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Research Paper

Improvement of diagnostic markers for resistance to *Globodera pallida* and application for selection of resistant germplasms in potato breeding

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Occurrence of pale potato cyst nematode, *Globodera pallida* (Stone) Behrens, was first recorded in Japan in 2015. Among several control measures, cultivation of resistant potato (*Solanum tuberosum* L.) varieties is the most effective in cost and environmental impact. As no *G. pallida*-resistant varieties have yet been developed in Japan, great emphasis is being placed on screening of germplasm possessing the resistance and development of the resistant varieties. In this study, we first improved previously reported DNA markers linked to the *G. pallida* resistance loci ($GpaIV^{s}_{adg}$ and *Gpa5*) and then used these to screen more than 1,000 germplasms to select several candidate germplasms with resistance. We performed inoculation testing on the selected candidates and identified several resistant germplasms to the Japanese *G. pallida* population. Furthermore, we developed a simultaneous detection method combining three DNA markers linked to *G. pallida* and *Globodera rostochiensis* (Wollenweber) Behrens resistance loci. We validated the ability of C237-I marker to select resistant allele of $GpaIV^{s}_{adg}$ and predict the presence of resistance in a Japanese breeding population. Resistant germplasms identified in this study could potentially be used to develop *G. pallida*-resistant varieties. The marker evaluation methods developed in this study will contribute to the efficient development of resistant varieties.

Key Words: potato, *Globodera pallida*, resistance, germplasms, DNA marker, marker assisted selection (MAS).

Introduction

The potato cyst nematodes (PCNs) are a significant threat to potato (*Solanum tuberosum* L.) cultivation worldwide. Plants infested with PCNs are stunted and may wilt, the leaves may yellow or display a dull color, and tuber yield is decreased compared to healthy plants. PCNs cause yield losses of 20%–70%, with worldwide losses estimated at 30% (Narabu *et al.* 2017, Oerke *et al.* 1994, Trudgill *et al.* 2014). Eggs within the cysts can survive more than ten years in the soil and can persist under unfavorable environmental conditions, making eradication of PCNs challenging (Grainger 1959). *Globodera rostochiensis* (Wollenweber) Behrens, the golden potato cyst nematode, and *G. pallida* (Stone) Behrens, the pale potato cyst nematode, are spreading worldwide and are subjected to stringent quarantine and regulatory procedures in many countries (OEPP/EPPO 2020, Pickup *et al.* 2018). Moreover, a new species, *G. ellingtonae* Handoo, Carta, Skantar, and Chitwood, has recently been described (Handoo *et al.* 2012).

Pathotype Ro1 of *G. rostochiensis*, was first identified in Japan in 1972 (Yamada *et al.* 1972) and has been spread in Hokkaido and other potato production areas. In contrast,

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G. pallida has been detected in Japan only recently. In 2015, mature females and cysts were observed on the roots of Ro1-resistant varieties collected from some fields in Abashiri, Hokkaido, northern prefecture. Detailed analysis of morphology and the ribosomal DNA (rDNA) sequence confirmed that the nematode was *G. pallida* (Narabu *et al.* 2016). Comparison of rDNA and the *cytochrome b* gene sequences with those of overseas populations revealed that the Japanese population of *G. pallida* is closely related to those found in Europe and North America (Ohki *et al.* 2018).

PCNs can be controlled by crop rotation, chemical soil disinfestation, use of trap crops, and cultivation of resistant varieties (Dandurand and Knudsen 2016, Wale *et al.* 2008). In infested regions of Japan, the measures taken to control *G. pallida*, include crop rotation, application of 1,3-dichloropropene, and cultivation of a trap crop (*S. peruvianum* L.) (Nakanishi 2020). Concern about the environmental effects, especially the impact on fisheries, has precluded fumigation with 1,3-dichloropropene in some fields. In certain low-temperature areas trap crops do not grow vigorously enough for their effect to be worthwhile against PCNs. Under these circumstances, cultivation of resistant varieties provides the most effective measure in terms of cost and environmental impact, but since no *G. pallida*-resistant varieties have been developed in Japan, great emphasis is placed on germplasm screening and resistant varieties development.

