mutated subsequent to diverging from their most recent common ancestor, MRCA). Because only four mutations distinguished the two isolates over the entire length of the 2,006,873 bp that were compared, the inferred yearly mutation rate was 1.99×10^{-8} mutations $\text{bp}^{-1} \text{ year}^{-1}$. Applying this rate to the two most divergent R3bv2 strains, 23-10BR and CFBP3858, which are separated by 5,370 SNPs, the MRCA of R3bv2 was inferred to have possibly existed as long ago as 67,125 years ago.

Inferred phylogeography of R3bv2. Within the tree of R3bv2 isolates (Fig. 1B), all South American isolates (23-10BR, Rs2, and NCPPB282) are on three of the four most basal branches. Therefore, these isolates can be considered more ancestral than all other R3bv2 isolates, which is typical of strains at the geographic origin of a pathogen (Goss et al. 2014). Moreover, the relatively large genetic distance between the South American isolates is suggestive of relatively high diversity of R3bv2 in South America, also expected for the geographic origin of a pathogen (Grünwald and Flier 2005; Stukenbrock and McDonald 2008). Therefore, the genome sequences analyzed here are in agreement with the previously assumed South American origin of R3bv2 (Denny 2006) and migration of R3bv2 out of South America to Africa, Europe, and Asia (Fig. 2).

The next objective of this study was to infer the direction of dissemination between Europe, Africa, and Asia. Since most strains isolated in Europe, Africa, and Asia were highly similar to each other and formed a single clade with bootstrap support of 100% (Fig. 1B), a single ancestor of these isolates may have been exported from South America some time before 1954 (the year of isolation of the earliest strain, CFBP4808) and then exchanged between Europe, Asia, and Africa. Interestingly, a small number of SNPs within this clade are informative and can provide some indication about the possible order of events during these exchanges (Fig. 2). First, CFBP4808 (isolated in Israel in 1954) is located on the most basal branch of the clade because at one SNP locus it retains the ancestral (Po82-like) allele whereas all other isolates in the same clade carry the derived allele. CFBP4808 may thus represent a very early derivative of the R3bv2 strain exported from South America and from which most other European, African, and Asian isolates are derived from. Second, the two isolates from Reunion Island (LNPV28.23 and JT516) share alleles at one SNP locus with the Egyptian isolate CFBP4578, possibly suggesting a Mediterranean origin of the Reunion outbreak. Third and finally, the Dutch isolate

TABLE 2. *Ralstonia solanacearum* race 3 biovar 2 isolates used in this study with references to papers in which strains were described previously

Strain name	Sequevar	Year of isolation	Geographic location of isolation	Pathogenicity ^a	Reference
IPO1609 ^b	1	1995	Netherlands	No	(Guidot et al. 2009)
UW551 ^b	1	2003	Kenya/Wisconsin	Yes	(Gabriel et al. 2006)
NCPPB909 ^b	1	1961	Egypt	Yes	(Smith et al. 1995)
23-10BR ^c	27	1981	Brazil	Unknown	This work
NCPPB282 ^c	2	1950	Colombia	Yes	This work
POPS2 ^c	1	1980	China	Unknown	This work
Rs2	1	2008	Bolivia	Yes	This work
CFBP4808 ^d	1	1954	Israel	No	(Cellier and Prior 2010)
CFBP4578 ^d	1	1966	Egypt	Yes	(Cellier and Prior 2010)
CFBP3927 ^d	1	1968	Greece	No	(Cellier and Prior 2010)
CFBP3884 ^d	1	1984	Sweden	No	(Cellier and Prior 2010)
CFBP3873 ^d	1	1992	Belgium	No	(Cellier and Prior 2010)
JT516 ^d	1	1993	Reunion	Yes	(Cellier and Prior 2010)
CFBP3858 ^d	1	1995	Netherlands	Yes	(Cellier and Prior 2010)
LNPV28.23 ^d	1	2004	Reunion	Yes	(Cellier and Prior 2010)
KZR-5 ^e	1	2004	Netherlands	Yes	(Stevens and van Elsas 2010)
PA1 ^e	1	2004	Netherlands	Yes	(Stevens and van Elsas 2010)

^a Pathogenicity results are from reported references, besides the two NCPPB isolates which are declared pathogenic by the National Collection of Plant Pathogenic Bacteria (NCPPB) and Rs2, which was determined to be pathogenic on potato and tomato by coauthor J. Castillo who collected Rs2 (data not shown).

