Sexual introgression of the late blight resistance gene *Rpi-blb3* from a Mexican wild diploid species *Solanum pinnatisectum* Dunals into potato varieties

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Abstract *Solanum pinnatisectum* Dunal (2*n*=2*x*=24) is a tuber-bearing Mexican wild species that has an extremely high-level of resistance to late blight (*Phytophthora infestans*). However, this species is distantly related and isolated from cultivated potato species by a strict sexual reproduction barrier. Previously, we successfully obtained triploid interspecific hybrids between chromosome-doubled *S. pinnatisectum* and a South American diploid clone carrying the *S* locus inhibitor gene (*Sli*). In this study, chromosome doubling of triploid hybrids was done to produce hexaploids used as the recurrent and donor parents in the successive backcrossing scheme. In a BC_2 population consisting of 198 genotypes, *P. infestans* was inoculated. Ninety-six genotypes were resistant, and 102 were susceptible. The presence of the *Rpi-blb3*-specific marker band perfectly matched the resistance phenotype, verifying that the *Rpi-blb3* gene controlled resistance. BC_1 and BC_2 progenies were relatively easy to reciprocally cross with cultivars. Therefore, the *Rpi-blb3* gene derived from *S. pinnatisectum* could be used in practical breeding to confer a high level of late blight resistance to potato varieties with aid of the gene-specific marker.

Keywords *Solanum pinnatisectum*, *Phytophthora infestans*, Late blight resistance, *Rpi-blb3*, Introgression

Introduction

Late blight caused by *Phytophthora infestans* (Mont.) de Bary is one of the most devastating diseases in potato. The disease was one of the reasons for the great Irish famine in the 1840s. Annually, there is 10–15% global potato production loss (Haverkort et al. 2009). Initial symptoms are small necrotic spots on foliage. Under favorable conditions lesions enlarge rapidly and destroy the entire plant in a few days.

Many resistant germplasms against late blight have been discovered from wild potato species or Andean landraces. Particularly, central Mexican species, consisting of 14 diploid and 10 polyploid species and four nothospecies (naturally occurring interspecific hybrids) according to the taxonomy of Spooner et al. (2004), is an attractive source of resistance (Neiderhauser and Mills 1953; Black and Gallegly 1957; Toxopeus 1960) because they have been coevolved with *P. infestans* in natural conditions in central Mexico a long time ago (Fry et al. 1993).

The Mexican hexaploid species *Solanum demissum* Lindl. is the most famous resistance source for the late blight, in which at least 11 race-specific resistance (*R*) genes (*R1–R11*) were found (Ross 1986). These *R*-genes were mapped on potato chromosomes (*R1* on chromosome 5, *R2* on chromosome 4, *R3a*, *R3b*, *R4*, *R6*, *R7*, *R10* and *R11* on chromosome 11, and *R8* and *R9a* on chromosome 9), and *R1*, *R2*, *R3a*, *R3b*, and *R8* were cloned (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009; Li et al. 2011; Vossen et al. 2016). To date, many cultivars (*S. tuberosum* L., $2n=4x=48$) carrying one or more *R* genes from *S. demissum* have been bred. These race-specific vertical *R*-genes initially supplied complete protection from the disease. However, the pathogen evolved quickly to overcome the resistance of these genes soon after the resistant varieties were released (Ross 1986; Fry and Goodwin 1997). Conferring race non-specific resistance is therefore required from new sources.

In a Mexican diploid species, *S. bulbocastanum* Dun., four late blight resistance genes were identified. The first *R* gene, named *RB*, was mapped on chromosome 8 (Naess et al. 2001) using second backcross populations (BC_2) derived from somatic fusion between *S. bulbocastanum* and *S. tuberosum* (Helgeson et al. 1998) and cloned (Song et al. 2003). The same gene was also cloned as *Rpi-blb1* in an F_1 population derived from an intraspecific cross between a susceptible and a resistant *S. bulbocastanum* parents (van der Vossen et al. 2003). *Rpi-bib2* was mapped on chromosome 6 and cloned from a complex hybrid including *S. acaule* Bitt., *S. bulbocastanum*, *S. phureja* Juz. et Buk. and *S. tuberosum*

(van der Vossen et al. 2005). Using this hybrid, *Rpi-apbt* was also mapped on chromosome 4 (Park et al 2005a; Oosumi et al. 2009). *Rpi-blb3* was also mapped and cloned on the chromosome 4 in an intraspecific *S. bulbocastanum* BC_1 population (Park 2005a, 2005b; Lokossou et al. 2009). Among 383 genotypes of 36 *S. bulbocastanum* accessions, 36% of the genotypes had *Rpi-blb1,* 9% of the genotypes had *Rpi-blb2* and 56% of the genotypes had *Rpi-blb3* (Lokossou et al. 2010). Furthermore, various homologues for *Rpi-blb1* and *Rpi-blb3* genes were found in some Mexican diploid and polyploid species closely related to *S. bulbocastanum*, while *Rpi-blb2* was only found in *S. bulbocastanum* (Lokossou et al. 2010).

Another Mexican diploid species, *S. pinnatisectum* Dunal, is one of the most distantly related species from the cultivated species, which is also known as an excellent source of late blight resistance and resistance to other pests and insects (Hawkes 1958, 1990; Kuhl et al. 2001; Ramon and Hanneman 2002; Chen et al. 2003). The first late blight resistance gene found in *S. pinnatisectum* was a single dominant resistance gene (*Rpi1*) mapped on chromosome 7 in a segregating population derived from a cross between a susceptible *S. cardiophyllum* Lindl. and a resistant *S. pinnatisectum* genotypes (Kuhl et al. 2001). Recently, a new resistance gene *Rpi2* was found in a BC_1 population derived from the backcrossing between a resistant *S. pinnatisectum* and a susceptible *S. cardiophyllum* genotypes and mapped on the chromosome 7 within a hotspot for resistance genes including *Rpi1* (Yang et al. 2017). Although these two genes have not been cloned, the linkage distance between *Rpi1* and *Rpi2* is estimated to be 5.4 to 5.9 cM (Yang et al. 2017). On the opposite arm of chromosome 7 a new resistance gene, designated *Pi-Blatt*, was mapped using a backcross population of resistant *S. pinnatisectum* with susceptible *S. pinnatisectum* (Nachtigall et al. 2017). Another gene, *Rpi-mch1*, was found in a population derived from an intraspecific cross of *S.* × *michoacanum* (Bitt.) Rydb., believed to be a nothospecies of *S. bulbocastanum* × *S. pinnatisectum* (Correll 1962; Hawkes 1990), and mapped on chromosome 7 close to the *Rpi1* gene (SĹiwka et al. 2012). Somatic hybrids have been successfully created using *S. pinnatisectum* (Sidorov et al. 1987; Ward et al. 1994; Menke et al. 1996; Thieme et al. 1997; Szczerbakowa et al. 2005; Polzerová et al. 2011; Sarker et al. 2011). In most somatic hybrids, male and female fertilities were low, and the degree of late blight resistance was lower than that of *S. pinnatisectum* (Szczerbakowa et al. 2005).