Due to plant quarantine laws and limited availability of inoculation testing facilities, evaluation of all germplasms by inoculation testing cannot be conducted. First, there is a need to narrow down candidate germplasms by several measures, such as selection by DNA markers, pedigree records, and information on databases. In a previous study, we employed three DNA markers for PCN-resistance genes/loci, *H1* and *Gro1-4* for *G. rostochiensis*, and *Gpa2* for *G. pallida* (Asano *et al.* 2012). We identified several varieties possessing *Gpa2* as candidates of *G. pallida*-resistant varieties. However, these varieties were susceptible to Japanese *G. pallida* because mature females and cysts were found on 'Aiyutaka' and 'Konayuki', both varieties possessing *Gpa2* (Asano *et al.* 2012, Ohki *et al.* 2018). The *Gpa2* locus is known to confer specific resistance to a small set of populations of *G. pallida*, D383, and D372, which can be differentiated from other *G. pallida* populations found in the Netherlands (van der Voort *et al.* 1997). Given these facts and the result of DNA sequences comparison (Ohki *et al.* 2018), the Japanese population is considered different from the populations to which *Gpa2* confers resistance. Virulence characteristics differ among PCN populations (Phillips and Trudgill 1998, Rigney *et al.* 2017), so screening of potato germplasms possessing resistance genes/quantitative trait loci (QTLs) to the *G. pallida* populations is a prerequisite to developing resistant varieties.

Several genes and QTLs conferring *G. pallida* resistance derived from other cultivated species and wild relatives have already been identified in potato (Bryan *et al.* 2002, 2004, Caromel *et al.* 2003, 2005, Finkers-Tomczak *et al.* 2009, Gebhardt and Valkonen 2001, Moloney *et al.* 2010, Sattarzadeh *et al.* 2006, Tan *et al.* 2009, van der Voort *et al.* 1997, 2000). Yet, though, many of these genes have not been introduced into commercial varieties or advanced breeding materials. Although introgression of diverse resistance genes into commercial varieties is an important task to prevent the breakdown of resistance genes, extensive backcrossing with cultivated species is required to eliminate undesirable donor traits, thus, it takes considerable time to bring such resistance resources into practical use. However, two sources of resistance to *G. pallida* have already been introduced into commercial potato varieties and are considered to be of practical use (Dalton *et al.* 2013). Quantitative resistance to *G. pallida* derived from the cultivated species *S. tuberosum* ssp. *andigena* 'CPC 2802' and the wild relatives *S. vernei* have been introgressed into some varieties and advanced breeding lines (Bradshaw *et al.* 1998, Bryan *et al.* 2002, 2004, van der Voort *et al.* 2000). The major loci associated with resistance in these germplasms have been identified as $GpaIV^{s}_{adg}$ and *Gpa5*, and potentially diagnostic markers, C237 and HC, have been developed for both loci (Achenbach *et al.* 2009, Moloney *et al.* 2010). In addition, it has been confirmed that the pyramiding of these QTLs confers a high level of resistance to multiple populations of *G. pallida* (Dalton *et al.* 2013, Rigney *et al.* 2017). These QTLs should be prospective candidates conferring resistance against the Japanese *G. pallida* population, thus, screening and using germplasms possessing these QTLs should be useful strategies for the rapid development of resistant varieties. However, our previous study indicated that these markers did not work in Japanese germplasms under the given experimental conditions. Original C237 is a cleaved amplified polymorphic sequence (CAPS) marker, which is said to produce band linked to $GpaIV^{s}_{adg}$ after Taq I digestion. There were polymorphisms among germplasms, however, a specific band linked to $GpaIV^{s}_{adg}$ could not be identified. Original HC is an allele-specific PCR marker amplifying bands when germplasm possess single-nucleotide polymorphism (SNP) linked to *Gpa5*. However, HC marker were amplified even in germplasms considered to be susceptible for *G. pallida* and could not obtain *Gpa5*-specific amplification (Asano *et al.* 2012).

In this study, we improved DNA markers for $GpaIV^{s}_{adg}$ and *Gpa5* based on published information. Using improved DNA markers (we named it C237-I and HC-I), we evaluated our germplasms to screen potential candidates with resistance. We also selected candidate germplasms based on previous research results, the information in databases, and pedigree records. We conducted inoculation testing against selected candidates and confirmed several germplasms that showed resistance to the Japanese *G. pallida* population. We also validated the ability of the C237-I marker to select for $GpaIV^{s}_{adg}$ and predict the presence of

resistance in a Japanese breeding population.

Further, we developed simultaneous detection of three DNA markers, N195 for *G. rostochiensis*, and C237-I and HC-I2 (improved the HC marker for simultaneous detection) for *G. pallida*. In Japan, there are fields infested with both *G. rostochiensis* and *G. pallida*. Therefore, the development of varieties having multiple resistance against both species is required. Simultaneous detection will contribute to the efficient selection of such varieties stacking several resistance genes in breeding programs.