^b Genome sequences were not obtained as part of this study but were publicly available.

^c DNA provided by C. Allen (University of Wisconsin, Madison, USA).

^d DNA provided by P. Prior (CIRAD, La Reunion, France).

^e DNA provided by P. Stevens (University of Groningen, The Netherlands).

Fig. 1. A, Maximum likelihood tree of all newly sequenced and a selection of published *Ralstonia solanacearum* genomes. The tree is based on single nucleotide polymorphisms (SNPs) identified by aligning reads against the genome of *R. solanacearum* Po82. The tree is midpoint rooted. Bootstrap values based on 1,200 nonparametric replicates are indicated at nodes. Branch lengths are proportional. The phylotype designations are indicated on appropriate branches. Phylotype IIB is highlighted using a higher weight. **B**, Maximum likelihood tree of *R. solanacearum* race 3 biovar 2 isolates based on SNPs when aligning reads against the genome of *R. solanacearum* Po82. Bootstrap values based on 1,200 nonparametric replicates are indicated at nodes. The tree is rooted on the genome of Po82. Because branch lengths are very different from each other, a cladogram is shown and the number of SNPs is indicated underneath each branch. The clade containing most European/African/Asian isolates is highlighted using a higher weight.

IPO1609 and the Swedish isolate CFBP3884 share alleles at two SNP loci and may thus be both derived from a recent ancestor imported into Northern Europe in the 1970s (Olsson 1976).

The European isolate IPO1609 and the Kenyan isolate UW551 (imported into the United States on geranium) are the only two isolates within the major European, African, and Asian clade having more than 100 private single-nucleotide variants. However, there is 100% bootstrap support for their placement within the same clade, strongly suggesting that they share the same common ancestor exported out of South America with the other isolates in the clade. Possible explanations for this high number of variants private to these two isolates are discussed below.

The Dutch isolate CFBP3858 differs at more than 4,000 SNP loci from all other European isolates and lies clearly outside of the main European, African, and Asian clade (Fig. 1B). Its ancestor was thus most likely imported into Europe independently from the ancestor of all other analyzed European isolates.

R3bv2 core effector repertoire. Repertoires of type III-secreted effectors are known to be important determinants of the host range of plant pathogens including *R. solanacearum* (Peeters et al. 2013). We therefore sought to define the core effector repertoire of the R3bv2 group. To do this, we aligned the R3bv2 genomes sequenced here (Table 1) and the previously sequenced genomes of IPO1609 and NCPPB909 against 58 *R. solanacearum* effectors cataloged in the R3bv2 genome UW551 by Peeters and colleagues (Peeters et al. 2013). Results are summarized in Table 3 and detailed coverage information for each effector in each genome is available in Supplementary Table 2. We considered an effector to

be present in a genome when sequencing reads from that genome covered more than 80% of the length of the UW551 ortholog. Using this cut-off, 31 effectors were found to be present in all analyzed R3bv2 genomes and not to have any frame shift or early stop codon in the gene regions covered by reads. We thus defined these 31 effectors as R3bv2 core effector repertoire. However, five of the 17 genome-sequenced isolates were reported in the literature to be nonpathogenic and two have not been tested for pathogenicity (Table 2). Therefore, we cannot exclude that five additional effector genes (*ripAV*, *ripJ*, *ripBK*, *ripY*, and *ripZ*), which are missing only from these isolates, may also be important for causing disease.

It is important to caution that we may have slightly underestimated the core effector repertoire because some effectors may have less than 80% coverage due to sequence divergence or biased sequencing coverage of the genome and not because of actual absence of genes. On the other hand, some of the effector genes with sequencing coverage between 80 and 100% may contain stop codons or frame-shift in the regions of the effectors not covered by reads and therefore not detected. However, only two (out of 915) of the effector sequences with over 80% coverage had any detected frame-shift mutation or early stop codons in any of the 17 analyzed genomes: *ripAX1*, which is truncated in NCPPB282, and *ripS3*, which is truncated in Rs2. We therefore have confidence that most of the 31 core effector genes are truly present and functional in the corresponding genomes.

