Use of *Solanum stenotomum* for introduction of resistance to bacterial wilt in somatic hybrids of potato

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Abstract

The wild tuber-bearing relative *Solanum stenotomum* was used as source of resistance to bacterial wilt caused by *Ralstonia solanacearum*. In order to transfer resistance, somatic hybrids between a dihaploid clone of potato (*S. tuberosum*) cv. BF15 and *S. stenotomum* were produced by electrofusion of mesophyll protoplasts. A total of thirty hybrid plants were regenerated. When transferred to the greenhouse, they exhibited a strong vigour and showed morphological intermediate traits, including leaf form, flowers and tuber characteristics. DNA analysis using flow cytometry revealed that 25 were tetraploids (4×; 48 chromosomes), three hexaploids (6×; 72) and two aneuploids (< 4×; 48). Their hybrid nature was confirmed by examining isoenzyme patterns for esterases, and analysis of DNA simple sequence repeat (SSR) markers. Analysis of chloroplast (ct) DNA microsatellites of fourteen somatic hybrids revealed that six hybrids possessed ctDNA of *S. stenotomum*, and eight contained *S. tuberosum* ct-type. Six tetraploid hybrid clones were evaluated for resistance to bacterial wilt by using race 1 and 3 strains of *R. solanacearum*, originating from Reunion Island. Inoculations were performed by an in vitro root dipping method. The cultivated potato was susceptible to both bacterial strains and died within a few days. Interestingly, all somatic hybrids tested showed a resistance level as high as that of the wild species.

Author Keywords: bacterial wilt; electrofusion; ***Ralstonia solanacearum***; ***Solanum stenotomum***; ***S. tuberosum***; somatic hybrids

Abbreviations: cfu, colony forming units; CPW salts, cell and protoplast washing solution; ctDNA, chloroplast DNA; dNTP, deoxynucleotides triphosphates; MS medium, Murashige and

Skoog medium; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat; VKM medium, V-47 medium and Kao and Michayluk medium

Article Outline

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is probably one of the most devastating bacterial disease. It is found worldwide, mainly in tropical and subtropical areas, but also in warm temperate countries and even in some cool temperate regions [15]. More than fifty botanical families were concerned including some economically important species such as bananas, potatoes and tomatoes [14]. The preliminary invasion of roots led to the colonisation of the stem, resulting in partial or complete wilting. Under tropical conditions, most plants were affected by race 1 of *R. solanacearum*. Potatoes and tomatoes could also be damaged by race 3, which occurred at high altitude in some tropical countries such as Peru or Reunion Island, and recently in north-western Europe [10] and [17].

Since agro-chemicals are costly and less effective, and sanitary cropping systems difficult to apply, control strategies of the disease have so far mainly consisted of plant breeding. Cultivars of tobacco and peanut resistant to bacterial wilt are commonly used. Besides, tolerant cultivars of potato were cultivated in Madagascar, Burundi, Rwanda, Zaire and Brazil [10] and [13]. However, more resistant or tolerant cultivars are needed to better control the disease.

Some wild or related cultivated species, which were found resistant or highly tolerant to bacterial wilt, have been used as potential source of resistance. Unfortunately, sexual hybrids of

potato with resistant genotypes of *Solanum chacoense*, *S. sparsipillum* and *S. multidissectum* revealed some traits of wildness such as a high glycoalkaloid content besides a moderate level of resistance to bacterial wilt [10]. Likewise, those of potato with *S. phureja*, a source of bacterial resistance commonly used in breeding programmes, displayed resistance which appeared temperature-sensitive [10] and [31]. Resistance to bacterial wilt has also been identified in *S. stenotomum*, another cultivated relative of potato, which originated from Peru and Bolivia, and could be crossed with *S. tuberosum* [16]. But, few interests have so far been attributed to *S. stenotomum* as a potential source of resistance against bacterial wilt in potato breeding programmes, although more than 80 % of plants evaluated did not show any wilting symptoms [20].

The introgression of resistance genes from wild *Solanum* species into *S. tuberosum* by classical breeding methods is time-consuming, laborious and may encounter difficulties because of sexual incompatibilities, particularly differences in ploidy level or in endosperm balance numbers. Therefore, somatic fusion is expected to provide a new possibility for increasing nuclear and cytoplasm genetic variability, and also a means of transferring desirable agronomic traits into potato. The potential use of somatic hybridisation has been demonstrated by the successful introduction of traits of resistance to viruses [11] and [37], to extreme climatic conditions such as frost [27], to fungi [21] and to insects [32] into the cultivated potato. Recently, traits of resistance against bacterial wilt has successfully been transferred from *S. commersonii* [18] and *S. phureja* [8] into potato through somatic hybridisation.

