Expression of *βhpmeh* gene in transgenic events of the potato variety Desiree increases resistance to bacterial wilt caused by *Ralstonia solanacearum*

Myriam, Izarra[1], Liliam Gutarra[1], Elizabeth Fernández[1], Eva Huamán, Marc Ghislain[1], Jan Kreuze[1*]

[1] International Potato Center (CIP), 1895 La Molina Avenue, Lima 15024, Peru.

*Corresponding Author: Jan Kreuze, j.kreuze@cgiar.org

Abstract

Bacterial wilt, caused by *the Ralstonia solanacearum* species complex (RSSC), severely affects many important crops and significantly limits potato production worldwide. RSSC bacteria can regulate the expression of their virulence factors, including extracellular polysaccharides and endoglucanases, through quorum sensing, which enables bacteria to sense their population density through signaling molecules and collectively switch on virulence factors when a threshold density is reached. 3-hydroxy palmitic acid methyl ester is the main quorum-sensing molecule in RSSC that can be hydrolyzed by β-hydroxypalmytate methyl ester hydrolase (βHPMEH). In this study, we evaluated the ability of *βhpmeh* transgenic plants of the potato variety Desiree to reduce bacterial wilt symptoms after artificial inoculation with two RSSC strains virulent on potato under controlled conditions and relate it to the expression of the *βhpmeh* gene. For each transgenic event, we analyzed the phenotypic response (wilt incidence, latent infection, and area under the disease progress curve). Real-time quantitative PCR was performed to determine the relative expression levels of *βhpmeh* in transgenic events. Several transgenic events were identified with reduced susceptibility to bacterial wilt compared to Desiree, and a resistance level similar or higher to that of potato variety Cruza 148, which is the most resistant variety available, and which was positively correlated with *βhpmeh* expression.

Keywords: *Ralstonia solanacearum* species complex, bacterial wilt, potato, quorum quenching.

Introduction

The *Ralstonia solanacearum* species complex (RSSC; E.F. Smith) is the second most important group of bacterial plant pathogens globally and the causal agent responsible for bacterial wilt in a broad range of crop plants such as potato, banana, cucurbits, eggplant, Eucalyptus, ginger, groundnut, mulberry, tobacco, tomato, and many ornamental plants (Mansfield *et al.*, 2012; Charkowski *et al.*, 2020).

RSSC pathogens were classified into five races and six biovars based on the host range of the pathogen and the ability of the pathogen to degrade specific carbohydrates (Denny & Hayward, 2001; Fegan & Prior, 2005; Ghorai *et al*., 2022). Genetic diversity distributes RSSC into four phylotypes (I, II, III, and IV) based on sequence analysis of the conserved endoglucanase (*egl*), DNA repair (*mutS*), hypersensitive response and pathogenesis B (*hrpB*), and intergenic spacer region (ITS) (Fegan & Prior, 2005). Phylotype I encompasses strains originating from Asia, while phylotype II is composed of two sub-clusters (IIA and IIB) found in the Americas, as documented by Fegan & Prior (2005). Phylotypes III and IV consist of strains from Africa and

Indonesia, respectively, as described by Paudel *et al.* (2020). RSSC has recently been reclassified into three distinct species, with phylotypes I and III representing *R. pseudosolanacearum*, phylotype II representing *R. solanacearum,* and phylotype IV representing *R. syzygii* (Safni *et al*., 2014). Each phylotype is subdivided into sequevars based on the *egl* gene (Fegan & Prior, 2005; Chesneau *et al.*, 2018; Ghorai *et al.*, 2022). Potato bacterial wilt (BW) is mainly caused by *R. solanacearum* race3/biovar 2A, which now belongs to phylotype IIB and sequevars 1 and 2 (Hayward, 1994).

For potatoes grown in the tropics, BW together with viruses form the most important constraints to production after late blight. Although moderately resistant varieties and potato wild species with various levels of quantitative resistance are known, to date, no immunity or complete resistance to tuber infection has been identified (Ferreira *et al.*, 2017). Characteristic symptoms of BW include wilting, stunting, and yellowing of the plant (Champoiseau *et al.*, 2009).

In potato, *Ralstonia solanacearum* enters primarily through wounds and natural root openings caused by soil friction, nematodes, or emerging roots (CABI, 2023). Initially, it colonizes the root cortex, where it multiplies in intercellular spaces and utilizes a Type III secretion system (T3SS) to deliver effector proteins that suppress plant defenses, facilitating its invasion of the xylem (Genin, 2010). T3SS activation is primarily induced by unidentified plant cell wall components and influenced by environmental factors such as soil composition, temperature, and moisture (Peeters *et al.*, 2013). Additionally, compounds like oleanolic acid further trigger T3SS activity, promoting bacterial colonization (Wu *et al.*, 2015).

Once inside the vascular system, *R. solanacearum* extensively colonizes the xylem, producing key virulence factors, including exopolysaccharides (EPS) and multiple extracellular cell wall-degrading enzymes (CWDE) (Clough *et al.*, 1997b; Flavier *et al.*, 1997a,b; Schell, 2000). EPS is abundantly secreted, obstructing water flow and causing wilting, while CWDE enhances virulence by facilitating invasion and vascular colonization (Saile *et al.*, 1997). The expression of these virulence factors is tightly regulated in response to environmental stimuli through a network that coordinates pathogenicity determinants (Valls *et al.*, 2006). A quorum sensing mechanisms regulate this process, with maximal expression occurring at high cell densities (Clough *et al.*, 1997b; Flavier *et al.*, 1997a). This density-dependent regulation, known as phenotype conversion, shifts *R. solanacearum* from a motile form, optimized for survival in soil and early plant invasion, to a non-motile EPS-producing phenotype, adapted for vascular colonization and systemic infection (Denny & Hayward, 2001; Hikichi *et al.*, 2007).

The phenotype conversion (*Phc*) regulon of *R. solanacearum* forms the core of the network that regulates genes involved in virulence (Clough *et al.*, 1997a). 3-Hydroxypalmitic acid methyl ester (3-OH PAME) is synthesized by the gene product of *phcB*, an S-adenosyl methionine-dependent methyltransferase that converts 3-hydroxypalmitic acid (3-OH PA) into a methyl ester, and then 3-OH PAME acts as a quorum-sensing signal molecule (i.e., a quormone) for post-transcriptional modulation of PhcA activity (Clough *et al.*, 1997a). PhcA, a LysR-type transcriptional regulator that is strictly controlled by 3-OH PAME, regulates the production of EPS and other virulence factors and displays a hypermotile phenotype (Fig. 1). When the concentration of 3-OH PAME is increases above 5 $nmol \cdot L^{-1}$, PhcA is activated, which increases the production of EPS and CWDE. The *phcA* mutants of this bacterium have decreased levels of EPS and extracellular proteins (EXPs), and are almost avirulent (Brumbley *et al.*, 1993). The *phcB* mutants are almost avirulent, but wild-type levels can be restored by exposure to 3-OH PAME (Flavier *et al.*, 1997a; Hikichi *et al.*, 2017). Thus, 3-OH PAME is essential for the network of reactions that regulates *R. solanacearum* virulence.