As mentioned above, some Mexican diploid species have a high level of broad-spectrum

resistance genes. However, these precious resistance traits have not been used in potato breeding programs because strict reproductive barriers exist between Mexican diploid species and cultivated potatoes (Hermsen and Rammana 1973; Helgeson et al. 1998; Jackson and Hanneman 1999). In tuber-bearing *Solanum* species, the interspecific crossing barrier is explained by the endosperm balance number (EBN) hypothesis (Johnston et al. 1980; Ehlenfeldt and Ortiz 1995). According to this hypothesis, a 2:1 ratio of maternal to paternal EBN in the endosperm is necessary for normal endosperm development (Johnston et al. 1980). Based on the assigned EBN and ploidy, the tuber-bearing *Solanum* species are classified into five crossing groups: the 2*x* (1EBN) group, including Mexican diploid species with exception of *S. verrucosum* Schltdl. and some South American species; the 2*x* (2EBN) group, including *S. verrucosum* and most South American species; the 4*x* (2EBN) group, including the Mexican tetraploid species (e.g., *S. stoloniferum* Schltdl.) and *S. acaule*; the 4*x* (4EBN) species, including the cultivated tetraploid potato *S. tuberosum*; the 6*x* (4EBN) group, including the Mexican hexaploid species (e.g., *S. demissum*) (Hanneman 1994). Interspecific hybridization between the same EBN species is generally successful. Between different EBN species, chromosome doubling, 2*n* pollen or haploids can help to adjust EBN values to the same (Johnston and Hanneman 1982). However, Mexican diploid species must have another isolation mechanism because chromosome-doubled plants of Mexican 2*x* (1EBN) species were not cross compatible with 2*x* (2EBN) *S. tuberosum* haploids (Novy and Hanneman 1991). Nevertheless, successful hybridizations involving the Mexican diploid species have been reported using *S. acaule* or *S. verrucosum* as bridging species (Dionne 1963; Hermsen 1966; Dinu et al. 2005; Jansky and Hamernik 2009). Therefore, currently available techniques for Mexican 2*x* (1EBN) species are limited to the use of bridging species, somatic hybridization, or cisgenesis (Kuhl et al. 2007; Haverkort et al. 2009).

In a previous study (Sanetomo et al. 2014), we successfully obtained triploid hybrids through sexual crossing between chromosome-doubled 4*x* (2EBN) *S. pinnatisectum* and a 2*x* (2EBN) clone carrying the *S* locus inhibitor gene (*Sli*). The *Sli* gene was originally identified as an inhibitor of *S*-locus-controlled self-incompatibility in pollen of diploid potatoes (Hosaka and Hanneman 1998). The obtained hybrids were triploid but had a high level of late blight resistance transferred from the parental *S. pinnatisectum*. In this study, we report successful introgression of the high level of resistance to cultivated potatoes by successive backcrossing. *Rpi-blb3* was detected as the responsible gene for late

blight resistant phenotype. For an on-going project to develop breeding clones with multiple disease and pest resistances, we performed crosses to incorporate the *Rpi-blb3* gene into potato varieties.

Materials and Methods

Plant materials

The plant materials used in this study are listed in Table 1. The following named varieties were also used as parents for crossing; Alowa, Alwara, Atlantic, Corolle, Early Rose, Haru-akari, Haruka, Koganemaru, Konafubuki, Nagasaki Kogane, Nishiyutaka, Ranran-chip, Saya-akane, Sayaka, Touya, Toyoshiro, and Zhong-Shu 3. Sanetomo et al. (2014) describe production of two triploid F_1 hybrids (11H43-1 and 11H43-2) from *S. pinnatisectum* (PI 275232).

in vitro chromosome doubling

Callus induction medium, consisted of Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), was supplemented with 2.5% sucrose, 1.0 mg/L thiamin hydrochloride, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine hydrochloride, 0.4 mg/L L-aspartic acid, 100.0 mg/L myo-inositol, 2.0 mg/L *trans*-zeatin, 1.0 mg/L IAA, and 2 g/L gellan gum. The regeneration medium consisted of the same components as the callus induction medium but IAA was removed. Both media were adjusted to pH 5.9 and autoclaved. Pieces of cut stems of F_1 hybrids (11H43-1 and 11H43-2) were incubated on callus induction medium approximately three weeks. Callus sections were transplanted on regeneration medium and subcultured every three weeks. Incubation conditions were 16 h light and 8 h darkness and a constant temperature at 20 °C.

Chromosome counting

Ploidy level of each plant was determined by counting the somatic chromosome number in root-tip cells

157 using the acetocarmine staining method.

158

159 Crosses

160

161 Crosses of two BC_1 plants derived from 3*x* F_1 hybrids × 2*x* genotypes were performed in 2012 and 2013.
162 Chromosome-doubled 6*x* F_1 hybrids were backcrossed to cultivated potatoes from 2015 to 2017. All
163 crosses were conducted in an ordinary manner in a pollinator-free greenhouse under 16 h daylength.
164 Berries were collected one month after pollination, and seeds were extracted after another month. For
165 seed extraction, matured berries were opened using a knife or squashed in tap water, and only plump
166 seeds were collected, dried, and stored in a refrigerator (4 °C) until use. Extracted seeds were soaked in
167 2,000 ppm gibberellic acid (GA_3) for 48 h and sown on a seedling tray.

168

169 Late blight resistance assay

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171 Seedlings were transplanted to potting soil in black vinyl pots with a diameter of 10.5 cm 3 weeks after
172 seed sowing and further grown for one month until inoculation of *P. infestans*. The *P. infestans* isolate
173 HU1304 (provided by S. Akino, Laboratory of Plant Pathology, Hokkaido University) was used, which
174 can overcome *R1*, *R3*, *R4*, *R5*, *R7*, *R8*, *R10* and *R11* genes. Inoculum was prepared on Rye A agar plate
175 medium for two weeks. Then, sporangia were collected and propagated on tuber slices of a susceptible
176 cultivar 'May Queen' for one week. Sporangia were collected in water and after a 3 h incubation at 4 °C,
177 sprayed on seedling plants. The inoculated seedling plants were maintained under a photoperiod of 16/8
178 h (day/night, 24 klx intensity) at 15–20 °C in a 100% moisture condition. One week later, a fungicide
179 was sprayed to stop the progression of disease. Plants with no symptoms were regarded as resistant
180 genotypes. For the resistance assay of a segregating population of *Rpi-blb3*, seedlings were grown and
181 propagated *in vitro*, transferred to potting soil in a greenhouse, and grown further. Whole plants were
182 inoculated with *P. infestans* HU1304. Because plant growth rate was considerably different by genotype,
183 four inoculation tests were conducted for different groups of plants. When typical symptoms on leaves
184 or stems or growing mycelium and sporangia were microscopically observed on the leaf surface, those
185 plants were regarded as susceptible. Plants with no symptoms were regarded as resistant. When the

symptoms were ambiguous, new plants transferred from *in vitro* stock were reevaluated.