In this study, somatic hybridisation through protoplast fusion was used as an alternative method for successful introgression of traits of resistance to bacterial wilt from *S. stenotomum* into cultivated potato. The resulting somatic hybrids were characterised and evaluated for resistance to race 1 and 3 strains of *Ralstonia solanacearum* originating from Reunion Island.

2. Results

2.1. Protoplast culture and plant regeneration

Two weeks after dilution of the fused protoplast suspension with fresh VKM medium, hundreds of microcolonies were observed. They rapidly developed into calli when transferred onto the solid growth medium. Early selection of putative somatic hybrid calli was based on their ability to grow faster and to regenerate earlier than the parental calli [34]. Two weeks later, only those of at least 2–3 mm in size were transferred onto the regeneration medium. After 5 weeks, 124 of 565 selected calli produced shoots. Only one shoot was excised from each regenerating callus, and multiplied on hormone-free MS medium. Since the parental lines were morphologically similar, the preliminary selection of putative hybrid plants was based on plant vigour through a faster growth in particular. Finally, 85 plants were retained for further determination of the ploidy level by using flow cytometry to achieve the selection.

2.2. Ploidy level of the selected putative hybrids

Flow cytometry was used to quantify DNA for determination of the ploidy level. Histograms were generated from nuclei of leaf cells, and the ploidy level was determined by comparing the position of dominant peaks corresponding to nuclei at G0-G1 phase of the cell cycle, between putative hybrids and parental plants (figure 1). The analysis revealed that thirty of 85 selected plants recovered from the fusion experiments had a ploidy level higher than that of diploids. Those plants were retained as they were putative somatic hybrids. The remaining plants, representing 64.7 % of the total, were diploids. Among the selected plants, 25 were tetraploids (4×; 48 chromosomes), three hexaploids (6×; 72) and two aneuploids (< 4×; 48) (figure 1). The ploidy level determined by flow cytometry was confirmed by chromosome counts made on root tips of a sample of the selected plants.

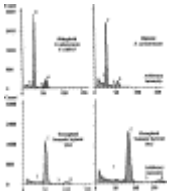 Full-size image (9K)

Figure 1. Histograms of relative nuclear DNA contents obtained by flow cytometry analysis of 10 000 DAPI-stained nuclei isolated from leaves of the diploid parents: *Solanum tuberosum* cv. BF15, *S. stenotomum*, and their tetraploid and hexaploid somatic hybrids. Fluorescence intensity is proportional to nuclear DNA quantity and the position of dominant peak reflects the ploidy level.

2.3. Morphological analysis

Both parental and hybrid plants were grown to maturity in the greenhouse. Most putative hybrid plants grew vigorously, and all overtopped the parental plants. Their morphology was relatively homogeneous. Hybrid leaves were well-developed and larger in size, compared with those from parents. Several traits of the putative hybrids were intermediate to those of the parents, including leaf forms, inflorescence, flower colour (figure 2). The hybrid stems were light purple in colour because of the presence of anthocyanin like *S. stenotomum*. Furthermore, in the greenhouse, the viability of pollen grain of the hybrid plants was not significantly different from that of the parental lines, estimated at 62–68 and 50–70 % respectively.

 Full-size image (214K)

Figure 2. Plant morphology. *S. tuberosum* (BF15), *S. stenotomum* (Sst) and their somatic hybrids (SH) (**A**). Leaves and flowers of *S. tuberosum* (**B** and **E**), *S. stenotomum* (**D** and **G**) and their somatic hybrid (**C** and **F**).

2.4. Isoenzyme analysis

The hybrid nature of the selected putative hybrids was confirmed by examining the electrophoretic patterns for esterases (figure 3). The isoenzyme system studied revealed differences between potato BF15 and *S. stenotomum*. They also distinguished somatic hybrids from the parents. All thirty putative somatic hybrids had identical banding patterns to those of a physical mixture of parental extracts.

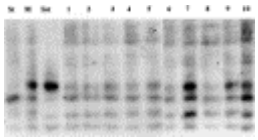 Full-size image (35K)

Figure 3. Electrophoresis banding patterns of esterases (EST) (EC 3.1.1.2.) for *S. tuberosum* cv. BF15 (St), *S. stenotomum* (Sst), a mixture of both parental extracts (M) and their somatic hybrids (lanes 1 to 10).