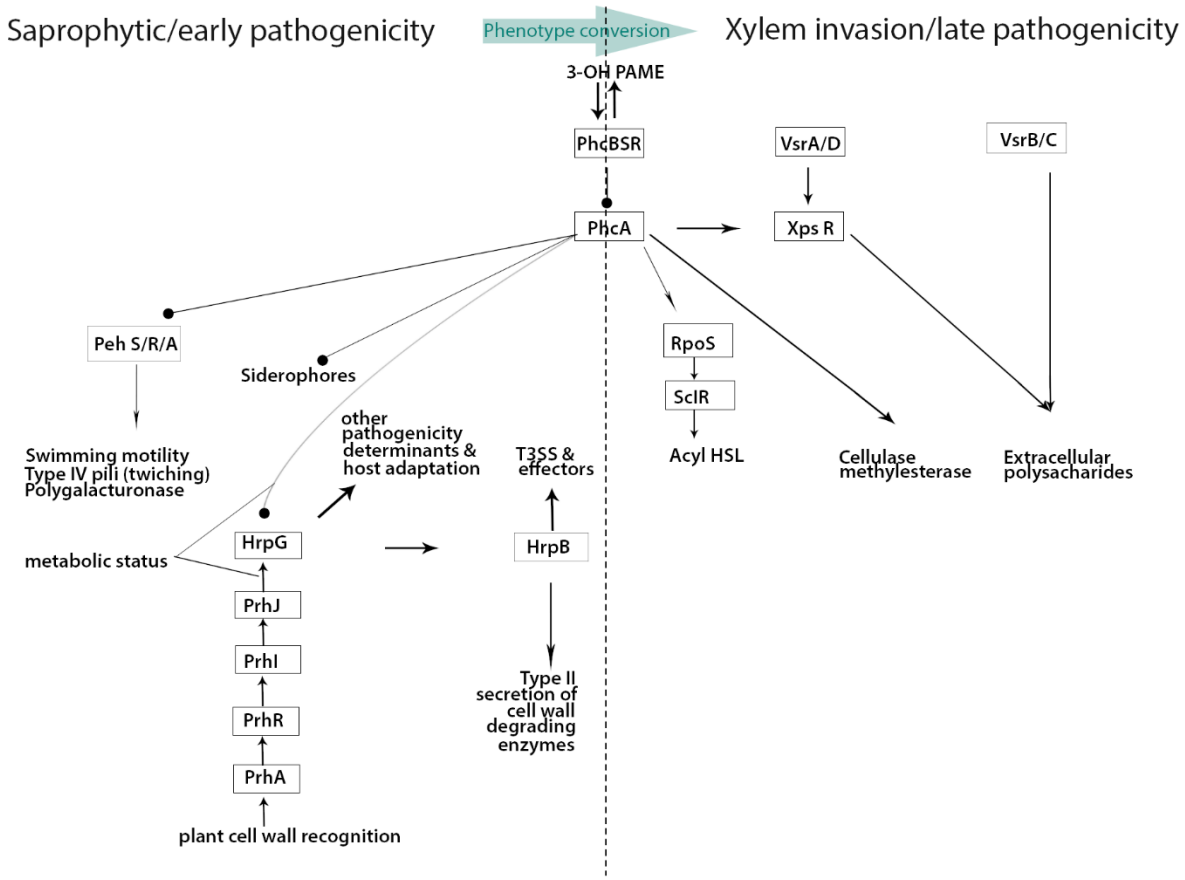

Figure 1. Simplified schematic of virulence regulation in R. solanacearum. PhcA is located at the center of the network and controls phenotypic conversion via quormone 3-OH PAME. Lines with arrowheads indicate stimulation and lines with circular heads indicate inhibition. T3SS: Type III secretion system.

Attempts to control the phenotype conversion by acting on the quorum have produced interesting results. Several quormones have been implicated in the regulation of bacterial virulence (Whitehead *et al.*, 2001). Indeed, other bacterial phytopathogens, such as *Agrobacterium tumefaciens, Pantoea stewartia, Erwinia carotovora, Pseudomonas syringae, and Pseudomonas aeruginosa*, exploit quorum-sensing mechanisms to control population-density-dependent attacks to overwhelm the host's defense responses (Whitehead *et al.*, 2001). These pathogens produce quormones in various chemical forms such as N-acyl homoserine lactone (AHL), fatty acid esters, and peptides. The expression of AHL-lactonase, which inactivates AHL by hydrolyzing its lactone bond (Dong *et al.*, 2000), in transformed *E. carotovora* significantly reduces AHL production and decreases the production of enzymes that degrade cell walls and virulence of this bacterium in various plants (Dong *et al.*, 2000). Plants expressing AHL lactonase exhibited significantly improved resistance to *E. carotovora* infection (Dong *et al.*, 2001). Such a 'quorum-quenching' method (Zhang, 2003), a technique for preventing the functioning of the quorum-sensing system, involves quormone-degrading enzymes and allows the use quormones as molecular targets for the control of bacterial pathogens through regulation of their virulence by quormones.

Almost two decades ago, an enzyme, β-hydroxypalmitate methyl ester hydrolase (βHPMEH), was purified from a soil-living bacterium, which exhibited high hydrolyzing activity towards the ester bond of 3-OH PAME and eliminated 3-OH PAME activity, thereby reducing the virulence of *R. solanacearum*. Crude enzyme extracts from transgenic *E. coli inhibited the in vitro* production of *R. solanacearum* EPS, indicating that βHPMEH inhibits the activation of virulence genes (Shinohara *et al.*, 2007).

In this study, we developed transgenic potatoes expressing βHPMEH and tested them for resistance to *Ralstonia solanacearum* under controlled greenhouse conditions. Two modes of expression were used: constitutive and tissue-specific. The 35S promoter of the cauliflower mosaic virus (CaMV) was used to drive strong constitutive expression as documented in many transgenic plants (Amack & Antunes, 2020). For tissue-specific expression, the GRP1.8 promoter, which drives xylem-specific expression of a glycine-rich protein (GRP), was selected. This promoter, originally isolated from bean and characterized in tobacco directs expression in the xylem parenchyma of young roots (Keller & Baumgartner, 1991). GRPs are structural cell wall proteins that contain an apoplastic secretion signal, enabling their secretion into the xylem and subsequent movement with the xylem sap (Lv *et al.*, 2009). Tissue-specific expression of a quorum-quenching system is desirable in this strategy to minimize pleiotropic effects on plant metabolism while targeting key infection sites. Resistance to *R. solanacearum* was assessed through phenotypic and genotypic analyses.