Pollen stainability

To determine the pollen viability, pollen grains were collected and stained with acetocarmine. At least 300 pollen grains were counted for stainability under a microscope.

Marker assay

A multiplex PCR method developed by Mori et al. (2011) was used to detect resistance genes to cyst nematode (*H1*), *Potato virus X* (*Rx1*), *Potato virus Y* (Ry_{chc}), and late blight (*R1* and *R2*). Note that for this method, Ohbayashi et al. (2010) originally developed the marker band of *R2*. However, Iketani et al. (2015) suggested that this marker band is not linked with *R2* but with the field resistance QTL reported by Leonards-Schipper et al. (1994). Gene-specific PCR markers for *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* were used following the method described in Lokossou et al. (2010). The amplified fragment was Sanger sequenced using a commercial provider (Takara Bio Inc., Kusatsu, Japan)

Results

Crosses of BC_1 plants from 3*x* F_1 hybrids

We previously reported that the two 3*x* F_1 hybrid clones, 11H43-1 and 11H43-2, were crossed with various 2*x* clones and 13 BC_1 plants were obtained (Sanetomo et al. 2014). Only two of the 13 BC_1 plants were flowered. These flowering plants (12H297-1 and 12H330-1) were crossed with one 4*x* cultivar and a superior 2*x* *Sli* gene donor (97H32-6) (Table 2). Because 12H330-1 was male-fertile, it was also used as a male parent. Although 83 berries were obtained from 314 pollinations, most berries contained no or only aborted seeds. A total of 51 seeds were obtained and sown *in vitro* on MS medium, but only 16 germinated. Nine plants were extremely deformed and died before flowering whereas seven

215 grew to maturity but never flowered.

216

217 Chromosome doubling of 3*x* F_1 hybrids

218

219 Pieces of cut stems of the 3*x* F_1 hybrids (11H43-1 and 11H43-2) were grown *in vitro* on callus induction
220 medium. Many shoots were regenerated via calli on regeneration medium. Six shoots from 11H43-1
221 (16H1 family) and 25 shoots from 11H43-2 (15H160 family) were excised from the same or different
222 calli and grown separately on MS medium. They were transplanted into the pot filled with potting soil
223 and placed in a shaded area with frequent watering for several days for acclimation. Then, these plants
224 were grown further in a greenhouse. Hexaploid F_1 hybrids exhibited thick and large leaves, large
225 flowers and vigorous growth, which were easily distinguished from 3*x* F_1 hybrids. Based on
226 chromosome counting and morphological inspection, 3 out of 6 and 18 out of 25 were determined as
227 hexaploids from 11H43-1 and 11H43-2, respectively (Fig. 1A, C–E). Pollen stainability was observed
228 for one 6*x* clone from 11H43-1 (37.8%) and 11 6*x* clones from 11H43-2 (a range of 48.0–84.9%).

229

230 Crosses of 6*x* F_1 hybrids

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232 Twenty-one 6*x* F_1 hybrids were crossed with either 2*x* or 4*x* genotypes. Because of the relatively high
233 pollen stainability of 6*x* F_1 hybrids, these hybrids were also crossed as pollen parents with 4*x* genotypes
234 (Table 3). The crosses of 6*x* F_1 hybrids used as female parents were successful with both 2*x* and 4*x*
235 genotypes. The crosses of 6*x* 15H160 family members (derived from 11H43-2) with 4*x* cultivars were
236 highly successful with the berry-setting rates of 40.9% when used as female parents and 38.2% when
237 used as male parents. However, the mean number of seeds per berry was much higher when 15H160
238 family members were used as male parents (53.6 seeds/berry) than when they were used as female parents
239 (10.4 seeds/berry). When the 6*x* 16H1 family members (derived from 11H43-1) were crossed as female
240 parents with either the 2*x* or 4*x* genotype, only six seeds in total were obtained from 31 pollinations.

241

242 Late blight resistance assay of 5*x* BC_1 hybrids

243

Five hybrid families (16H133–137) derived from crosses between 6*x* F_1 hybrids and 4*x* cultivars were chosen for the late blight resistance assay (Table 4). The chromosome number of these hybrid plants was not known except for one plant (16H133-13), which certainly had 60 chromosomes (Fig. 1B, F). All these hybrid plants were tentatively regarded as 5*x* BC_1 plants. The seeds of three families (16H133, 16H134 and 16H135), derived from crosses of 15H160 family members used as male parents, germinated with a frequency of 97.5–98.6%. One hundred eighty-five seedlings were grown and subjected to the late blight resistance assay of which 95 were selected as resistant. A slightly higher selection percentage (60.0%) was obtained in the 16H135 family. This might be because Saya-akane, the parent of the 16H135 family, was thought to have a late blight resistance QTL (Iketani et al. 2015). Two families (16H136 and 16H137) were derived from crosses of 15H160 family members used as female parents with 10H17 used as the male parent. Of the 200 seeds sown, only 26 germinated and 15 of them grew to adult plants. These plants showed extremely abnormal morphology (*i.e.* thick and dark green leaves, no or little internodes, very slow growth, and appearing similar to hyperpolyploids). Although 14 of the plants were resistant to *P. infestans*, these were discarded from further use.

Crosses of late blight resistant 5*x* BC_1 plants with 4*x* cultivars

Of the 95 resistant 5*x* BC_1 genotypes, 55 were pollinated with pollen from three cultivars (Table 5). A total of 1073 flowers produced 184 berries with average berry-setting rates of 31.4% from 16H133, 6.4% from 16H134 and 21.2% from 16H135 family females. Because only 16H133 family members showed sufficient pollen stainability (an average of 54.5%), they were used as pollen parents on 318 flowers of 4 cultivars. The total of 318 flowers set 60 berries (berry-setting rate of 29.7%).

Allele mining

From our collections, three late blight resistant varieties, nine wild species accessions and four breeding clones, including the 3*x* F_1 hybrid (11H43-2) and its parental 4*x* *S. pinnatisectum* (11H2-21), were surveyed by gene-specific primer pairs for *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*. The *Rpi-blb1*-specific primer pair Blb1F/Blb1R amplified an 820 bp fragment only from *S. stoloniferum* PI 275248 (Fig. 2).