2.5. Microsatellite amplification

Several pairs of primers leading to nuclear microsatellites polymorphisms between different potato genotypes were described by Milbourne et al. [22]. Among these primers, STM0015 gave the best distinct patterns between the parental lines. Parental patterns can be distinguished by specific bands, about 193 and 190 bp in size for *S. tuberosum* cv. BF15 and *S. stenotomum*, respectively. The thirty putative somatic hybrids obtained showed similar patterns with both specific parental bands (figure 4A).

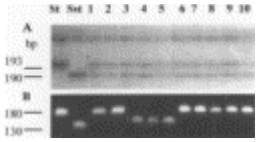 Full-size image (4K)

Figure 4. Electrophoresis profiles of PCR amplification products. **A**, Nuclear microsatellite patterns: DNA from *S. tuberosum* (St), *S. stenotomum* (Sst) and their somatic hybrids (lanes 1 to 10) was amplified using the primers set, STM0015 (5'-GATTGTGAGAAGGCACTGA-3'; 5'-CACTTGATATACTAGTGTGTTTGG-3'). Fractionation was carried out on 6 % polyacrylamide denaturing gel. **B**, Chloroplast microsatellite patterns: DNA from the same genotypes as above was amplified using NTCP6 primers (5'-GGTTCGAATCCTTCCGTC-3' and 5'-GATTCTTTCGCATCTCGATTC-3'). Fractionation was carried out on 1.8 % agarose gels. Left of picture, 100-bp DNA ladder (Biolabs).

Polymorphic simple sequence repeat (SSR) markers in chloroplast genomes of *Solanum* plants were recently described [3]. Several pairs of primers given by these authors have been used to distinguish the chloroplast (ct) genomes of the parents and to characterise the ct genome type of the corresponding hybrids. The NTCP6 primers led to the distinction between the two parents of the fusion experiments. An unique and specific ctDNA band of about 180 and 130 bp was amplified with the cultivated (as expected from Bryan et al. [3]) and wild species, respectively. The fourteen somatic hybrids examined showed the ctDNA pattern of either one or the other parent. The ctDNA of potato was found in eight somatic hybrids and the remaining six hybrids possessed the *S. stenotomum* ct-type (figure 4B).

Taking into account the intermediate morphology, the ploidy level and the analysis of nuclear and chloroplast genomes of the selected plants by examining isoenzymes and DNA microsatellite markers, it was concluded that the thirty selected plants were somatic hybrids between *S. tuberosum* and *S. stenotomum*.

2.6. Evaluation of resistance to bacterial wilt

As expected, *S. tuberosum* cv. BF15 was found to be very susceptible to race 1 and 3 strains, showing 94.3 and 100 % of wilted plants at 30 d (d30) after inoculation respectively. *S. stenotomum* appeared resistant to race 1 strain with no wilting at d30, and rather tolerant to race 3 strain, causing only 30 % of wilted plants (table I).

Table I. Disease indices and disease incidence recorded 30 d after root inoculation by race 1 and 3 strains of *R. solanacearum*. Disease indices are the weighed average of the disease index. Disease index ranges from 0 to 4: 0, no wilted leaves; 1, up to 25 % wilted; 2, up to 50 % wilted; 3, up to 75 % wilted; and 4, plants entirely wilted. Disease incidence is the percentage of inoculated plants displaying a disease index of 4. Values followed by the same letter are not significantly different at $P = 0.05$.

Genotype	Race 1 strain		Race 3 strain	
Somatic hybrids	Disease indices	Disease incidence	Disease indices	Disease incid
BS2 (2n = 4×)	0.04 b	0.00 b	0.76 b	16.7 c
BS31 (2n = 4×)	0.10 b	0.00 b	0.64 b	16.7 c
BS33 (2n = 4×)	0.11 b	0.00 b	0.65 b	16.7 c
BS37 (2n = 4×)	0.10 b	0.00 b	0.74 b	30.6 b
BS42(2n = 4×)	0.09 b	0.00 b	0.69 b	27.8 b
BS44 (2n = 4×)	0.04 b	0.00 b	0.63 b	13.9 c
BF15 (2n = 2× = 24)	0.98 a	94.3 a	1.00 a	100.0 a
S. stenotomum (2n = 2× = 24)	0.10 b	0.00 b	0.74 b	30.6 b
Non-infected controls	0.00 b	0.00 b	0.00 c	0.00 c