Materials and Methods

Constructs and generation of transgenic events

The gene constructs used in this study (pPAMEH and pGRP1.8-PAMEH) have been described previously (Fernández *et al.*, 2015). They comprise the plant codon-optimized gene of βHPMEH, preceded by the GRP signal peptide for extracellular targeting to the apoplast and a viral translational enhancer signal, under the control of the constitutive 35S or xylem-specific GRP1.8 promoters. Potato transformation was performed following the *Agrobacterium tumefaciens* organogenesis protocol described by Medina-Bolivar et al. (2003) with minor modifications for the variety Desiree. Pathogen-free plants of the potato variety Desiree (accession number CIP800048) were obtained from the potato germplasm collection of the International Potato Center (CIP). Plantlets were cultured in liquid propagation medium (4.3 $g \cdot L^{-1}$ Murashige and Skoog (MS) salts, 0.4 $mg \cdot L^{-1}$ thiamine, 2 m $g \cdot L^{-1}$ glycine, 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$ pyridoxine, 0.1 $mg \cdot L^{-1}$ gibberellic acid, 2% sucrose, and pH 5.6) during 4 weeks in a growth room at 14–16°C, 70% relative humidity, and 16 h photoperiod (2,000 lx). In parallel, *A. tumefaciens* carrying the plasmid pPAMEH or pGRP1.8-PAMEH were cultivated on Luria–Bertani (LB; 1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5, and 2.5 $g \cdot L^{-1}$ of agar) supplemented with 100 $mg \cdot L^{-1}$ kanamycin at 28°C for 48 h. A single bacterial colony was used to inoculate 3 mL LB liquid medium containing 100 $mg \cdot L^{-1}$ kanamycin and 100 $mg \cdot L^{-1}$ rifampicin at 28°C for 20 h in a water bath shaker (250 rpm). Leaves with the petiole (1.5–2 cm) were cut from the top third of the *in vitro* plantlets and co-cultivated with *A. tumefaciens* at a final concentration of 5.9×10^7 $cells \cdot mL^{-1}$ (bacterial concentration was estimated from a standard curve by absorbance at 620 nm) in 25 ml of co-culture medium (4.6 $g \cdot L^{-1}$ of MS salts, 2% sucrose, and 50 µM acetosyringone) at 22°C for 24 h in the dark. The explants were then taken out from liquid medium and briefly dried on sterile filter paper before transferring them to semi-solid regeneration medium (4.6 $g \cdot L^{-1}$ MS salts, 0.02 $mg \cdot L^{-1}$ gibberellic acid, 0.02 $mg \cdot L^{-1}$ naphthalene acetic acid, 2 $mg \cdot L^{-1}$ zeatin riboside, 2% sucrose, pH 5.6 and 4 $g \cdot L^{-1}$ of agar) without antibiotic for one day. Subsequently, the explants were transferred (ten per Petri dish) to a semi-solid regeneration medium containing 100 $mg \cdot L^{-1}$ kanamycin and 200 $mg \cdot L^{-1}$ carbenicillin and transferred onto fresh medium every 2 weeks. After one month, regenerants started to appear from *Agrobacterium-infected* leaf explants, and 6-week old putative transgenic regenerants (only one regenerant per explant) were screened for *nptII* (Km-F/R), *pameh 241* (F/R), *35S-pameh* (F/R), and *GRP1.8-pameh* (F/R) genes as described in Fernández et al. (2015). The regenerants were propagated in test tubes containing semi-solid regeneration medium at 15–18°C under artificial light (16 h light/ 8 h dark). Leaves from the putative transgenic regenerants were confirmed to be transgenic on a highly selective medium: leaves were laid on kanamycin selective callus-inducing medium (4.6 $g \cdot L^{-1}$ MS salts, 20 $g \cdot L^{-1}$ D-mannitol, 0.5 $g \cdot L^{-1}$ 2-(N-morpholino)-ethane sulfonic acid, 0.5 $g \cdot L^{-1}$ polyvinylpyrrolidone (40,000), 200 $mg \cdot L^{-1}$ L-glutamine, 40 $mg \cdot L^{-1}$ adenine, 0.1 $mg \cdot L^{-1}$ naphthalene acetic acid, 0.1 $mg \cdot L^{-1}$ 6-benzylaminopurine, 0.5 $mg \cdot L^{-1}$ nicotinic acid, 0.5 $mg \cdot L^{-1}$ pyridoxine, 2 $mg \cdot L^{-1}$ glycine, 2% sucrose, adjusted to pH 5.8, and 2 $g \cdot L^{-1}$ gelrite). The sterilized medium was supplemented with 1 $mg \cdot L^{-1}$ zeatin riboside, 0.1 $mg \cdot L^{-1}$ naphthalene acetic acid, and 200 $mg \cdot L^{-1}$ kanamycin. Untransformed potato leaves from the variety Desiree were included as controls. After one month, the presence or not of a callus confirmed or not the transgenic nature of the leaf.

Testing for Bacterial wilt resistance

Transgenic events were tested against *R. solanacearum* isolate CIP-204 (Bv2A Race 3, phylotype IIB sequevar 1 of *Ralstonia solanacearum*) under greenhouse conditions in separate batches (referred to as experiments hereafter) using three plants per event and the events showing the least amount of disease and infection were selected for more extensive testing. Selected transgenic events were evaluated with 15 repetitions per event, placed in three separate trays (five plants per tray) and tested either with CIP-204 or CIP-475 (Bv2A Race 3, phylotype IIB sequevar 1) of *R. solanacearum*.

For each experiment, *in vitro* plantlets were transplanted into Jiffy-Strips 7 (Jiffy Products [NB] Ltd, Canada) containing 20 g of Promix Bx substrate: sand mixture) and maintained under greenhouse conditions at 18-20°C. Approximately two weeks later when the plants were 10-15 cm long and had adequate roots, they were transplanted in 3.5 inch-diameter pots containing 85 g of promix Bx substrate (Premier Tech, Canada). 50 mL solution of fertilizer (N–P–K: 6-7-22) was applied at the time of planting. Plants were irrigated daily, except for one day prior to inoculation.

For plant inoculation, bacterial suspensions were prepared by culturing isolates for 48 h at 30°C in modified Kelman's medium (MKM) without 2,3,5 triphenyl tetrazolium chloride (TZC). Bacterial cells were harvested in sterile distilled water, and the bacterial concentration was evaluated by measuring the absorbance at 600 nm (OD_{600}) using a spectrophotometer. The absorbance reading at $OD_{600} = 0.1$ corresponded to a bacterial concentration of 2×10^8 cells·mL^{-1}. Two weeks after transplanting, the plants were inoculated by pouring 40 mL of the bacterial suspension (to obtain a final concentration of 4×10^7 cells·g^{-1} soil) into the pots. Plants were maintained until one month after inoculation, during which temperatures ranged from 24 to 26°C. Non-transgenic plants of the potato varieties Desiree and Cruza 148 were used as the susceptible and moderately resistant controls, respectively. The latter has been found to have a degree of resistance to bacterial wilt (French & Lindo, 1982).

For each transgenic event, wilt incidence was recorded twice a week after the first symptom of wilting began to appear in any of the plants until wilting was complete. Stems of events that exhibited no wilt symptoms were analyzed to detect latent infections. An approximately 5-cm-long piece of stem near the base of the plant was cut from the symptomless plants, disinfected with 70% alcohol, and rinsed with sterile distilled water. The stem fragments were placed in plastic bags, weighed, and crushed using a wooden rolling pin. The samples were homogenized using 3 ml of sterile citrate extraction buffer (0.1 M citric acid, 0.1 M sodium citrate, pH 5.6) per gram of stem tissue. Stem extract was used to assess for the presence of *R. solanacearum* in post-enrichment NCM-ELISA tests (Priou *et al.*, 1999), and positive samples were confirmed by isolating *R. solanacearum* on MKM medium with TZC (French *et al.*, 1995).

Data were subjected to analysis of variance (ANOVA) using the F-test ($p < 0.05$). Differences in the level of resistance between the best events were measured as percentages of wilt, the area under the disease progress curve (AUDPC), the percentage of symptomless plants with latently infected stems, and the total percentage of infected plants (wilted plants + symptomless plants with latently infected stems) and analyzed using the Scott–Knott Clustering Algorithm for Single Experiments method detailed by Scott & Knott (1974), and the Kruskal-Wallis test and multiple comparisons of treatments (Conover, 1999), using the Agricola package (De Mendiburu, 2012) of R software (R Core Team, 2019). The AUDPC of the transgenic events was used to summarize the progression of disease severity over time and was compared to that of the untransformed controls: moderately resistant (Cruza 148) and susceptible (Desiree).

Inoculation, sampling and processing for RT-qPCR

Gene expression analysis was performed on the leaves of the transgenic plants one month after inoculation with isolate CIP-204 inoculation with sterile water. For inoculation, the plant roots were damaged with a scalpel and 40 mL of the bacterial suspension was immediately poured into the soil (the final concentration of each isolate was 5×10^7 cells·g^{-1} soil). The plants were maintained under the same conditions as those described above. Three leaf or root samples were collected as biological replicates, washed with distilled water, immediately frozen in liquid nitrogen, and stored at -70°C until processing.