Materials and Methods

Plant materials and DNA extraction

We evaluated 1,143 germplasms, including wild relatives, other cultivated species, landraces, modern varieties, and lines derived from crosses among these germplasms. To improve DNA markers and determine polymerase chain reaction (PCR) conditions, standard germplasms were chosen based on previous research results. For the improvement of C237, 'Eden' and '12601ab1' were used, both of which were previously reported to possess $GpaIV^{s}_{adg}$ (Moloney *et al.* 2010). Development of a novel CAPS marker for *Gpa5* was conducted using 'Innovator', which is known to possess *Gpa5* (Achenbach *et al.* 2009, Sattarzadeh *et al.* 2006). To confirm the new markers' specificity, 'Elles', 'Irida', and 'Sassy' were used. These varieties were recorded as *G. pallida*-resistant varieties by breeder's evaluation (the European Cultivated Potato Database: https://www.europotato.org/, the Potato Variety Database: http://varieties.ahdb.org.uk/, personal communication). 'Irish Cobbler' and 'May Queen' were used as susceptible varieties.

To test the suitability of the C237-I marker for selection of *G. pallida* resistance, 27 F_1 progeny derived from 'Eden' (C237-I +) and 'Hokkai 106' (C237-I –) was used. The 27 lines were selected from 143 F_1 progeny at secondary individual clonal selection test by evaluating agronomical traits, such as tuber number, shape, and size, while selection for resistance to *G. pallida* was not conducted. At line selection test, genotypes of the 27 lines were determined, and tubers were subjected to inoculation testing.

Potato leaves were harvested and stored at –30°C. Total DNA was isolated from the leaves by the hexadecyltrimethylammonium bromide method (Doyle and Doyle 1987).

DNA marker assay

The total volume in the PCR assay was 10 µl, comprising 2 µl template DNA, 5 µl gene amplification reagent (Ampdirect Plus, Shimadzu Corp., Kyoto, Japan), 0.25 units Taq DNA polymerase (BIOTAQ HS; Bioline, London, UK), and the related primer pair with concentrations shown in **Table 1**. To establish PCR condition, 20 ng/µl DNA was used. however, for germplasm screening, DNA was used without adjustment of concentration. Thermal cycling was performed using 96-well or 384-well thermal cyclers (Veriti, Applied Biosystems, Life Technologies, Carlsbad, CA). The PCR conditions for C237-I consisted of one cycle of 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, and finally one cycle of 5 min at 72°C. The PCR conditions for HC-I consisted of one cycle of 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and finally one cycle of 7 min at 72°C. The PCR product of HC-I was digested with restriction endonuclease Hind III. Three DNA markers, N195, C237-I, and HC-I2, were detected simultaneously using the following conditions; one cycle of 10 min at 94°C, followed by 37 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, and finally one cycle of 7 min at 72°C. After PCR cycling, the PCR product of multiplex PCR was digested with Hind III. All PCR products were separated by electrophoresis on 1.2%

Table 1. Information on primers used in this study

DNA marker	Target locus	Primer name	Primer sequence (5′→3′)	Expected size (bp)	Individual PCR		Multiplex PCR
					Conc. (µM)	Annealing temp. (°C)	Conc. (µM)
C237-I	$GpaIV^{s}_{adg}$	contig237_fwd*	GCAGTCCTAATTGCACGTAACA	138	0.2	55	0.2
		C237-SR2	CGATCAATACCATATGGTCA		0.2		0.2
HC-I	*Gpa5*	BA87d17t3 F	GTAGTACATCAACATACATTTTGCGG	490	0.2	56	–
		HC R*	GCCTTACTTCCCTGCTGAAG		0.2		–
HC-I2		BA87d17t3 F	GTAGTACATCAACATACATTTTGCGG	458	–	–	0.2
		BA87d17t3 R2	GGTGACTAAGATGGAAATCAGAG		–	–	0.2
N195	*H1*	N195-09	TGGAAATGGCACCCACTA	337	–	–	0.063
		N195-06	CATCATGGTTTCACTTGTCAC		–		0.063
GBSS	*GBSS1*	gbss-03	ATAGGAATGTCAAGTGGTAGCG	441	0.2	55	0.05
		gbss-04	TAGGCAAGAGAATCAAAATCAG		0.2		–
		GBSS1 R2	GACAAGGTTGATTTGGTGTCTAG	641[a]	–	–	0.1

[a] Expected size when combined gbss-03 in multiplex PCR.
* Sequence from Moloney *et al.* (2010) and Achenbach *et al.* (2009).

Breeding Science
Vol. 71 No. 3 **BS**

agarose gels for individual PCR and 2% for multiplex PCR in 1× TAE buffer and visualized with SYBR Safe DNA gel stain (Invitrogen, Life Technologies, Carlsbad, CA) and UV.