The six tetraploid somatic hybrids, which had been selected for evaluation of resistance to bacterial wilt race 1, expressed a resistance level at least similar to that of the wild parent. When inoculations were performed with race 3 strain, the final wilting rate reached approximately 30 % for the wild species and for two hybrids BS37 and BS42. The final wilting rate of the four remaining hybrids (BS2, BS31, BS33 and BS44) was significantly lower than that of the wild species (table I). However, based on the disease indices at d30, all hybrids were not significantly different from the wild species. Yet, when comparisons were performed on percentages of plants with disease indices above 2 (up to 50 % wilted plants) recorded at d23 and d30, BS31 was revealed to be the hybrid with the significantly slowest evolution (table II).

Table II. Percentages of plants displaying a disease index above 2 recorded after 23 and 30 d after root inoculation by race 3 strain of *R. solanacearum*. Disease index is ranging from 0 to 4: 0, no wilted leaves; 1, up to 25 % wilted; 2, up to 50 % wilted; 3, up to 75 % wilted; and 4, plants entirely wilted. Values followed by the same letter are not significantly different at $P = 0.05$.

Genotype	d23	d30
S. stenotomum (2n = 2× = 24)	41.7 a	72.2 a
BS2 (2n = 4×)	8.3 b	80.6 a
BS31 (2n = 4×)	13.9 b	52.8 b
BS33 (2n = 4×)	30.6 a	66.7 a
BS37 (2n = 4×)	30.6 a	52.8 b
BS42(2n = 4×)	30.6 a	72.2 a
BS44 (2n = 4×)	30.6 a	52.8 b

Bacterial populations (expressed as logarithms of cfu·g^{-1} fresh weight) recovered from roots of apparently healthy looking plants were higher than 6 whatever the bacterial strain or the plant clone. However, significant differences were recorded between both parental lines and also between the parents and hybrids. No bacterial populations were recovered within stems of the wild parent and hybrids plants inoculated with race 1 strain, whereas high populations (8.61) were found in the susceptible cultivated parent. When plants were inoculated with race 3 strain, stems of the wild species and somatic hybrids were admittedly colonised, but bacterial

populations were found to be lower than 10^4 cfu·g^{-1} fresh weight. In contrast, high populations were found within stems of *S. tuberosum* inoculated by race 3 (table III).

Table III. Estimation of populations of *R. solanacearum* within roots and stems of apparently healthy looking plants, 15 d after root inoculation by race 1 or 3 strains. Bacterial populations were expressed as the logarithm of cfu·g^{-1} fresh weight. Values followed by the same letter are not significantly different at $P = 0.05$.

	Race 1 strain		Race 3 strain	
	Root	Stem	Root	Stem
BF15 (2n = 2× = 24)	8.27 a	8.61 a	7.92 a	7.79 a
S. stenotomum (2n = 2× = 24)	7.29 b	0.00 b	7.02 b	3.31 b
BS2 (2n = 4×)	6.95 c	0.00 b	6.34 c	3.32 b
BS31 (2n = 4×)	6.98 c	0.00 b	6.10 c	3.04 b

3. Discussion

In this study, somatic hybrids between *S. tuberosum* and *S. stenotomum* have successfully been produced by using electrofusion of protoplasts. The preliminary selection of putative hybrids was based on the difference in cultural behaviour of calli, particularly callus growth and ability to regenerate shoots early. A similar method was successfully used previously to recover somatic hybrids of eggplant [34] and potato [27] and [32]. The suitable characterisation of somatic hybrids was a necessary prerequisite for the exploitation of protoplast fusion in crop improvement. Numerous precocious markers, such as isoenzyme analysis [4]; [32] and [38], nuclear DNA analysis with RFLP [21] and [25] and RAPD markers [1]; [37] and [40] were used for selection and identification of somatic hybrids. In this study, characterisation of somatic hybrids was performed by using simple sequence repeats. Such markers, locus-specific and co-dominant, were more effective than RAPD-based polymorphism assays for identification of polymorphism in plant genomes [22]; [26]; [29] and [30]. When used for the analysis of intra-specific somatic hybrids of potato, microsatellites did not show any variation due to in vitro culture [26]. In this study, STM0015 primers allowed the identification of inter-specific hybrid structures. Furthermore, ctDNA analysis by microsatellite amplification indicated that among the fourteen somatic hybrids, which had been examined, eight had potato ct-type and six *S. stenotomum* ct-type. Chloroplast DNA appeared to be randomly distributed in the somatic hybrids, but the small number of somatic hybrids examined in our experiments did not allow to conclude statistically. Nevertheless, biased plastid transmission has already been reported in tomato and eggplant [19] and [34].