These samples were collected on the 22nd day after inoculation to ensure sufficient root growth. Frozen leaf or root samples were ground with a mortar and pestle in liquid nitrogen, and 100 mg of the powder was used for total RNA extraction using TRIzol Reagent (Invitrogen®) following the manufacturer's protocol. RNA extraction was performed with three experimental replicates for each sample.

Real-time quantitative PCR

The expression of the *βhpmeh* gene was assessed using real-time quantitative PCR (RT-qPCR) and the *ef1α* gene as reference gene (Nicot *et al.*, 2005). RNA extraction was cleaned with DNase treatment using Turbo DNA-free (Ambion®), following the manufacturer's protocol. The reaction mixture was prepared in a total volume of 10 µL containing 4 µL of template (50 ng), 0.4 µM of each primer, and Power SYBR® Green PCR Master Mix (Applied 274 Biosystems® StepOne Real-Time PCR System). The expression of the *βhpmeh* gene was determined using the primers (pameh150Fw 5'-GCT ATG TTC GCT GCC CAA GG-3' and pameh150Rv 5'- CGT TCC TTC CGG TAC CAA CG-3') and the *ef1α* gene was amplified using the primers ef1αFw 5'- ATT GGA AAC GGA TAT GCT CCA -3' and ef1αRv 5'- TCC TTA CCT GAA CGC CTG TCA -3' (Nicot *et al.*, 2005). The following PCR program was used: 95°C for 10 min, 95°C for 15 sec, and 55°C for 1 min for 40 cycles, followed by 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec (melting curve analysis). The *ef1α* gene was used to normalize the expression levels, and differential expression was determined using REST® 2009 software. No Ct values were obtained for non-transgenic events but were set at 40 cycles (the number of cycles run and thus the maximum theoretical detection limit) to enable comparison by relative expression levels. Differences between RT-qPCR results were tested for statistical significance by ANOVA and Tuckey's multiple comparison test. The significance values (pH1) were calculated using the REST software, which evaluates the probability of the hypothesis that there are differences in gene expression between the analyzed groups (H1), is true.

Results

Bacterial wilt resistance evaluation

Three hundred and sixty transgenic events (197 events with *GRP1.8-pameh*, and 163 transgenic events with the *35S-pameh*) were produced and evaluated from explants infected with *Agrobacterium* containing the corresponding gene construct. All transgenic events were screened for BW resistance using isolate CIP-204 in five experiments (*Exp 1 to 5*) with 3 repetitions per genotype (Figure 2). Of the 360 events tested, 24 did not develop any wilt symptoms, 174 had low to high wilting symptoms, whereas 162 were fully susceptible (Table S1). This large-scale screening allowed to eliminate the transgenic events which were as susceptible as the control Desiree. The bioassay data were not further considered because of the small number of repetitions.

Sixty-eight transgenic events were selected from this large-scale screening. Sixty-two were selected based on a reduced incidence of wilt and no latent infection compared to the control. Four events with latent infections and two highly susceptible were included as well. The 68 events were evaluated in seven experiments (*Exp 6 to 12*) with 15 repetitions per genotype using isolate CIP 204 (Figure 2). Twenty-two of these transgenic events were additionally evaluated with another strain of *R. solanacearum*, strain CIP-475. No differences in virulence or aggressivity were observed between the two strains on the susceptible control variety Desiree. Bioassay data were thus combined.

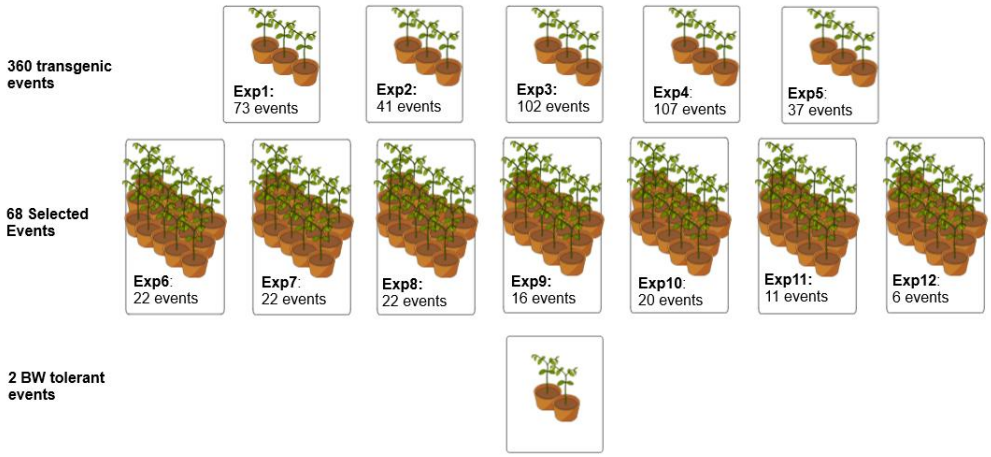

Figure 2. Schematic representation of screening transgenic events for BW resistance in a total of 12 experiments from an initial large-scale screening of 360 events in five experiments with (3 plants per genotype), followed by a screening of 68 selected transgenic events in seven experiments *Exp* 6 to 12 (15 plants per event) which resulted in identifying 22 highly confident resistant transgenic events of which 2 are as resistant as Cruza 148 to bacterial wilt disease, inoculated with CIP-204 or CIP-475 (*Exp* 8).

In the experiments (*Exp 6-10*, *12*) with 15 plants, statistical analysis was performed to identify the most resistant plants, and the detailed results are presented in supplementary table (Table S2). The Scott–Knott test was used in *Exp 6*, *7*, *9*, and *10*. In *Exp 8*,*11* and *12*, the Kruskal-Wallis test was used. The latter test, a rank-based nonparametric test, was chosen because it can detect differences between groups with a small number of samples. The mean ranks values in Kruskal-Wallis are compared to determine if the differences are statistically significant among them. In all experiments, except for *Exp 8*, the resistant control Cruza 148 showed significantly reduced wilt, AUDPC, and total infected plants compared to the non-transgenic Desiree.

Exp 6 (22 events) resulted in one resistant event (GRP 3.11) with 6.7% wilting symptoms, 6.7% latent infection (13.3% total infected plants) and the lowest AUDPC (113.3) values, similar to the moderately resistant control Cruza 148 (wilting :6.7%, latent infection: 20%, total infected plants: 26.7%, and AUDPC: 100), followed by event II 4.16, with 53.3% of total infected plants. The bioassay data for these three genotypes were statistically significant.

The *Exp 7* (22 events) did not show any significant differences between events or controls in wilt and AUDPC results, but there was a significant reduction in the total percentage of infected plants (wilted + latently infected) for nine events compared to the non-transgenic Desiree. Because wilt incidence was relatively low in this experiment compared to the others, the conditions were likely unfavorable for disease development.

The *Exp 8* (22 events), with RSSC strain CIP-475, showed one event (GRP 3.11) with significantly reduced mean ranks of wilt incidence, AUDPC, and total infected plants compared to non-transgenic Desiree. Similar to the result from *Exp 6*, the event II 4.16 presented AUDPC values significantly lower than those of Desiree. When analyzing the total % of infected plants, the events GRP 3.11, GRP 2.18, GRP 2.3, GRP 2.6, and GRP 1.5, had significantly lower infection rates than Desiree.

The *Exp 9* (16 events) showed five events (GRP 7.28, GRP 7.15, GRP 9.7, GRP 6.48, and GRP 5.14) with significantly reduced total infected plants compared to Desiree.

The *Exp 10* (20 events) resulted in four events (II 10.29, II 4.23, II 8.5, and II 5.8) with significantly reduced wilt percentage and AUDPC (except for II 5.8), two of which (II 5.8, and II 10.29) were also reduced in total infected plants.