The R3bv2 core effectors were then compared against the effector repertoires predicted in non-R3bv2 sequenced *R. solanacearum* genomes at the RalstoT3E database (Peeters et al. 2013) revealing that 11 of the 31 R3bv2 core effectors represent core effectors of the

Fig. 2. Inferred routes of dissemination of *Ralstonia solanacearum* race 3 biovar 2 strains. Isolate details are listed in Table 2. European isolates are not placed exactly over their country of isolation because of space limitations. Map from Luke Roberts (<http://lukeroberts.deviantart.com/>) released under a Creative Commons Attribution-Noncommercial 3.0 license.

TABLE 3. Summary of *Ralstonia solanacearum* race 3 biovar 2 (R3bv2) core effector analysis^a

R3bv2 core effectors	R3bv2 noncore effectors
<u>ripAB</u> , <u>ripAC</u> , <u>ripAD</u> , <u>ripAE</u> , <u>ripAI</u> , <u>ripAN</u> , <u>ripAP</u> , <u>ripAR</u> , <u>ripAY</u> , <u>ripBI</u> , <u>ripB</u> , <u>ripCI</u> , <u>ripE1</u> , <u>ripF1</u> , <u>ripF2</u> , <u>ripG2</u> , <u>ripG3</u> , <u>ripG4</u> , <u>ripG5</u> , <u>ripG6</u> , <u>ripG7</u> , <u>ripH1</u> , <u>ripH2</u> , <u>ripI</u> , <u>ripN</u> , <u>ripS1</u> , <u>ripU</u> , <u>ripV1</u> , <u>ripV2</u> , <u>ripW</u> , <u>ripX</u>	<i>ripA2</i> , <i>ripAA</i> , <i>ripAJ</i> , <i>ripAL</i> , <i>ripAM</i> , <i>ripAO</i> , <i>ripAQ</i> , <i>ripAS</i> , <i>ripAT</i> , <i>ripAV</i> , <i>ripAX1</i> , <i>ripBH</i> , <i>ripD</i> , <i>ripE2</i> , <i>ripJ</i> , <i>ripM</i> , <i>ripO1</i> , <i>ripQ</i> , <i>ripR</i> , <i>ripS7</i> , <i>ripT</i> , <i>ripY</i> , <i>ripA4</i> , <i>ripH3</i> , <i>ripS3</i> , <i>ripZ</i>

^a Core effectors of the *R. solanacearum* species complex are underlined.

entire *R. solanacearum* species complex: *ripE1*, *ripF1*, *ripG6*, *ripH1*, *ripW*, *ripAB*, *ripAC*, *ripAI*, *ripAN*, *ripAR*, and *ripAY*. Interestingly, no effector was present exclusively in R3bv2.

Screening for resistance genes against R3bv2 effectors.

Agrobacterium-mediated transient expression of T3E proteins in the leaves of plants carrying corresponding resistance gene results in the induction of defense responses usually associated with programmed cell death known also as HR (Bent and Mackey 2007). Therefore, 27 R3bv2 effector genes, including 17 of the R3bv2 core effectors, were expressed in two eggplant genotypes, 20 tomato genotypes, 5 pepper genotypes, and 22 lettuce genotypes to determine the possible presence of resistance genes in these plant genotypes. Both host and nonhost plants for R3bv2 were used in this screen because *R* genes capable of recognizing pathogen effectors are expected to underpin both race-level resistance and nonhost resistance. We used a panel of plant genotypes selected to maximize diversity with regards to resistance to different pathogens (Wroblewski et al. 2009), and correspondingly maximize the diversity of underlying *R* genes. An additional five genes that had been previously predicted to encode effectors but were not identified as such by Peeters and colleagues (Peeters et al. 2013) were also included: UCD163 (a predicted membrane-bend lytic murein transglycosylase B); UCD190 (a predicted PopC-like putative type III effector similar to *RipAC*); UCD191 (HpaP, a putative type III secretion substrate specificity switch protein which was recently found not to be secreted nor translocated (Lohou et al. 2014)); UCD197 (a putative type III effector); UCD203 (a conserved protein of unknown function). All effector genes had been previously cloned from an R3bv2 strain from Bolivia (Wroblewski et al. 2009). While the results for 10 of the analyzed effectors for tomato, pepper, *N. benthamiana*, and lettuce were already reported (Wroblewski et al. 2009) and have been accessible in the CHARGE database (http://charge.ucdavis.edu/charge_db/chargedb_index.php), these results were analyzed here for the first time in the context of the R3bv2 core effectorome.