The response of the parental lines to inoculation with *Ralstonia solanacearum* varied with the strain used. Whatever the strain, *S. tuberosum* cv. BF15 was highly susceptible, as all plants inoculated were wilted within 4 weeks. A high density of bacterial population was recovered from roots and stems of BF15 (10^7–10^8 cfu·g^{-1} fresh weight). In contrast, *S.*

stenotomum and all the somatic hybrids inoculated with race 1 strain did not show any wilted plants after 4 weeks. Moreover, no bacterial population was recovered from the stems of the wild species and from both hybrid clones tested, BS2 and BS31. Therefore, *S. stenotomum* and its somatic hybrids with potato are considered resistant to the race 1 strain, although high bacterial populations were found within roots. The mechanism of resistance involved in *S. stenotomum* and its somatic hybrids could concern the diffusion of bacterial population from roots to stems through collar and/or the capability to multiply within the stems. These results are in agreement with those for tomato regarding the relationship between cultivar resistance or tolerance to bacterial wilt and density of bacterial population inside the stems [12] and [14]. When inoculated with race 3 strain, the wilting rate reached 30 % for the wild parent and two somatic hybrids. For the four remaining hybrids, the wilting rate was significantly lower but also significantly different from zero. No significant difference could be recorded between disease indices of the wild parent and hybrids. Bacterial populations recovered from stems of the wild species and both somatic hybrids BS2 and BS31 were significantly lower than those found in BF15 stems: the difference was of more than three logarithmic units. Therefore, *S. stenotomum* and all hybrids could not be considered resistant to race 3 strain, since bacterial populations passed through collar and colonised stems, but rather tolerant to race 3 strain. If bacterial populations within stems could remain at such a low level without further multiplication, the probability for further wilting would be rather low or even null. In this case, *S. stenotomum* and somatic hybrids could be considered resistant rather than tolerant. Relationships between bacterial population within the plant and the establishment of the wilt disease were also observed for *Arabidopsis thaliana* inoculated with *R. solanacearum* [6]. Restricted colonisation of 'resistant' plants by *R. solanacearum* was reported to be related to the production of tyloses and other non-specific physical barriers [28]. Moreover, it was shown that potato resistance against bacterial wilt was highly enhanced when genes encoding for both biotic and abiotic resistance were combined together [7]. Resistance to race 1 was reported to be more stable than resistance to race 3 [31], which was dependent on environmental conditions, particularly temperature [13].

Taking into account the results obtained in this study together with previous work on the introgression of bacterial resistance [5]; [8] and [18], it is concluded that somatic fusion appears to be a valid means of introducing traits of resistance from wild into cultivated potatoes, and to complement and supplement conventional breeding methods for the improvement of potato. Moreover, traits of resistance to bacterial wilt, which had been introgressed into cultivated crops like eggplant by using protoplast fusion, were found to be stable in field conditions [5]. The somatic hybrids of potato and *S. stenotomum* obtained herein showed a similar level of resistance to *R. solanacearum* rather than to the wild parent. Nevertheless, further detailed evaluation of the somatic hybrids under field conditions is needed to confirm the stability of introgressed resistance to bacterial wilt, to appreciate their agronomic properties, and also to validate their potential exploitation in breeding programmes of potato.

4. Methods

4.1. Plant materials

A dihaploid clone of *Solanum tuberosum* L. (2n = 2× = 24 chromosomes) cv. BF15, obtained from the Institut National de Recherche Agronomique (Ploudaniel, France) and a clone of *S. stenotomum* (2n = 2× = 24 chromosomes) (PI234013) from the International Potato Centre (Lima, Peru) were used. Plants were propagated by subculturing leafy node cuttings, at 4-week intervals, on MS basal medium [24] containing vitamins [23], 20 $g \cdot L^{-1}$sucrose and 7 $g \cdot L^{-1}$ agar [4]. Environmental conditions were 14 $h \cdot d^{-1}$ illumination at 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 20 °C and 60 % relative humidity.