The *Exp 11* (10 events) resulted in one event (II 10.31) with significantly reduced mean ranks of wilt, AUDPC or infection, including the control Cruza 148. However, the Desiree control did not behave as expected and was not among the most wilted genotypes. Hence, the analysis using relative AUDPC to Desiree did not include the data from *Exp 11*.

The *Exp 12* (six events) resulted in two events (II 6.3 and II 4.16) with significantly reduced mean ranks of AUDPC and wilt percentage, and one event II 10.29 as resistant in terms of total wilt percentage, compared to Desiree.

Twenty-two transgenic events were finally selected from the second level screening based on the criteria of having gone through at least three independent BW resistance evaluations (experiments). Indeed, wilting symptoms varied significantly from experiment to experiment as evidenced from the bioassay data of the controls. One of the experiments (*Exp 11*) has not been included in the BW resistance analysis because the Desiree control did not perform as expected. We therefore selected further our sample of transgenic events for those with at least three bioassay datasets that supported significant increase in resistance, which resulted in 22 transgenic events with highly confident bioassay data (Table 1).

The further analysis of BW resistance assay data conducted on the 22 transgenic events with highly confident BW resistance data resulted in the identification of 2 transgenic events, GRP 3.11 and II 4.16 with an average AUDPC relative to the AUDPC of the susceptible control Desiree of 23% and 31% respectively (Table 1). This is similar to the value of the moderately BW resistant variety Cruza 148 (27%). Most of the rest of the transgenic events were almost as susceptible as Desiree (Figure 3). A statistical analysis was conducted on the bioassay data and is presented hereafter.

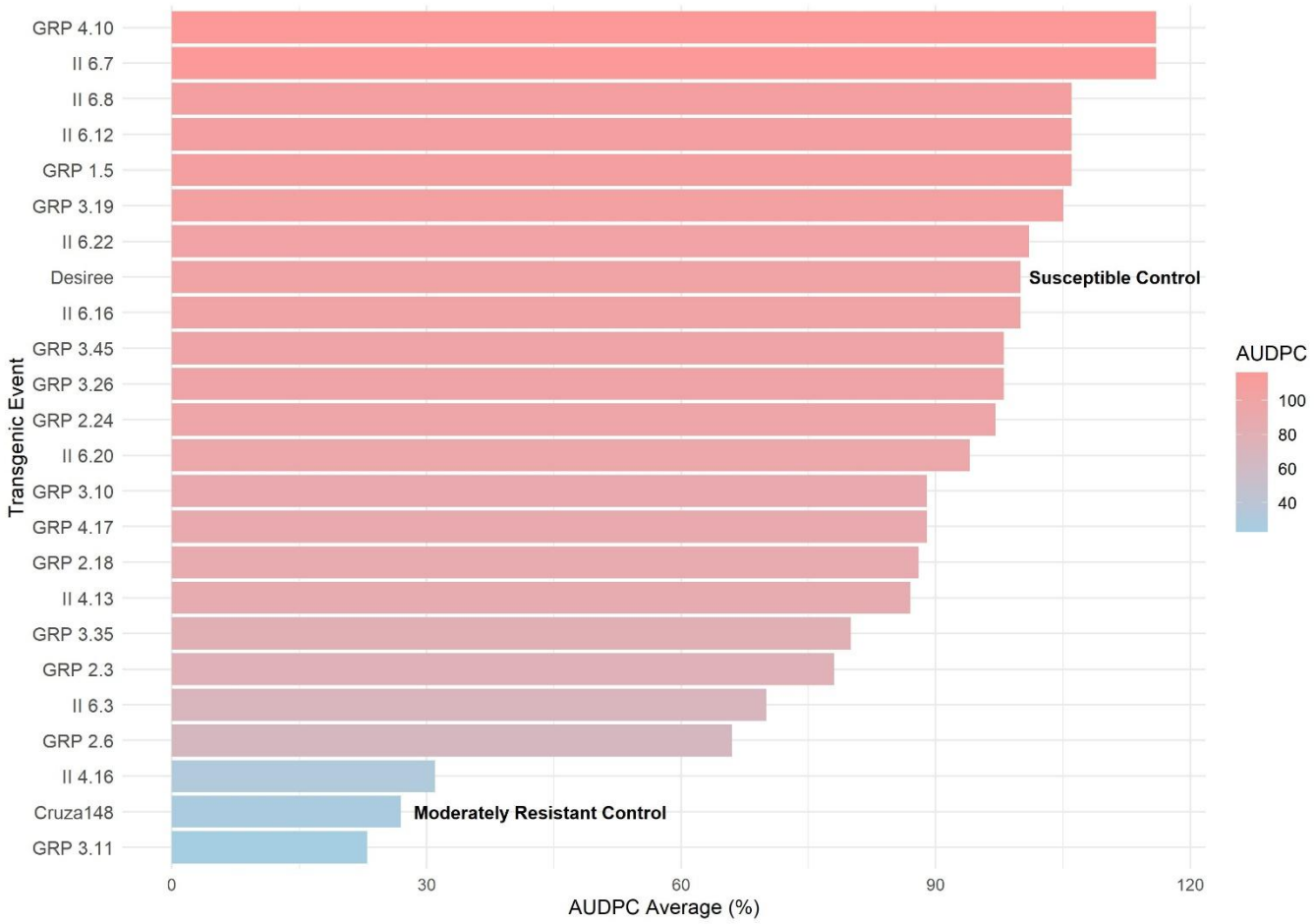

Figure 3. Disease progression across transgenic events based on the average area under disease progress curve (AUDPC) from *Exp 6-10, Exp 12*. Desiree and Cruza 148 were used as susceptible and moderately resistant controls, respectively.

βhpmeh gene expression

RNA was extracted from leaves from plants one month after inoculation from *Exp* 12 (GRP 3.45, GRP 3.11, II 4.16, II 6.3, GRP 7.15, and II 10.29) and were analyzed using RT-qPCR. Expression levels from transgenic plants were analyzed relative to GRP 7.15, chosen because of its lowest expression level in the experiment (Figure 4). All measurements were normalized to *ef1α* before performing relative expression comparisons. Events II 6.3, II 4.16, and II 10.29 were identified as the events with highest levels of *βhpmeh* expression and correlated with lowest level of wilting in the same plants, and II.6.3 had a significantly higher level of expression than the rest. Also, the pH1 values (<0.05) support that this event (as well as II.10.29 and II.4.16) had significantly higher expression compared to the non-transgenic control (Figure 4).