The 1/1' primer pair, which is specific for the functional allele of *Rpi-blb1*, also amplified a 213 bp fragment only from the same *S. stoloniferum* accession. The *Rpi-blb2*-specific primer pair Blb2F/Blb2R amplified no fragment from any sample. The *Rpi-blb3*-specific primer pair Blb3F/Blb3R amplified a 618 bp fragment from 11H43-2 and 11H2-21. Out of the 55 resistant 5*x* BC_1 genotypes, 51 had this 618 bp fragment. An example of 19 genotypes of the family 16H133 is shown in Fig. 3A. The amplified fragment from 11H2-21 was Sanger sequenced and showed 100% homology with the reported sequence of *Rpi-blb3* (GenBank: FJ536326.1, Lokossou et al. 2010).

Correlation between late blight resistance and a specific marker to *Rpi-blb3*

Because the resistance was suspected to be due to the function of *Rpi-blb3*, segregating populations were evaluated by late blight resistance assay and marker analysis. The 17H149 family consisted of 98 genotypes derived from a cross of 16H133-13 (2*n*=60) as a female parent with a susceptible variety Nagasaki Kogane (2*n*=48). On the other hand, the 17H150 family consisted of 99 genotypes derived from the reciprocal cross. In the 17H149 family, 41 genotypes were resistant whereas 57 were susceptible (Table 6). In the 17H150 family, 52 genotypes were resistant whereas 47 were susceptible. Total numbers of resistant and susceptible phenotypes fitted to a 1:1 ratio (P=0.4332) which indicated that one copy of a dominant gene was segregating in the population. For all 197 plants, a marker assay was applied using the *Rpi-blb3*-specific primers. All resistant plants had the 618 bp fragment and none of the susceptible plants had the fragment, which indicated a perfect match between the phenotype and the genotype (Fig. 3B).

Late blight resistance assay of BC_2 hybrid progenies

Late blight resistance was assayed by a whole plant inoculation test for 19 BC_2 families in addition to the 17H149 and 150 families described above (Table 7). Resistant and susceptible genotypes were segregated in all families, and the selection rates ranged from 20.0 to 79.2%, with an average of 45.1%. An overall ratio of 197 resistant:240 susceptible genotypes was slightly biased from 1:1 Mendelian ratio (P=0.040).

302

303 Backcrosses of BC_2 plants with various 4*x* genotypes

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305 Of the BC_2 plants, 17 genotypes from the 17H149 family and 13 genotypes from the 17H150 family were
306 chosen because these genotypes carried marker bands not only for *Rpi-blb3* but also for *H1* and Ry_{chc} (Fig.
307 3B). To accumulate multiple disease resistance genes, these 30 genotypes were crossed as female
308 parents with 14 4*x* genotypes (Table 8). All genotypes except 10H17 were long-day adapted selections
309 from F_1 or BC_1 hybrids of *S. tuberosum* ssp. *andigena* Hawkes (Table 1). Twelve male genotypes
310 produced 109 berries and mostly several hundreds of seeds. Their overall mean berry-setting rate was
311 36.5%. Of the 30 genotypes, 12 from 17H149 and six from 17H150 families were chosen as male
312 parents because they showed over 60% pollen stainability. These genotypes were crossed with ten
313 named varieties and three breeding clones to introgress the *Rpi-blb3* gene. A total of 29 berries were
314 obtained from six varieties and two breeding clones. Their overall mean berry-setting rate was 17.6%.
315 From most of the successful cross combinations, over 100 seeds were obtained.

316

317

318 **Discussion**

319

320 In the previous study (Sanetomo et al. 2014) triploid interspecific hybrids were successfully obtained by
321 crossing between chromosome-doubled 4*x* *S. pinnatisectum* and 2*x* clones carrying the *S* locus inhibitor
322 gene (*Sli*). Although the hybrids were triploid, they were crossable as females with cultivated diploids.
323 In this study, because the triploid hybrids possess an extremely high level of resistance to *P. infestans*, we
324 attempted to introgress the resistance into the cultivated potato gene pool. Unexpectedly, backcrossed
325 progeny from the triploid hybrids could not proceed to further generations. However, hexaploid plants
326 obtained by *in vitro* chromosome doubling from the triploid hybrids were easily backcrossed three times
327 to 4*x* varieties. The 3*x* F_1 hybrids were most likely 2EBN because they were derived from a cross
328 between a chromosome-doubled 4*x* (2EBN) clone of *S. pinnatisectum* and a 2*x* (2EBN) *Sli* gene donor
329 clone. By *in vitro* chromosome doubling, the EBN of 3*x* F_1 hybrids was doubled and became 4EBN
330 similar to the EBN of *S. tuberosum*. Simultaneously, male-sterile 3*x* F_1 hybrids (Sanetomo et al. 2014)

recovered pollen fertility by chromosome doubling. These hybrids resulted in successful crosses with *S. tuberosum* and their backcrosses. Therefore, use of the *Sli* gene, combined with ploidy manipulation for the EBN adjustment would provide new opportunities to use germplasms of sexually isolated Mexican diploid species, at least for *S. pinnatisectum* (Sanetomo et al. 2014).

Several resistance genes to *P. infestans* have been identified in *S. pinnatisectum*; *Rpi1* (Kuhl et al. 2001), *Rpi2* (Yang et al. 2017), *Rpi-mch1* (Śliwka et al. 2012), *Rpi-blb3* (Lokossou et al. 2010), and *Pi-Blatt* (Nachtigall et al. 2017). Among these genes, only *Rpi-blb3* was cloned (Lokossou et al. 2009), and the gene-specific PCR maker is available for (Lokossou et al. 2010). Fortunately, the resistance to *P. infestans* segregating in our backcross progenies was identified as *Rpi-blb3* by the presence of the gene sequence and its 100% correlation with the resistant phenotype. The *Rpi-blb3* gene exhibited a broad spectrum of resistance (Lokossou et al. 2010). *Rpi1*, *Rpi2*, *Rpi-mch1* and *Pi-Blatt* were all mapped to potato chromosome 7. In contrast, *Rpi-blb3* was located on potato chromosome 4 in a major late blight resistance gene cluster containing *R2*, *Rpi-abpt*, and *R2-like* (Li et al. 1998; Park et al. 2005a) and *Rpi-mcd1* (Tan et al. 2008). In the same resistance gene cluster, *Hero* conferring resistance to all known pathotypes of *Globodera rostochiensis* and partial resistance to *G. pallida* (Ernst et al. 2002) and Ny_{tbr} conferring resistance to *Potato virus Y* (Celebi-Toprak et al. 2002) are located. *Rpi-blb3* was found in most *S. bulbocastanum* accessions and in a wide variety of other Mexican species: *S. brachistotrichum*, *S. cardiophyllum*, *S. hjertingii*, *S. pinnatisectum*, and *S. stoloniferum* (Lokossou et al. 2010). Moreover, using the *Rpi-blb3*-specific primers, 22 different sequence variants were obtained from *S. bulbocastanum* and related species, suggesting that the *Rpi-blb3* gene must be very important for Mexican species to survive against *P. infestans* (Lokossou et al. 2010).