The overall severity of the cell death elicited by transient expression of the effectors across many replicates was demarcated

into the broad categories of “Strong,” “Intermediate,” and “None” (Figure 3 provides examples). Specifically, 15 of the screened effectors elicited an HR-like plant response on at least one genotype tested suggesting the presence of underlying effector-detecting *R* genes (Table 4, see detailed results in Supplementary Table 3, which also lists all plant genotypes tested). Eight of these fifteen effectors were core R3bv2 effectors: *ripB1*, *ripE1*, *ripG6*, *ripH2*, *ripX*, *ripV1*, *ripV2*, and *ripAB*. Therefore, the genotypes on which these effectors elicited cell death potentially harbor *R* genes capable of possibly providing durable resistance to R3bv2.

Of special interest, six effector genes, including five core effectors (*ripE1*, *ripH2*, *ripV1*, *ripV2*, and *ripX*), elicited substantially stronger cell death in eggplant genotype MM853 than in genotype MM738 (Table 4). Because MM853 is resistant to R3bv2, whereas MM738 is susceptible (Lebeau et al. 2011), the eggplant genotype MM853 possibly has *R* genes that recognize one or more of these core effectors.

DISCUSSION

Because of its impact on world-wide potato production (Elphinstone 2005) and because of its status as Select Agent in the United States (CDC and APHIS), R3bv2 has been the focus of many studies. For example, the phylogenetic relationship between R3bv2 and other *R. solanacearum* strains has been determined (Castillo and Greenberg 2006; Cellier et al. 2012; Wicker et al. 2012), the genomes of a small number of R3bv2 strains have been sequenced (Gabriel et al. 2006; Guidot et al. 2009), and these genome sequences allowed the prediction of virulence genes (Peeters et al. 2013). However, the geographic origin and intercontinental routes of dissemination of R3bv2 are not well known (Cellier et al. 2012) and, even more importantly, the identification of resistance genes effective against R3bv2 is still in its infancy (Carmeille et al. 2006; Lebeau et al. 2013; Siri et al. 2009). Here we present progress in these two areas.

An estimated timescale for R3bv2 emergence and diversification.

While yearly mutation rates and divergence times for human pathogen species have recently been inferred, for example (Cui

Fig. 3. Examples of the broad categories of plant responses to effectors used for classification of cell death elicited by *Agrobacterium*-mediated transient expression and reported in Table 4.

et al. 2013; Morelli et al. 2010a; Nübel et al. 2010), our knowledge of divergence times for bacterial crop pathogens is preliminary at best (Vinatzer et al. 2014) making it impossible to determine the time scale of bacterial crop pathogen evolution.

Mutation rates can only be inferred reliably if there is a statistically significant correlation between years of isolation and the genetic distance from the MRCA (Nübel et al. 2010), which requires a relatively large number of closely related isolates being collected over many years. Only then it is likely that the isolates actually evolved from each other and accumulated mutations during the time span of collection.

Surprisingly, 11 closely related R3bv2 isolates collected over a time span of 50 years did not accumulate more than seven mutations (in the 36% of the genome that could be compared with high confidence) and isolates collected in similar years were as different from each other as isolates collected 50 years apart (Table 2). Therefore, a mutation rate could not be inferred based on the accumulation of mutations over time. Instead, a minimal mutation rate based on the most similar isolates collected 50 years apart was inferred and found to be as low as 1.99×10^{-8} bp⁻¹ year⁻¹. This is lower than the mutation rates recently calculated for human pathogens (mostly in the range from 3×10^{-5} to 3×10^{-7} ; (Morelli et al. 2010a) and references therein). The reason could be that *R. solanacearum* bacteria may grow, and thus mutate, very slowly in soil and surface water populations, which often constitute the inoculum sources of outbreaks (Álvarez et al. 2008; van Elsas et al. 2000) and most of the mutations that get fixed during fast pathogen replication during plant infection may constitute a sink rather than a source according to the model proposed by Sokurenko et al. (2006).