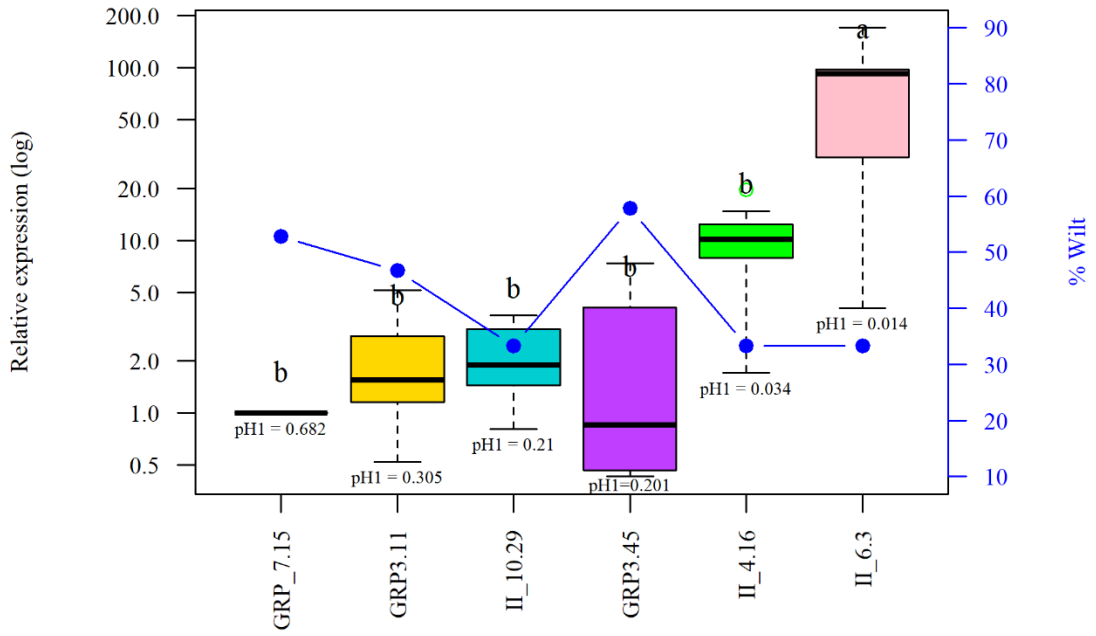

Figure 4. Box plot of the relative expression of *βhpmeh* gene in leaves of transgenic events relative to GRP 7.15 (value=1) one month after inoculation with CIP-204 (experiment 12). Expression levels were normalized to those of *ef1α*. The phenotypic results (%) are plotted on the 2nd axis. Statistically significant differences in expression between different events, as determined by Tukey's multiple comparison test (α: 0.05), are shown by different letters above each box plot. The significance values (pH1) for expression as compared to non-transgenic Desiree are shown below each box plot.

Additionally, we analyzed the relative expression of *βhpmeh* in root samples from transgenic events 22 days after inoculation with CIP204, using GRP 2.18 as a reference (Figure 5). This experiment was conducted independently (Experiment 13) due to the use of a distinct tissue for expression analysis. A subset of 9 randomly selected transgenic events (II 10.31, GRP 9.7, II 5.19, GRP 6.48, II 5.8, GRP 7.28, GRP 6.58, II 4.13, and II 10.18) that were not part of the twenty-two transgenic events previously selected was included. Overall, the analysis comprised 12 events harboring the GRP promoter (GRP 3.45, GRP 3.10, GRP 2.3, GRP 4.17, GRP 2.18, GRP 3.35, GRP 7.15, GRP 6.48, GRP 6.58, GRP 9.7, GRP 3.11, and GRP 7.28) and 9 events harboring the 35S promoter (II 5.8, II 6.16, II 4.13, II 6.3, II 10.29, II 5.19, II 4.16, II 10.31, and II 10.18). Since root sampling was destructive, phenotypic assessment could not be performed on the same plants. Among the tested events, II 10.18 (excluded from Experiment 12 due to logistical reasons) exhibited the highest relative expression.

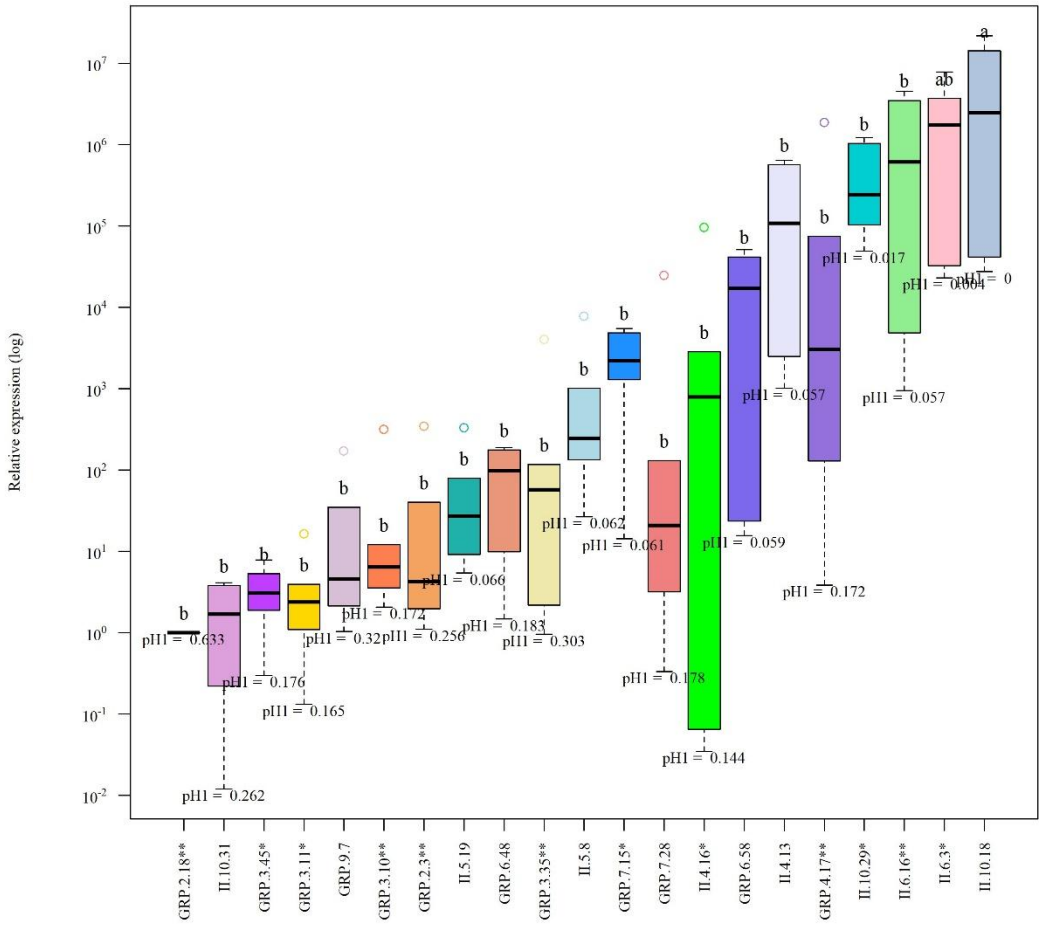

Figure 5. Relative expression of the *βhpmeh* gene in roots of transgenic events 22 days after inoculation with CIP204, relative to GRP 2.18 (colored whisker plots). The expression levels were normalized to those of *ef1-a*. Non-transgenic 'Desiree' was used as a susceptible control. Statistically significant differences in expression between different events, as determined by Tukey's multiple comparison test (α: 0.05), are shown by different letters above each box plot. *Events included in *Exp* 12 and in the 22 selected events, **Events belonging to the 22 selected events. The significance values (pH1) for expression as compared to non-transgenic 'Desiree' are shown below each box plot.

Discussion

In this study, we evaluated the ability of the *βhpmeh* gene transferred into potato to confer bacterial wilt resistance using artificial inoculation with *R. solanacearum*. Two gene constructs were evaluated, one using the root-specific promoter GRP 1.8 from bean, and the other using the constitutive promoter 35S from CaMV. The highly susceptible variety Desiree was used for producing 360 transgenic events and bioassays were done in 12 experiments including the moderately resistant variety Cruza 148 in each experiment.

Screening for bacterial wilt resistance is notoriously difficult because of the varying aggressiveness of the pathogen between experiments and the difficulties standardizing environmental condition in the soil (e.g. temperature, humidity, artificial injuries). This explains why bioassay data and statistical significance were analyzed for each experiment and were not pooled together. After a large-scale screening of 360 transgenic events, 68 of them were more extensively evaluated for BW resistance. Out of these, 22 transgenic events evaluated in least three experiments were considered as having highly confident BW resistance data. Two

transgenic events, driven by one or the other promoter, were found to be as resistant to BW as the moderately resistant control Cruza 148. Few transgenic events displayed intermediate levels of BW resistance between Cruza 148 and the susceptible variety Desiree while a large group was equally susceptible as Desiree. In one of the experiments, gene expression of the *βhpmeh* gene in leaves revealed a correlation with level of resistance to bacterial wilt. The differences in expression in roots for transgenic events between one or the other promoter was not significant.