4.2. Protoplast isolation, electrofusion and plant regeneration

The protocol for protoplast isolation was derived from Chaput et al. [4]. Leaves from 4-week-old in vitro plants were scarified and placed in an enzyme solution containing CPW salts [9], 1 % (w/v) cellulase R-10 (Yakult, Tokyo, Japan), 0.05 % (w/v) pectolyase Y-23 (Sheishin, Tokyo, Japan), 0.5 M mannitol and 0.05 % (w/v) 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.5). After overnight digestion in darkness at 27 °C, protoplasts were separated from undigested material through metallic sieves (100-µm mesh), and the resulting suspension was purified and rinsed by centrifugation in successively 0.6 M sucrose, and 0.5 M mannitol + 0.5 mM $CaCl_2$ solutions. Prior to electrical fusion, the protoplasts were suspended in the last washing solution, and the density for both species adjusted to $4.0 \cdot 10^5$ protoplasts$\cdot mL^{-1}$. Electrical fusion experiments were performed as described by Sihachakr et al. [35]. Briefly, the movable multi-electrodes were placed into a 15 × 5 mm Petri dish containing 600–800 µL of a mixture (1/1) of protoplasts from both fusion partners. Protoplasts were aligned for 15 s by the application of an AC-field at 230 $V \cdot cm^{-1}$ and 1 MHz. Subsequently, one square pulse developing 1.2 $kV \cdot cm^{-1}$ was applied for 40 µs to achieve protoplast fusion. After electrical treatments, the mixture of fused protoplasts was progressively diluted with 6 mL VKM medium [2], supplemented with 250 $mg \cdot L^{-1}$ polyethylene glycol 6000 (PEG), 0.2 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 $mg \cdot L^{-1}$ zeatin, 1 $mg \cdot L^{-1}$ α-nathalenacetic acid (NAA), 0.18 M mannitol and 0.18 M glucose as osmotic agents, and 0.05 % (w/v) MES. The pH of the medium was adjusted to 5.8 prior to sterilising by filtration (0.22-µm filter, Millipore). Cultures were kept in darkness for 7 d, afterwards they were exposed to light. On day 15, cultures were diluted 8 times with fresh VKM medium supplemented with 2 $mg \cdot L^{-1}$ 6-benzylaminopurine (BAP) and 0.1 $mg \cdot L^{-1}$ 2,4-D. Two weeks later, in order to accelerate callus growth, the cultures were transferred onto the growth medium, composed of MS basal medium containing vitamins [23] and [24], 0.5 $mg \cdot L^{-1}$ NAA, 0.5 $mg \cdot L^{-1}$ zeatin, 20 $g \cdot L^{-1}$ sucrose and solidified with 7 $g \cdot L^{-1}$ agar. Calli of 3–4 mm in diameter were then transferred to the regeneration medium, which were identical to the growth medium except for growth regulators replaced by 2 $mg \cdot L^{-1}$ zeatin and 0.1 $mg \cdot L^{-1}$ indol-3-acetic acid (IAA). Emerging shoots were excised from callus and multiplied by subculturing leafy node cuttings on hormone-free MS medium. Both parental and selected hybrids plants were transferred to the greenhouse. Environmental conditions were 14 $h \cdot d^{-1}$ illumination at 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 20 °C and 60 % relative humidity for in vitro cultures and 16 $h \cdot d^{-1}$ illumination at 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 25–30 °C and 70–85 % relative humidity in the greenhouse.

4.3. Determination of ploidy level and pollen viability

Flow cytometry was used for the determination of ploidy level. About 1 cm^2 leaf material from in vitro plants was chopped with a razor blade in 1 mL buffer containing CPW salts [9], 0.5 M mannitol, 0.25 % (w/v) PEG, 0.5 % (w/v) Triton X-100, 0.25 % (v/v) mercaptoethanol at pH 6.5–7. Crude samples were filtered through a 40-µm mesh nylon and stained with 4,6 diamidino-2-phenylindole (DAPI, 5 $\mu g \cdot mL^{-1}$). DNA analysis was performed on a PARTEC CA II flow cytometer (Chemunex, Maisons-Alfort, France) equipped with a 100-W mercury lamp (type HBO). Blue fluorescence at 455 nm was recorded as a function of DNA content. The DNA distribution was analysed, by using DPAC software, on histograms generated from at least 10^4 nuclei. The dihaploid parental plants were used as external references to calibrate fluorescence scale. Pollen viability was evaluated by staining pollen grains with fluorescein diacetate (FDA, 5 $\mu g \cdot mL^{-1}$). Samples of at least 250 pollen grains each were observed under UV light. Viability was expressed as the percentage of pollen grains with a fluorescent cytoplasm.