However, at this point we cannot exclude the possibility that we underestimated the mutation rate because of ascertainment bias

arising from considering only the 36% of the genome for which we could be confident of the genotype in all our sequenced genomes. By definition, this 36% that is represented in all the sequenced genomes represents part of the core genome rather than the variable genome. Examining this portion of the genome has the advantage that it is less likely to have undergone horizontal transfer and thus reflects the 'true' phylogeny in addition to the advantage of high confidence in the nucleotide sequence in all isolates. However, if there is an over-representation of vertically inherited SNPs in the noncore (variable) portion of the genome, then conversely this implies SNPs are under-represented in the core-genome, including the 36% on which we based our phylogenetic analysis. The high number of private variants in the UW551 and IPO1609 genomes are likely due to sequencing errors in these two draft-quality genomes that were obtained previously by relatively low coverage Sanger sequencing (Gabriel et al. 2006; Guidot et al. 2009). This is in contrast to the situation for the genomes that were sequenced to greater depth by next-generation sequencing methods and for which accuracy benefited from consensus of multiple overlapping independent sequencing reads. Alternatively, UW551 and IPO1609 may be derived from outbreaks during which rates of bacterial replication were high.

Estimated frequency and direction of R3bv2 dissemination.

The phylogeographic studies undertaken with the plague pathogen *Yersinia pestis* are an impressive example of how whole genome sequencing of bacteria allows reconstruction of historic routes of pathogen dissemination (Morelli et al. 2010b). While the number of analyzed R3bv2 isolates in our study is much lower than the number of *Y. pestis* isolates used in the above study, we can still make some conclusions by combining the microbial forensics evidence derived from our data with previous results about the origin and dissemination of R3bv2.

TABLE 4. Elicitation of cell death by transient expression of *Ralstonia solanacearum* race 3 biovar 2 (R3bv2) effectors on eggplant (MM738 and MM853), *Nicotiana benthamiana*, tomato (20 genotypes), pepper (5 genotypes), and lettuce (22 genotypes)

Effector (UCD-ID) ^a	Effector name ^b	Lettuce ^c	<i>Nicotiana</i> ^c	Pepper ^c	Tomato ^c	MM853	MM738
163	Not a Rip ^d	None	None	None	None	None	None
164	RipD	Inter.	None	None	None	None	None
166	RipAA	Inter.	Strong	Strong	Inter.	Inter.	Inter.
167	RipW	None	None	None	None	None	None
168	RipV2	None	None	None	Inter.	Inter.	None
169	RipAY	None	None	None	None	None	None
170	RipOI	None	None	None	None	None	None
171	RipAQ	None	None	None	None	None	None
172	RipG5	None	None	None	None	None	None
173	RipAB	None	None	None	Inter.	None	None
175	RipG7	None	None	None	None	None	None
176	RipH2	Strong	None	None	Inter.	Strong	None
177	RipAJ	None	None	None	None	Inter.	None
178	RipG4	None	None	None	None	None	None
179	RipE2	None	None	None	None	None	None
181	RipF2	None	None	None	None	None	None
182	RipAT	Strong	None	Strong	Strong	Strong	Strong
184	RipG6	None	None	None	Inter.	None	None
185	RipAP	None	None	None	None	None	None
187	RipAV	Strong	None	Strong	Strong	Strong	Strong
188	RipV1	Strong	None	Inter.	None	Inter.	None
190	not a Rip ^d	None	None	None	None	None	None
191	not a Rip ^d	None	None	None	None	None	None
192	RipAO	None	None	None	None	None	None
194	RipBI	Inter.	None	Inter.	Strong	Inter.	Strong
195	RipE1	None	None	None	Inter.	Inter.	None
196	RipAS	None	None	None	None	None	None
197	not a Rip ^d	None	None	None	None	None	None
198	RipG3	None	None	None	None	None	None
199	RipN	None	None	None	None	None	None
202	RipX	Inter.	None	None	None	Inter.	None
203	not a Rip ^d	None	Inter.	None	Inter.	None	None

^a UCD designations are from the University of California-Davis CHARGE database (http://charge.ucdavis.edu/charge_db/chargedb_index.php).

^b Bolded effectors are part of the R3bv2 core effector repertoire.

^c Intermediate and strong cell death responses for tomato, pepper, and lettuce indicate that cell death was observed in at least one of the tested genotypes.

^d Not an experimentally confirmed effector.