Our results are consistent with other approaches used to interfere with quorum sensing of *R. solanacearum* to reduce disease. Wang *et al.* (2023) achieved high levels of 3-OH PAME degradation using *Pseudomonas* strains with 3-OH PAME degrading activity and demonstrated their ability to suppress the virulence factors of *R. solanacearum* in *C. equisetifolia*, peanut, and tomato plants. Achari and Ramesh (2015) studied other bacteria with 3-OH PAME degrading activity, such as *Stenotrophomonas maltophilia* and *Rhodococcus corynebacterioides,* that also had the ability to decrease bacterial wilt incidence in eggplant seedlings. However, the identity of the genes involved in these enzymatic activities has still not been determined.

Having reached similar level of BW resistance of Cruza 148 in bioassays, it is likely that the best transgenic events with the *βhpmeh* gene will also display moderate resistance under field conditions. From a disease epidemiology viewpoint, moderate resistance is actually a threat to spreading the disease unconciously because these events like Cruza 148 would be latent carriers of *R. solanacaearum*. This disease spread was evidenced in Ethiopia with significant damages on the national potato production (Abdurahman *et al.*, 2017). Therefore, combining the *βhpmeh* gene with other genes providing BW resistance might be more prudent to achieve complete resistance without latent infection that could spread the disease through vegetatively propagated seed.

Currently the best candidate gene to be pyramided with the *βhpmeh* gene is the *Arabidopsis thaliana* receptor kinase essential for bacterial EF-Tu perception, called *EFR* (*EF*-Tu *r*eceptor) which activates a common set of signaling events and defense responses (Zipfel *et al.,* 2006). Transgenic potato plants with the *EFR* gene displayed BW resistance under controlled conditions (Boschi *et al.*, 2017). Targeting to the tissues that the pathogen actually infects, the quorum-quenching and/or pathogen-associated molecular pattern (PAMP) receptors strategies can result in synergistic effect providing complete BW resistance.

Yet, the number of identified *R. solanacearum* PAMPs and plant pattern recognition receptors (PRRs) is limited, and there is a need to discover new PAMPs and their corresponding PRRs to improve our understanding of bacterial diseases and host resistance (An & Zhang, 2024). Transgenic expression of several immune receptors from *Nicotiana benthamania* have shown promising results towards bacterial pathogen resistance (Schultink *et al.*, 2017, 2019). A stack of *EFR*, *Roq1*, and *Jim2* is currently under investigation for conferring complete resistance to bacterial wilt (Mwangi *et al.*, 2024).

Understanding the interactions and role of QS signals, PAMPs, and PRRs is crucial for developing effective and durable resistance strategies against *R. solanacearum* in crop plants (Huet, 2014; An & Zhang, 2024).

In conclusion, this study provides evidence for a useful approach, through quorum quenching mediated by βHPMEH enzyme that can contribute to control bacterial wilt disease in potato. Quorum quenching has advantages compared to conventional antibacterial approaches, primarily because of its reduced probability of generating bacterial resistance (Wang et al., 2022). Disruption of quorum sensing can make it more difficult for bacteria to develop resistance because it targets a fundamental aspect of bacterial physiology rather than focusing on specific molecular targets.

Author contributions

M. Izarra: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.
L. Gutarra: Supervision, Data curation, Methodology, Formal analysis, Investigation.
E. Fernandez: Data curation, Methodology, Formal analysis, Investigation

E. Huaman: Investigation
M. Ghislain: Formal analysis, Writing – review & editing.
JF. Kreuze: Conceptualization, Project administration, Supervision, Formal analysis, Investigation, Methodology, Writing – review & editing.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

Not applicable.

Ethics declaration

Not applicable.

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Tables

Table 1. List of transgenic events (n=68), and 2 controls that were tested more than once (sorted by the average of total infected plants).

N	Transgenic events	Repetitions	Experiment (Exp)	Wilt		Symptomless plants with latently infected stems (%)[****]	Infected plants (wilting and symptomless) (%)	Average of infected plants (%)
				% plants	AUDPC[***]			
1	II 10.31 [a,**]	15	Exp 9	6.7	1046.7	13.3	20.0	20.0
2	GRP 3.11 [a,b,*,**]	15	Exp 11	6.7	113.3	6.7	13.3	26.7
		15	Exp 11	26.7	320.0	6.7	33.3	
		15	Exp 10	6.7	60.0	0.0	6.7	
		15	Exp 12	46.7	856.7	6.7	53.3	
3	GRP 7.28 [a,**]	14	Exp 9	28.3	336.7	0.0	28.3	28.3
4	II 10.18 [a,**]	15	Exp 11	33.3	126.7	0.0	33.3	33.3
5	GRP 6.58 [a,**]	15	Exp 11	33.3	620.0	6.7	40.0	40.0
6	II 10.29 [a,*,**]	15	Exp 10	40.0	503.3	13.3	53.3	43.3
		15	Exp 12	33.3	670.0	0.0	33.3	
7	GRP 7.15 [a,*,**]	15	Exp 9	40.0	570.0	0.0	40.0	46.4
		15	Exp 12	52.8	1145.8	0.0	52.8	
8	II 4.44	15	Exp 11	46.7	493.3	0.0	46.7	46.7
9	II 11.3	15	Exp 11	46.7	400.0	0.0	46.7	46.7
10	GRP 6.48 [a,**]	15	Exp 9	46.7	826.7	0.0	46.7	46.7
11	II 4.16 [a,b,*,**]	15	Exp 6	40.0	576.7	13.3	53.3	50.6
		15	Exp 7	11.1	133.3	11.1	22.2	
		15	Exp 8	73.3	730.0	7.1	80.0	
		15	Exp 12	33.3	430.0	13.3	46.7	
12	GRP 9.7 [a,**]	15	Exp 9	53.3	690.0	0.0	53.3	53.3
13	GRP 8.10	15	Exp 11	33.3	700.0	20.0	53.3	53.3
14	GRP 6.54	15	Exp 11	53.3	436.7	0.0	53.3	53.3
15	II 5.11	15	Exp 11	53.3	423.3	0.0	53.3	53.3
16	II 6.29	15	Exp 11	60.0	933.3	0.0	60.0	60.0