Among resistance genes identified to date from Mexican diploid species, the *Rpi-blb2* gene from *S. bulbocastanum* was introgressed to *S. tuberosum* through bridge-crosses of *S. acaule* with *S. bulbocastanum* (Hermsen 1966; Hermsen and Ramanna 1973), which finally resulted in the resistant varieties Bionica and Toluca after 46 years of breeding efforts (Haverkort et al. 2009). The *RB/Rpi-blb1* gene cloned from the *S. bulbocastanum* was transferred to *S. tuberosum* by somatic hybridization (Helgeson et al. 1998) or *Agrobacterium*-mediated transformation (Song et al. 2003; van der Vossen et al. 2003; Halterman et al. 2008). All transgenic lines containing *RB* exhibit strong foliar resistance, whereas *RB*-containing tubers do not exhibit increased resistance (Halterman et al. 2008). Shandil et al.

(2017) crossed the *RB*-transgenic Katahdin (SP951) with two popular Indian susceptible cultivars and found that the level of late blight resistance varied greatly within the F_1 progeny, i.e., a few F_1 genotypes possessing the *RB* transgene showed resistance to late blight, whereas some others were completely susceptible even possessing the *RB* transgene. Consequently, they indicated that expression level of the *RB* gene might be dependent on the genotypic background of the recipient genotype, specifically combinations with pathogenesis-related gene (PR) alleles, such as the *Sgt1* gene (Bhaskar et al. 2008; Shandil et al. 2017). As mentioned above, the use of *Rpi-blb1* and *Rpi-blb2* genes have entered the commercialization pipeline (Vleeshouwers et al. 2011). Some other resistances to *P. infestans* might have been incorporated from Mexican diploid species to *S. tuberosum* through somatic hybridization (Thieme et al. 1997, 2008, 2010; Szczerbakowa et al. 2005; Polzerová et al. 2011; Sarker et al. 2011; Tiwari et al. 2013; Luthra et al. 2016). The *Rpi-blb3* gene was incorporated singly or together with *Rpi-sto1* and *Rpi-vnt1.1* by transformation to potato variety Desiree (Zhu et al. 2012; Haesaert et al. 2015), and plants with single *R* genes showed a lower level of resistance than plants with multiple *R* genes (Haesaert et al. 2015). Overall, this report is the first in which the *Rpi-blb3* gene was transferred to *S. tuberosum* by normal backcrossing, which therefore becomes available for conventional breeding programs.

The genome constitution of the chromosome-doubled *S. pinnatisectum* could be arbitrarily designated $P^1P^1P^2P^2$, whereas that of the clone possessing *Sli* could be AA. The genome AP^1P^2 would be the most likely constitution for the obtained 3*x* F_1 hybrids, and for their 6*x* F_1 hybrids, the genome would be $AAP^1P^1P^2P^2$. We observed the segregation of late blight resistance in the 5*x* BC_1 and BC_2 populations (Tables 4, 7). This suggests that the original *S. pinnatisectum* parent was heterozygous for the resistance gene, which is consistent with other reports (Kuhl et al. 2001; Chen et al. 2003; Nochtigall et al. 2017). Even so, the resistance allele would be expected in 5*x* BC_1 plants with much higher transmission frequencies than the observed ones (Table 4). Furthermore, in the crosses of 6*x* F_1 plants with 4*x* genotypes, reciprocal differences were remarkably found in the number of seeds per berry, germination rates of the obtained seeds, morphology of the progenies and appearance frequencies of resistant plants (Tables 3, 4). Therefore, during the process of going from 6*x* F_1 hybrids to the 5*x* BC_1 and in the subsequent progenies, some genetic disorders, such as multivalent chromosome formation and abnormal disjunction at meiosis, pollen competition, and post-fertilization irregularity, might occur.

Further investigation is necessary to exploit the genetic disorders and to clarify whether the *Rpi-blb3* gene is recombined into the *S. tuberosum* chromosome.

In conclusion, we successfully introduced a broad-spectrum late blight resistance gene, *Rpi-blb3*, to potato by sexually breaking a strict reproductive barrier using the *Sli* gene combined with an EBN adjustment via ploidy manipulation. Fertile 6*x* F_1 hybrids were easily backcrossed, resulting in abundant BC_3 seeds that will be used in conventional breeding to develop new varieties with durable late blight resistance. Because a gene-specific marker is available for *Rpi-blb3*, marker-assisted selection can be applied in practical breeding and to our efforts to stack multiple disease and pest resistances into single clones in a multiplex condition (Table 8), which would be beneficial for breeders. Although, to date, the *Sli* gene has only successfully broken a reproductive barrier with *S. pinnatisectum* (Sanetomo et al. 2014; unpublished data), exploiting the functional mechanism of the *Sli* gene will shed new light on efficient germplasm enhancement activities.

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626

627 **Table 1.** Plant materials used for crossing

Accession identity	Note (pedigree, among others)
3*x* F_1 hybrid	
11H43-1	4*x S. pinnatisectum* (PI 275232) × 97H32-6 (Sanetomo et al. 2014)
11H43-2	4*x S. pinnatisectum* (PI 275232) × 97H32-6 (Sanetomo et al. 2014)
BC_1 from 3*x* F_1 hybrid	
12H297-1	11H43-2 × 97H32-6
12H330-1	11H43-2 × 2*x Sli* bulked pollen
6*x* F_1 hybrid	
16H1	*in vitro* chromosomes doubled from11H43-1 (3 regenerates)
15H160	*in vitro* chromosomes doubled from11H43-2 (18 regenerates)
2*x* genotype	
97H32-6	A superior *Sli* donor (Phumichai et al. 2006)
15H140-3	*Sli* (heterozygous) and embryo spot marker (homozygous) combined
15H143-5	*Sli* (heterozygous) and embryo spot marker (homozygous) combined
4*x* breeding clones	
Saikai 35	TD0101 × Sakurafubuki, possessing *H1* and Ry_{chc} genes (Mori et al. 2012)
10H17	Saikai 35 × Pike, possessing marker bands for *H1*, *R1*, *Rx1* and Ry_{chc} genes (Mori et al. 2012)
12H187-1	*S. tuberosum* ssp. *andigena* PI 246545 × 10H17
14H163-1	*S. tuberosum* ssp. *andigena* PI 161350 × 10H17
14H164-3S	*S. tuberosum* ssp. *andigena* PI 161683 × 10H17
15H71-2H	10H17 × *S. tuberosum* ssp. *andigena* PI 473293
16H53-2	10H17 × *S. tuberosum* ssp. *andigena* PI 473265
16H57-2S	10H17 × *S. tuberosum* ssp. *andigena* PI 473270
16H60-2S	10H17 × *S. tuberosum* ssp. *andigena* PI 473285
16H81-1	10H17 × *S. tuberosum* ssp. *andigena* PI 498291