R3bv2 is generally assumed to have first emerged in the Andes of South America (Denny 2006). In particular, French (1979) found biovar 2 in virgin soil in the Amazon basin and he suggests that potatoes brought from the Peruvian highlands and cultivated in these soils centuries ago got infected there and that infected potatoes were brought back later to the Peruvian Highlands leading to the emergence of brown rot. From South America, R3bv2 is assumed to have been exported with potatoes to the rest of the world and molecular data in agreement with this hypothesis were obtained previously by pulsed field gel electrophoresis and rep-PCR (Smith et al. 1995), microarray analysis (Cellier et al. 2012), and multilocus sequence analysis (Wicker et al. 2012). However, these previous studies were either based on markers that do not allow determination of ancestral versus derived relationships (Cellier et al. 2012; Smith et al. 1995) or were based on a small number of loci (Wicker et al. 2012). Here instead we analyzed over one third of the entire length of R3bv2 genomes using SNPs, which are informative of ancestral versus derived relationships. This approach identified much higher genetic diversity among genomes of South American origin compared with genomes of European origin and the phylogenetic tree in Figure 1B clearly shows that all but one of the isolates of European, African, and Asian origin are derived from the more ancestral South American isolates. This is so far the strongest evidence of a South American origin of R3bv2. Moreover, our estimated time since the MRCA of R3bv2 (over 50,000 years ago) clearly places the origin of R3bv2 before the domestication of potato and suggests that the ancestor of R3bv2 was a pathogen of wild potatoes and/or other Solanaceae in South America.

In regard to the phylogeography of R3bv2 outside of South America, Cellier et al. (2012) had found that R3bv2 isolates were clustered geographically based on phylogeny and population structure. One R3bv2 group was found to be associated with African and the Indian Ocean region, one group with the Mediterranean regions, and one group with Northern Europe. Although the northern European isolates IPO1609 (Netherlands) and CFBP3884 (Sweden) cluster together also based on SNPs, for the rest our data do not support geographic differentiation of populations. In particular, the Dutch isolates Pa1 and KRZ-5 do not share any alleles exclusively with IPO1609 or CFBP3884. Therefore, our data do not support any separate Northern European population. We thus conclude frequent exchange of R3bv2 between regions, which is in agreement with the pulsed field gel electrophoresis and rep-PCR results that also revealed almost identical isolates from Northern Europe, the Mediterranean region, and Africa (Smith et al. 1995).

As for the temporal order of transfers, it is interesting to note that isolate CFBP4808 was collected in the Mediterranean region in 1954 (the earliest isolate outside of South America) and is at the same time also the most ancestral isolate of the main clade outside of South America. This agrees with the fact that R3bv2 caused outbreaks in the Mediterranean region starting in the 1920s and that R3bv2-contaminated potatoes were imported from that region into the rest of Europe, possibly being the source of the outbreaks observed in northern Europe starting in the 1980s (Grousset et al. 1998; Janse 1996). Our data also support a Mediterranean origin of the Reunion Island population. Since the only Chinese and the only Kenyan isolates sequenced here are also nested within the European clade, either a Mediterranean or Northern European origin of the R3bv2 populations in these geographic areas is likely as well (Fig. 2).

The core effector repertoire of R3bv2. Bart and colleagues suggested that presence of an effector across all isolates may indicate that the effector is essential for the pathogen to cause disease and that resistance genes against such pathogen core effectors may provide durable resistance (Bart et al. 2012). While this hypothesis still needs to be empirically tested, it is plausible. We thus determined here the core effector repertoire R3bv2 consisting of 31 effectors and the core effector repertoire of *R. solanacearum* overall consisting of 13 effectors.

The 31 core effectors can now be prioritized to identify corresponding resistance genes for breeding potato, tomato, and pepper cultivars with durable disease resistance. We expect the members of the R3bv2 core effector repertoire to be particularly important to cause disease on the common R3bv2 hosts potato and tomato while the *R. solanacearum* core effector repertoire should consist of the basic tool set used by *R. solanacearum* to cause disease on all its hosts.

The five effectors only missing from either nonpathogenic isolates or from the isolate 23-10BR of unknown pathogenicity remain for now of uncertain importance for pathogenicity because we cannot exclude that loss of the missing effectors contributed to loss of pathogenicity. However, at least in the case of isolate IPO1609, the missing effector genes *ripJ* and *ripAV* were not found to be the cause of the observed loss of pathogenicity (González et al. 2011). It is also important to point out that even the nonpathogenic isolates were isolated from diseased plants and that their loss of pathogenicity is probably not due to adaptation to a nonpathogenic life style. These isolates may have lost pathogenicity during plant infection just before isolation or even during culturing after isolation. In fact, the latter is frequently observed in *R. solanacearum* (Kelman 1954).