17	GRP 5.14	15	Exp 9	63.3	972.5	0.0	63.3	63.3
18	GRP 2.3 [a,b,**]	15	Exp 6	86.7	1563.3	0.0	86.7	64.5
		15	Exp 7	40.0	573.3	0.0	40.0	
		15	Exp 8	60.0	1100.0	7.1	66.7	
19	GRP 2.6 [b]	15	Exp 6	66.7	1046.7	0.0	66.7	66.7
		15	Exp 7	46.7	560.0	20.0	66.7	
		15	Exp 8	66.7	1123.3	0.0	66.7	
20	GRP 7.32	15	Exp 9	66.7	1253.3	0.0	66.7	66.7
21	II 6.3 [a,b, *,**]	15	Exp 6	93.3	1426.7	0.0	93.3	68.3
		15	Exp 7	33.3	540.0	6.7	40.0	
		15	Exp 8	86.7	1443.3	7.1	93.3	
		15	Exp 12	33.3	670.0	13.3	46.7	
22	II 4.13 [a,b **]	15	Exp 6	86.7	1663.3	0.0	86.7	68.9
		15	Exp 7	40.0	760.0	0.0	40.0	
		13	Exp 8	80.0	1124.4	0.0	80.0	
23	GRP 3.35 [a,b **]	15	Exp 6	73.3	1346.7	6.7	80.0	68.9
		15	Exp 7	26.7	390.0	0.0	26.7	
		14	Exp 8	100.0	1734.2		100.0	
24	GRP 2.18 [a,b, **]	15	Exp 6	100.0	2010.0		100.0	71.1
		15	Exp 7	46.7	700.0	0.0	46.7	
		15	Exp 8	60.0	936.7	7.1	66.7	
25	II 6.20 [b]	15	Exp 6	86.7	1490.0	0.0	86.7	72.2
		15	Exp 7	40.0	830.0	13.3	53.3	
		14	Exp 8	70.0	1529.2	7.1	76.7	
26	GRP 3.45 [a,b,*,**]	15	Exp 6	100.0	2076.7		100.0	72.8
		15	Exp 7	40.0	610.0	0.0	40.0	
		15	Exp 8	93.3	2030.0	0.0	93.3	
		15	Exp 12	57.8	1105.6	0.0	57.8	
27	GRP 3.10 [a,b,**]	15	Exp 6	93.3	1603.3	0.0	93.3	73.3
		15	Exp 7	33.3	470.0	0.0	33.3	
		15	Exp 8	93.3	1750.0	0.0	93.3	
28	GRP 11.2	15	Exp 9	73.3	1500.0	0.0	73.3	73.3
29	GRP 6.52	15	Exp 9	73.3	1243.3	0.0	73.3	73.3
30	II 5.8 [a,**]	15	Exp 10	73.3	1440.0	0.0	73.3	73.3
31	GRP 4.17 [a,b,**]	15	Exp 6	86.7	1723.3	6.7	93.3	77.8
		15	Exp 7	26.7	473.3	13.3	40.0	
		15	Exp 8	100.0	1600.0		100.0	
32	GRP 3.19 [b]	15	Exp 6	86.7	1273.3	0.0	86.7	77.8
		15	Exp 7	60.0	1200.0	13.3	73.3	

		15	Exp 8	73.3	1686.7	0.0	73.3	
33	GRP 2.24 [b]	15	Exp 6	93.3	1676.7	0.0	93.3	77.8
		15	Exp 7	53.3	1036.7	6.7	60.0	
		14	Exp 8	80.0	1151.7	0.0	80.0	
34	II 12.3	15	Exp 10	80.0	1100.0	0.0	80.0	80.0
35	GRP 6.12	15	Exp 9	73.3	870	6.7	80.0	80.0
36	GRP 5.2	15	Exp 9	80.0	1560.0	0.0	80.0	80.0
37	II 5.19 [a,**]	15	Exp 11	80.0	33.3	0.0	80.0	80.0
38	II 8.5	15	Exp 10	60.0	930.0	20.0	80.0	80.0
39	II 6.7 [b]	15	Exp 6	93.3	1883.3	0.0	93.3	80.0
		15	Exp 7	66.7	1303.3	0.0	66.7	
		15	Exp 8	80.0	1396.7	0.0	80.0	
40	GRP 3.26 [b]	15	Exp 6	93.3	1776.7	0.0	93.3	80.0
		15	Exp 7	46.7	770.0	0.0	46.7	
		13	Exp 8	93.3	1493.3	7.1	100.0	
41	II 6.22 [b]	15	Exp 6	100.0	1913.3		100.0	82.2
		15	Exp 7	46.7	606.7	0.0	46.7	
		15	Exp 8	100.0	1773.3		100.0	
42	II 6.8 [b]	15	Exp 6	100.0	1873.3		100.0	84.4
		15	Exp 7	40.0	633.3	13.3	53.3	
		15	Exp 8	100.0	1996.7		100.0	
43	GRP 1.5 [b]	15	Exp 6	100.0	1993.3		100.0	84.4
		15	Exp 7	66.7	1033.3	13.3	80.0	
		15	Exp 8	66.7	1230.0	7.1	73.3	
44	II 6.12 [b]	15	Exp 6	100.0	1913.3		100.0	84.5
		15	Exp 7	66.7	846.7	0.0	66.7	
		15	Exp 8	86.7	1620.0	0.0	86.7	
45	GRP 4.10 [b]	15	Exp 6	100.0	2096.7		100.0	86.7
		15	Exp 7	60.0	1140.0	6.7	66.7	
		14	Exp 8	80.0	1449.2	14.3	93.3	
46	II 2.10	15	Exp 10	86.7	1403.3	0.0	86.7	86.7
47	II 4.23	15	Exp 10	73.3	870.0	13.3	86.7	86.7
48	GRP 8.15	15	Exp 9	86.7	1970.0	0.0	86.7	86.7
49	GRP 6.53	15	Exp 9	80.0	1583.3	6.7	86.7	86.7
50	II 4.5	15	Exp 10	86.7	1813.3	0.0	86.7	86.7
51	II 6.16 [a,b,**]	15	Exp 6	100.0	1920.0		100.0	88.9
		15	Exp 7	53.3	746.7	20.0	73.3	
		15	Exp 8	86.7	1480	7.1	93.3	

52	GRP 5.3	15	Exp 9	93.3	1913.3	0.0	93.3	93.3
53	GRP 7.18	15	Exp 9	93.3	2076.7	0.0	93.3	93.3
54	GRP 5.37	15	Exp 9	93.3	1750.0	0.0	93.3	93.3
55	GRP 9.8	15	Exp 9	86.7	1746.7	6.7	93.3	93.3
56	II 6.48	15	Exp 10	93.3	2066.7	0.0	93.3	93.3
57	II 11.22	15	Exp 10	93.3	1856.7	0.0	93.3	93.3
58	II 3.32	15	Exp 10	93.3	1763.3	0.0	93.3	93.3
59	II 11.5	15	Exp 10	86.7	1613.3	6.7	93.3	93.3
60	II 11.2	15	Exp 10	93.3	1590.0	0.0	93.3	93.3
61	II 14.4	15	Exp 10	93.3	1903.3	0.0	93.3	93.3
62	II 13.2	15	Exp 10	93.3	1953.3	0.0	93.3	93.3
63	II 4.32	15	Exp 10	86.7	1530.0	6.7	93.3	93.3
64	II 5.22	15	Exp 10	93.3	2030.0	0.0	93.3	93.3
65	II 6.11	15	Exp 10	86.7	1320.0	13.3	100.0	100.0
66	II 3.28	15	Exp 10	100.0	2333.3		100.0	100.0
67	II 6.45	15	Exp 10	100.0	1143.3		100.0	100.0
68	II 10.24	15	Exp 10	100.0	2173.3		100.0	100.0
	Cruza148	15	Exp 6	6.7	100.0	20.0	26.7	31.1
		15	Exp 7	20.0	263.3	0.0	20.0	
		15	Exp 9	46.7	606.7	0.0	46.7	
		15	Exp 10	53.3	793.3	0.0	53.3	
		15	Exp 11	6.7	943.3	0.0	6.7	
		15	Exp 12	26.7	440.0	6.7	33.3	
	Desiree	15	Exp 6	93.3	1523.3	0.0	93.3	77.1
		15	Exp 7	60.0	976.7	0.0	60.0	
		15	Exp 8	80.0	1536.7	0.0	80.0	
		15	Exp 9	80.0	1700.0	6.7	86.7	
		15	Exp 10	93.3	2056.7	0.0	93.3	
		15	Exp 11	33.3	686.7	6.7	40.0	
		15	Exp 12	80.0	1760.0	6.7	86.7	

[a]: Events tested in RT-qPCR

[b]: 22 selected events

[*] leaf samples

[**] root samples

[***] AUDPC: Area under disease progress curve

[****] Transgenic events with 100% wilting were not tested for latent infection