16H85-1	10H17 × *S. tuberosum* ssp. *andigena* PI 546025
16H102-2	(*S. tarijense* PI 473244 × *S. tuberosum* ssp. *andigena* PI 473294) × 10H17
15H10-15	H98A11 × Schwalbe
16H171-2	(*S. tuberosum* ssp. *andigena* PI 161350 × 10H17) × Konafubuki
16H171-3	(*S. tuberosum* ssp. *andigena* PI 161350 × 10H17) × Konafubuki
16H12-22	Alwara × 10H17
16H12-29	Alwara × 10H17

628

629

630 **Table 2.** Crosses of two BC_1 plants, 12H297-1 and 12H330-1, derived from 3*x* F_1 hybrid clones

Female	Male	Flowers	Berries	Rate %	Seeds	Grown
12H297-1	Koganemaru	11	0	0.0	-	-
12H297-1	97H32-6	58	35	60.3	0	-
12H330-1	97H32-6	93	41	26.7	30	7
97H32-6	12H330-1	129	7	5.4	21	9

631

632

633 **Table 3.** Crosses of 6*x* F_1 hybrids (16H1 and 15H160 families, derived by *in vitro* chromosome
634 doubling from 11H43-1 and 11H43-2, respectively)

Female parent[1)]	Male parent[1)]	No. of flowers	No. of berries	Berry-setting rate (%)	No. of seeds	Seeds per berry
6*x* F_1 × 2*x*						
16H1 (2)	15H143-5	7	3	42.9	1	0.33
16H1 (1)	97H32-6	4	3	75.0	1	0.33
15H160 (7)	15H140-3	41	15	36.6	6	0.40
15H160 (10)	15H143-5	79	22	27.8	7	0.32
15H160 (8)	97H32-6	48	26	54.2	24	0.92
6*x* F_1 × 4*x*						
16H1 (1)	10H17	5	5	100.0	2	0.40
16H1 (1)	Saikai 35	4	4	100.0	2	0.50
16H1 (2)	Touya	11	1	9.1	0	-
15H160 (8)	10H17	138	81	58.7	1384	17.09
15H160 (11)	Atlantic	54	17	31.5	15	0.88
15H160 (12)	Konafubuki	65	32	49.2	55	1.72
15H160 (8)	Saikai 35	31	13	41.9	78	6.00
15H160 (10)	Saya-akane	38	0	0.0	-	-
15H160 (5)	Sayaka	21	4	19.0	2	0.50
15H160 (6)	Touya	27	6	22.2	63	10.50
4*x* × 6*x* F_1						
Saikai 35	16H1 (2)	5	0	0.0	-	-
10H17	15H160 (3)	35	14	40.0	946	67.57
Atlantic	15H160 (4)	65	30	46.2	1084	36.13
Konafubuki	15H160 (3)	16	2	12.5	84	42.00
Saya-akane	15H160 (4)	24	12	50.0	997	83.08
Touya	15H160 (2)	12	0	0.0	-	-

635 [1)] The number of regenerates used for crossing is shown in parentheses.

636

637 **Table 4.** Germination rates of hybrid seeds obtained from crosses of 6*x* F_1 and 4*x* cultivars and the
638 result of the inoculation test with *P. infestans*

Family	Pedigree	No. of seeds sown	No. of germinated plants (%)	No. of plants grown	No. of resistant plants (%)
16H133	10H17 × 15H160-38	74	73 (98.6)	60	21 (35.0)
16H134	Atlantic × 15H160-16	80	78 (97.5)	60	35 (58.3)
16H135	Saya-akane × 15H160-19	80	78 (97.5)	65	39 (60.0)
16H136	15H160-8 × 10H17	110	12 (10.9)	7*	7 (100)
16H137	15H160-22 × 10H17	90	14 (13.3)	8*	7 (87.5)

639 * Extremely abnormal phenotypes observed.
640
641

642 **Table 5.** Crosses of late blight resistant 5*x* BC_1 plants of the families 16H133, 16H134, and 16H135

643 with 4*x* varieties

Female parent*	Male parent*	No. of flowers	No. of berries	Berry-setting rate (%)
16H133 (16)	Nagasaki Kogane	159	70	45.9
16H133 (13)	Haru-akari	150	12	10.1
16H133 (12)	Haruka	80	31	38.2
16H134 (20)	Nagasaki Kogane	263	17	10.9
16H134 (12)	Haru-akari	109	0	0.0
16H134 (10)	Haruka	100	6	8.2
16H135 (19)	Nagasaki Kogane	130	44	43.9
16H135 (6)	Haru-akari	71	3	2.9
16H135 (2)	Haruka	11	2	16.7
Nagasaki Kogane	16H133 (16)	216	39	18.3
Haru-akari	16H133 (8)	68	7	8.1
Haruka	16H133 (3)	16	2	13.9
Toyoshiro	16H133 (5)	18	12	78.7

644 * No. of genotypes in parentheses.

645

646

647 **Table 6.** Segregation of late blight resistance and *Rpi-blb3* in the17H149 family (16H133-13 ×
648 Nagasaki Kogane) and 17H150 family (Nagasaki Kogane × 16H133-13)

Family	*Rpi-blb3*	Resistant	Susceptible	χ^2 test for 1:1
17H149	+	41	0	*P*=0.1060
	-	0	57	
17H150	+	52	0	*P*=0.6153
	-	0	47	
Total		93	104	*P*=0.4332

649

650

651 **Table 7.** Whole plant inoculation test of BC_2 progenies against *P. infestans*

Family	Pedigree	Total	Resistant	Susceptible	Selection rate (%)
17H157	16H133-5 × Nagasaki Kogane	30	15	15	50.0
17H158	16H133-6 × Nagasaki Kogane	30	22	8	73.3
17H160	16H133-17 × Haru-akari	10	4	6	40.0
17H161	16H133-17 × Haruka	22	14	8	63.6
17H162	16H134-15 × Nagasaki Kogane	26	7	19	26.9
17H163	16H134-19 × Nagasaki Kogane	30	7	23	23.3
17H164	16H134-23 × Nagasaki Kogane	9	3	6	33.3
17H165	16H134-29 × Nagasaki Kogane	30	9	21	30.0
17H166	16H134-30 × Nagasaki Kogane	20	4	16	20.0
17H167	16H135-3 × Nagasaki Kogane	3	1	2	33.3
17H168	16H135-6 × Nagasaki Kogane	30	6	24	20.0
17H169	16H135-10 × Nagasaki Kogane	15	4	11	26.7
17H170	16H135-16 × Nagasaki Kogane	26	15	11	57.7
17H171	16H135-21 × Nagasaki Kogane	30	18	12	60.0
17H172	16H135-34 × Nagasaki Kogane	7	2	5	28.6
17H174	Nagasaki Kogane × 16H133-6	30	17	13	56.7
17H175	Nagasaki Kogane × 16H133-9	43	24	19	55.8
17H176	Touya × 16H133-17	22	6	16	27.3
17H177	Toyoshiro × 16H133-19	24	19	5	79.2