Assessment of resistance to G. pallida

Resistance to *G. pallida* was tested using two inoculation methods, cup or pot test or both. Since most germplasms are conserved as genetic resources and commercial seeds are not distributed, the number of seeds available for inoculation testing is limited. Also, strict plant quarantine laws meant that we could not test all candidates by both methods simultaneously in our limited facilities. Therefore, we selected several representative germplasms from candidates based on pedigree information. Evaluations were divided into three occasions and conducted between 2016–2017. If germplasm showed resistance, the evaluation was repeated, while if it was susceptible, the germplasms were only assessed once. For the cup test, a tuber was planted in a transparent plastic cup containing 125 ml soil (four replicates). *G. pallida* suspension (1 ml) containing 1,000 infective eggs and juveniles per ml was added to each cup. The cups were incubated at 20°C for approximately 70 days with appropriate watering, after which newly formed cysts were counted from outside the cup. Five tubers per genotype were planted separately in 12-cm vinyl pots containing 500 ml soil for the pot test. After germination, 5,000 eggs were inoculated to each pot. After approximately 70 days of growth in a greenhouse at 20°C, newly formed cysts were separated from the soil and roots according to the floating cyst sieving method (Mizukubo and Futai 2014, Sakata *et al.* 2021) and counted separately for each pot. The mean cyst number over the five replicates was established for each genotype. *G. pallida* used in this study were collected in Abashiri and propagated on susceptible varieties. The degree of susceptibility was quantified as a score of 1–9 based on relative susceptibility (RS) of tested germplasm to the standard variety. Score 1: RS > 100, 2: RS = 50.1–100, 3: RS = 25.1–50, 4: RS = 15.1–25, 5: RS = 10.1–15, 6: RS = 5.1–10, 7: RS = 3.1–5, 8: RS = 1.1–3, 9: RS < 1. Where RS = cyst number formed on test germplasm/cyst number formed on standard variety × 100. We tentatively set score four as the threshold for resistance because some varieties categorized as partially resistant scored four in the Potato Variety Database (http://varieties.ahdb.org.uk/). We judged the resistance according to the following criteria. RS = 1–3; susceptible, RS = 4–6; moderately resistant, RS = 7–9; resistant.

Statistical analysis

The statistical analysis was performed using JMP statistical software v.13.0.0 (SAS Inc., Canada). Since assumptions of homoscedasticity were not met such that parametric tests could be used, the Wilcoxon rank-sum test was conducted to identify significant differences between genotypes (C237-I + or C237-I –).

Results

Improvement of DNA markers for GpaIV$^{s}_{adg}$ and Gpa5

DNA marker C237 is a CAPS marker for *GpaIV$^{s}_{adg}$*, and its amplicon sequence has been published (Moloney *et al.* 2010). Based on this sequence, we designed primers detecting SNP's presence reported to exhibit diagnostic potential for the QTL. To avoid erroneous judgment due to PCR error, a marker amplifying the *granule-bound starch synthase 1* (*GBSS1*) gene was combined with C237-I. *GBSS1* is shared in all potato species (Spooner *et al.* 2008); thus, it functions as a positive control to check whether the PCR was performed correctly or not (Asano *et al.* 2012, Mori *et al.* 2011). The expected size band (160 bp) was amplified explicitly in 'Eden' and '12601ab1' by improved DNA marker C237-I (**Fig. 1A**). Before evaluating all germplasms, several germplasms underwent a preliminary evaluation, and a positive band was obtained in some germplasms, including *S. tuberosum* ssp. *andigena* '553-4', 'Musamaru', 'Ehud', and 'Astarte'. The presence of target SNP in these materials was confirmed by direct sequencing (data not shown).

The SNP marker HC was developed to specifically detect haplotype c linked with a high level of *G. pallida* resistance. The two pairs of forward and reverse primers were designed on SNPs BA87d17t3-snp212 (T/C), in which haplotype c has allele T, and on snp444 (T/C) (Sattarzadeh *et al.* 2006). Since, snp212 is a recognition site for the restriction enzyme Hind III in haplotype c, we newly developed a CAPS marker using this SNP. We used the forward primer, BA87d17t3 F, with the original reverse primer, HC R, to amplify the region that included snp212.