Several plant genotypes contain putative resistance genes against core R3bv2 effectors. Unfortunately, most *R. solanacearum* effectors for which sources of resistance had been previously identified are missing from R3bv2, for example *ripAA* and *ripP1* (Poueymiro et al. 2009), *ripP2* (Deslandes et al. 2003), and *rip36* alias *ripAX2* (Nahar et al. 2014). Therefore, the R3bv2 core effectors identified here need to be given priority in identifying new sources of resistance for breeding/engineering resistance to R3bv2.

Toward this goal, a collection of cloned R3bv2 effectors was previously used in *Agrobacterium*-mediated expression to identify potential sources of resistance among tomato, pepper, and lettuce accessions and *N. benthamiana* (Wroblewski et al. 2009). We extended the search here to two eggplant genotypes: MM853 found to be resistant to most *R. solanacearum* strains, including R3bv2 strains, and the susceptible MM738 genotype (Lebeau et al. 2011).

Combining all results, an interesting picture arose. First of all, none of the five tested *R. solanacearum* core effectors triggered strong cell death in any of the tested genotypes: two *R. solanacearum* core effectors did not trigger any HR-like response in any genotype (*ripAY* and *ripW*), two triggered an intermediate response in only one plant genotype each (*ripAB* in the tomato accession M82 and *ripG6* in the tomato accession V121), and *ripE1* triggered an intermediate response in two genotypes (the resistant eggplant genotype MM853 and the tomato genotype VF36). This almost complete absence of HR-like responses to *R. solanacearum* core effectors suggests that these effectors have been maintained in all *R. solanacearum* strains because plants have not evolved effective resistance against them.

Six of the twelve tested R3bv2 core effectors did not trigger any response in any host genotype and two triggered only intermediate responses (*ripV2* and *ripX*). However, *ripH2*, *ripAT*, *ripV1*, and *ripBI* triggered intermediate or even strong HR-like responses in multiple genotypes of several plant species, revealing that promising sources of resistance may be available for these R3bv2 core effectors.

Particularly promising is the result that the R3bv2 core effector gene *ripH2* triggered a strong HR-like cell death response in the resistant genotype MM853 but not in the susceptible genotype MM738. *ripH2* is a member of the HLK effector family and *ripH1*-2-3 alleles from the phylotype I strain OE1-1 were demonstrated to be functionally redundant and mildly involved in virulence on tomato and eggplant (Chen et al. 2014). *RipH2* is thus an excellent candidate effector that is possibly recognized by a cognate *R* gene in genotype MM853, which could be mapped in a cross with the susceptible genotype MM738, cloned, and expressed in tomato and/or potato to confer resistance to R3bv2 strains. The multiple lettuce and tomato accessions that also reacted with a strong cell death response to the R3bv2 core effector genes *ripH2*, *ripV1*, and *ripBI* may be very promising resistance sources as well.

Interestingly, the two effector genes that triggered strong cell death responses in the largest number of plant accessions are the noncore effectors *ripAT* and *ripAV*. One possible explanation is that these effectors are in fact missing from some R3bv2 isolates because ancestors of these isolates were exposed to plants carrying cognate resistance genes and loss of these effectors thus increased fitness on such plants.

In summary, we have found several promising resistance sources for R3bv2, the resistance genes of which could be mapped and cloned. Importantly, *Agrobacterium*-mediated transient expression could be used for mapping the underlying resistance genes in segregating progenies. This would be very useful since employing actual R3bv2 isolates for this purpose would be very difficult in the United States due to the regulatory restrictions and the risk of accidental spread of R3bv2.

In conclusion, we have shown here how genome sequencing of multiple isolates of a Select Agent and transient expression assays of its effector genes can give new insight into pathogen evolution and dissemination and can constitute a tool for identifying new promising sources of disease resistance. Importantly, this research was done without having to actually do any research with the Select Agent R3bv2 itself, avoiding regulatory hurdles and minimizing the risk of accidental pathogen spread.

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