652

653

654 **Table 8.** Crosses of the genotypes selected from the BC_2 families 17H149 and 17H150 possessing the

655 *H1*, Ry_{chc}, and *Rpi-blb3* genes with various 4*x* genotypes

4*x* genotype used as	Resistance genes[1)]	No. of BC_2 parents	No. of flowers	No. of successful BC_2 parents	No. of berries	No. of seeds[2)]
Male						
10H17	*H1*, *R1*, *Rx1*, Ry_{chc}	2	5	2	3	>200
12H187-1	*H1*, *R1*, *Rx1*, Ry_{chc}	3	9	1	1	11
14H163-1	*H1*, *R1*, *Rx1*, Ry_{chc}	9	41	6	9	>386
14H164-3S	*H1*, *Rx1*, Ry_{chc}	1	5	1	1	56
15H10-15	*Rx1*	1	3	0	0	-
15H71-2H	*H1*, *R1*, *Rx1*, Ry_{chc}	10	30	6	12	>451
16H53-2	*H1*, *R1*, *Rx1*, Ry_{chc}	13	29	7	11	>448
16H57-2S	*H1*, *Rx1*, Ry_{chc}	16	43	9	18	>890
16H60-2S	*H1*, *R1*, *Rx1*, Ry_{chc}	4	8	2	2	140
16H81-1	*H1*, *R1*, *Rx1*, Ry_{chc}	22	64	14	28	>686
16H85-1	*H1*, *Rx1*, Ry_{chc}	7	19	0	0	-
16H102-2	*H1*, *R1*, *Rx1*, Ry_{chc}	7	23	6	12	>563
16H171-2	*Rx1*, Ry_{chc}	13	17	8	9	>666
16H171-3	*H1*, *Rx1*, Ry_{chc}	1	3	1	3	>100
Female						
10H17	*H1*, *R1*, *Rx1*, Ry_{chc}	8	21	3	4	>246
16H12-22	*Rx1*, Ry_{chc}	5	14	2	2	121
16H12-29	*H1*, *Rx1*, Ry_{sto}	2	4	0	0	-
Alowa	*H1*	3	6	0	0	-
Alwara	Ry_{sto}	3	7	2	2	107
Corolle	*R1*, *H1*	7	28	1	1	92

Early Rose	None	7	27	5	9	415
Haruka	*H1*, *R1*, *Rx1*	7	25	0	0	-
Konafubuki	Ry_{chc}	1	6	1	2	>100
Nishiyutaka	None	1	1	0	0	-
Ranran-chip	*H1*, *R1*, *Rx1*	1	6	1	4	>100
Sayaka	*H1*, *R1*, *Rx1*	2	5	0	0	-
Zhong-Shu 3	None	5	15	2	5	>200

[1)]Resistance genes that can be assayed by the multiplex PCR method developed by Mori et al. (2011) or by a PCR detection method of Ry_{sto} (Song and Schwarzfischer 2008).

[2)]The number of seeds from a cross combination that produced more than 100 seeds was recorded as >100.

661 Figure Legends

662

663 **Fig. 1.** Morphology of whole plants and somatic chromosomes in root tip cells. A, 6*x* F_1 hybrid
664 (15H160-38); B, 5*x* BC_1 plant (16H133-13)**;** C, D, aceto-carmine-stained 6*x* F_1 hybrids (16H1-8 and
665 15H160-11, respectively, 2*n*=72); E, F, DAPI-stained 6*x* F_1 hybrid (15H160-38, 2*n*=72) and 5*x* BC_1
666 hybrid (16H133-13, 2*n*=60), respectively. Photographs of DAPI-stained chromosomes were provided
667 courtesy of S. Kikuchi (Chiba University, Japan)

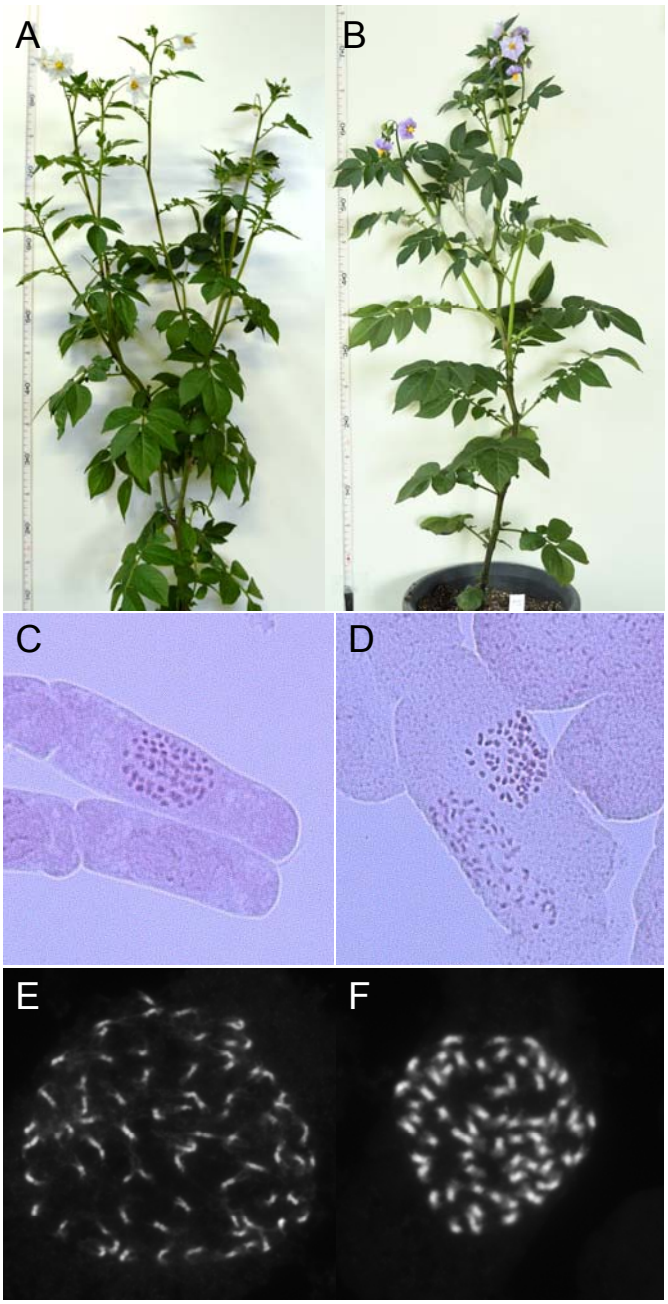
668

669 **Fig. 2.** Survey of late blight resistant genotypes using gene-specific markers for *Rpi-blb1*, *Rpi-blb2*, and
670 *Rpi-blb3*. M, *Hin*dIII-digested lambda DNA; 1, W553-4; 2, Hanashibetsu; 3, Saya-akane; 4, Konahime;
671 5, *R2* differential; 6, *S. demissum* (PI 186551); 7, 11H43-2; 8, 11H2-21 (4*x S. pinnatisectum*); 9–10, *S.*
672 *verrucosum* (PI 195170 and PI 275260, respectively); 11–16, *S. stoloniferum* (PI 249929, PI 283101, PI
673 251740, PI 255547, PI 184770, and PI 275248, respectively)