4.4. Isoenzyme analysis

Leaf extracts were prepared from in vitro-grown plants, according to Chaput et al. [4]. Twenty microlitres of each sample was loaded on polyacrylamide gels (running gel: 7.5 % acrylamide + 0.2 % bisacrylamide; stacking gel: 4.5 % acrylamide + 0.12 % bisacrylamide), and electrophoresis performed for 3 h at 4 °C and 20 mA. Staining for esterases (EC 3.1.1.2) was done as described previously [33].

4.5. DNA analysis

Total DNA was extracted from leaf tissue with a DNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Simple sequence repeat polymorphism analysis was performed using several primer sets described by Milbourne et al. [22]. Microsatellite markers were amplified by PCR using 30 ng DNA, 10× Taq Polymerase buffer including 1.5 mM $MgCl_2$, 0.3 mM each dNTP, 0.2 µM primer (Genaxis) and 1 unit Taq DNA Polymerase (Appligene) in a total volume of 25 µL. All amplifications were performed in a Touch Gene thermal cycler using the procedure of Provan et al. [29]. Amplification products were added with a loading buffer containing formamide and denatured at 80 °C for 5 min. Samples were loaded on vertical denaturing gels (6 % polyacrylamide, 8 M urea) for 2 h at 40 W. DNA bands were stained with a silver staining kit (Promega). A similar procedure was used for amplification of chloroplast microsatellites. Primers and the thermal cycling profile were as described by Bryan et al. [3]. Amplification products were electrophoresed onto 1.8 % agarose gels, stained with ethidium bromide and then photographed on an UV box with Polaroid 665 films.

4.6. In vitro tests for resistance to bacterial wilt

Two strains of *Ralstonia solanacearum*, both originating from Reunion Island, G14* (race 1, biovar 3; isolated from *Pelargonium asperum*) and PDT-5* (race 3, biovar 2; isolated from potato), and provided by CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Saint-Pierre, Reunion Island) were used to inoculate

parental lines and somatic hybrids. Cultures of *R. solanacearum* were routinely grown (24 h, 28 °C) on basal medium, i.e. YPGA medium (yeast extract, 7 $g \cdot L^{-1}$; peptone, 7 $g \cdot L^{-1}$; glucose, 7 $g \cdot L^{-1}$; agar, 15 $g \cdot L^{-1}$; pH 7.2). Evaluation of the susceptibility to bacterial wilt was performed on in vitro plants using root inoculation with a suspension containing 10^7 colony forming units (cfu) per millilitre. Four-week-old vitroplants were used for in vitro tests. Freshly cut roots were dipped for 30 min either in the bacterial suspension (inoculated plants) or sterile water (control plants). After inoculation, plants were kept in MS medium liquid and placed in a culture room (14 $h \cdot d^{-1}$ illumination at 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 20 °C and 60 % relative humidity). The tests for bacterial resistance were made by using 36 plants per clone, distributed in three replicates. Plants were observed weekly and symptoms recorded using disease indices ranging from 0 to 4: 0, no wilted leaves; 1, up to 25 % wilted; 2, up to 50 % wilted; 3, up to 75 % wilted; and 4, plants entirely wilted. Disease indices were calculated, according to Winstead and Kelman [39]. Disease incidence was also evaluated 15 and 30 d after inoculation (d15 and d30, respectively) by estimating the percentage of entirely wilted plants (disease indices = 4). Data on disease indices and disease incidence recorded on d15 and d30 were subjected to statistical analysis using a GStat test [36]. On d15, three healthy looking plants of the parental lines, BF15 and *S. stenotomum*, and two somatic hybrids, BS2 and BS31, which had been inoculated, were sampled and washed three times in sterile 10 mM Tris buffer (pH 7.2) to remove superficial bacterial populations. Roots and stems of each plant were separately crushed in 5 mL Tris buffer. An aliquot of the resulting suspension and its suitable dilutions were spread onto modified YPGA medium plates (two replicates) and incubated at 28 °C for 3 d. Data on bacterial populations were subjected to analysis of variance (ANOVA), and means separation done by using Duncan's multiple-range test.

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