674

675 **Fig. 3.** Marker assay for late blight resistant genotypes of the 16H133 family (A) and resistant (R) and
676 susceptible (S) genotypes of 17H149 and 17H150 families (B). The multiplex PCR method developed
677 by Mori et al. (2011) detects *R1*, *Rx1*, *R2*, Ry_{chc}, and *H1* with a positive marker *GBSS* simultaneously.
678 The *Rpi-blb3*-specific primer pair Blb3F/Blb3R amplified a 618 bp fragment from genotypes with
679 *Rpi-blb3*

680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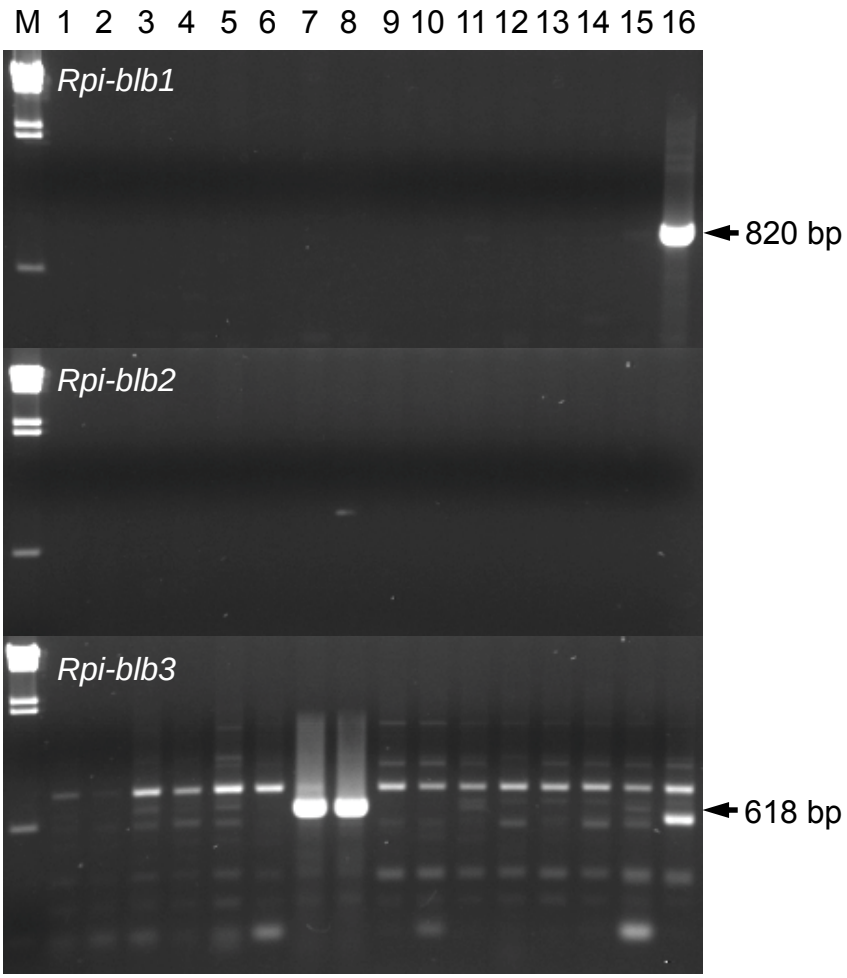
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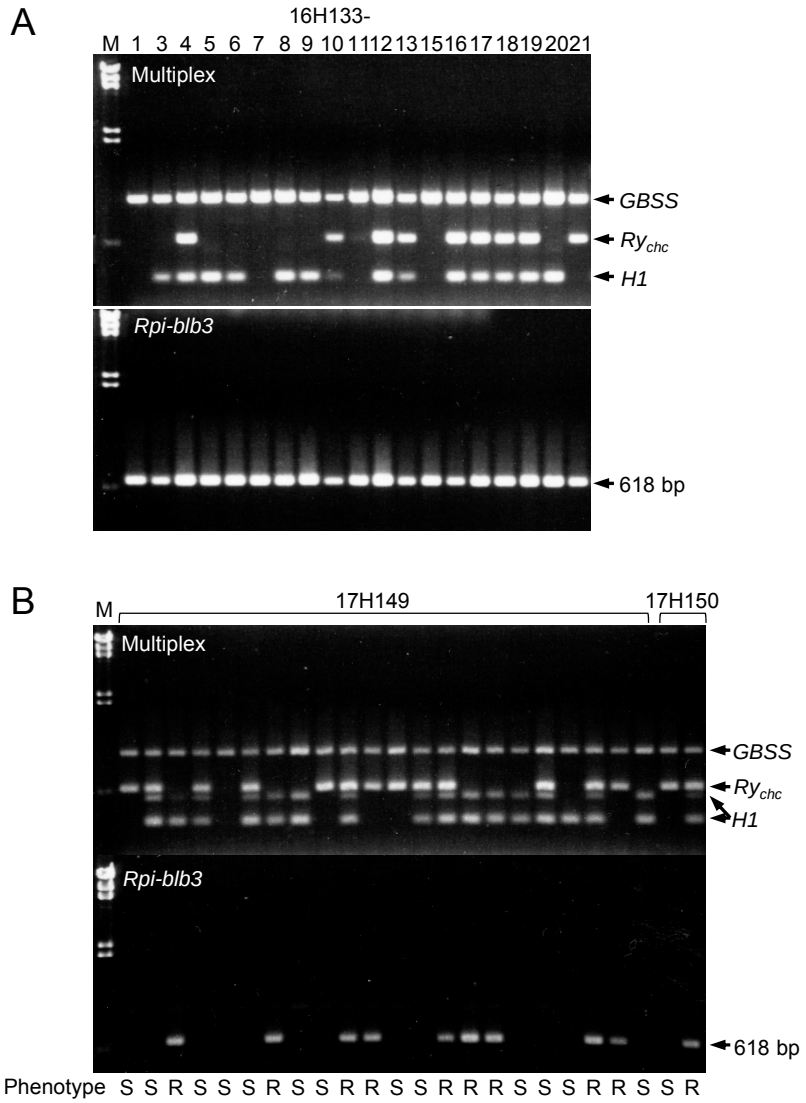
Rpi-blb2
Rpi-blb3
618 bp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Multiplex
GBSS
Ry chc
H1
Rpi-blb3
618 bp
B
M
17H149
17H150
Multiplex
GBSS
Ry chc
H1
Rpi-blb3
618 bp
Phenotype S S R S S S R S S R R S S R R R S S S R R S S R