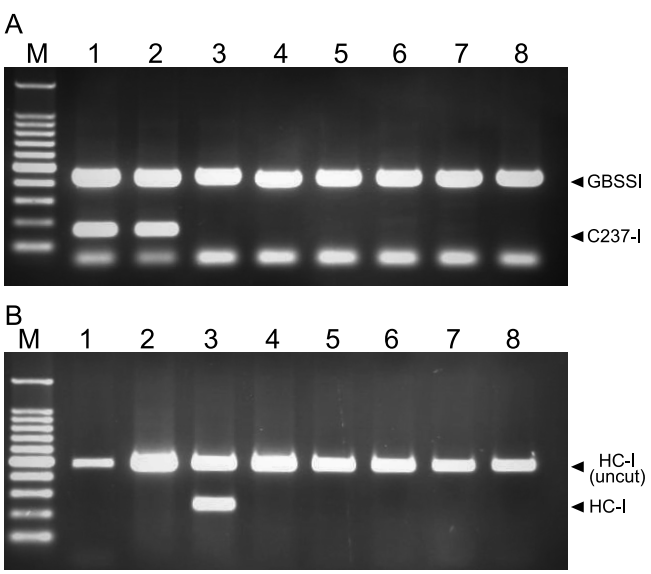

Fig. 1. Improvement of DNA markers for *G. pallida* resistance locus. Improved DNA markers for *GpaIV$^{s}_{adg}$* (A) and *Gpa5* (B). M: 100 bp marker, 1: 'Eden', 2: '12601ab1', 3: 'Innovator', 4: 'Sassy', 5: 'Elles', 6: 'Irida', 7: 'Irish Cobbler', 8: 'May Queen'.

The combination of the newly developed forward primer and the original reverse primer obtained a 490 bp DNA fragment in all tested germplasms. After digestion with Hind III, a novel band was observed only in 'Innovator'. Theoretically, digestion with Hind III produces 238-bp and 257-bp bands; however, because the size difference between the two bands is small, what appeared to be a single band was obtained around 250 bp (**Fig. 1B**). The digested band was not observed even in 'Irida' and 'Elles', varieties that are categorized as *G. pallida*-resistant in the European Cultivated Potato Database (https://www.europotato.org/) or by breeder's evaluation; their resistance is considered to be derived from '$(VT_n)_2$ 62-33-3'.

Evaluation of the germplasms by improved markers

A total of 1,143 germplasms were evaluated for the presence of these improved markers. Of them, 45 germplasms were positive for C237-I. Most of these germplasms have *S. tuberosum* ssp. *andigena* in their pedigree. 'Tunika', which possesses the *H1* gene derived from *S. tuberosum* ssp. *andigena* 'CPC1673', has been frequently used as an *H1* resource in Japan (Asano *et al.* 2012), and its progeny account for more than one-third of the 45 germplasms. Eleven germplasms positive for C237-I were the progeny of different *S. tuberosum* ssp. *andigena* germplasms, which have been used to introduce favorable traits, such as resistance to late blight and tolerance to low-temperature sweetening, during Japanese potato breeding. Six of the C237-I-positive germplasms had different species in their pedigree, including *S. phureja*, *S. chacoense*, *S. vernei*, and *S. boliviense*. One germplasm was the progeny of 'Eden'. The origin of C237-I in two germplasms was unclear (**Table 2**).

We found 12 germplasms were positive for the HC-I marker. Among them, nine derived from *S. vernei*, and one each from *S. boliviense* and *S. tuberosum* ssp. *andigena*. The origin of HC-I in one germplasm was unclear. 'W792224-1', the progeny of *S. boliviense*, was positive for both markers (**Table 3**).

Neither DNA marker was detected in some germplasms, including 'Irida', '$(VT_n)_2$ 62-33-3', 'Starter', 'Elles', and 'Darwina', which are all categorized as *G. pallida*-resistant in the European Cultivated Potato Database or by breeder's evaluation.

Inoculation testing for candidate germplasm

We selected some representative germplasms for inoculation testing based on pedigree information. Some germplasms whose marker was presumed to be derived from the same lineage were excluded from inoculation testing to reduce samples for the test. Among 45 and 12 germplasms positive for C237-I and HC-I, 22 and 11 germplasms (in total, 32 germplasms as both markers were detected in W792224-1) were selected for inoculation testing, respectively. Also, we selected four germplasms utilizing information in the European Cultivated Potato Database, pedigree records, and breeder's evaluations. Irish Cobbler, which is negative for both markers, was highly susceptible at all test times in both methods. Among germplasms positive for C237-I, germplasms derived from 'Tunika' did not show resistance. Alternatively, some lines derived from other *S. tuberosum* ssp. *andigena* germplasms ('233', '234', and '553-4') showed moderate resistance with a score 4–6. 'W792224-1', which was positive for C237-I and HC-I, scored 5 by cup test but scored 3 by pot test. 'Eden' and '12601ab1', which were used to develop the C237 marker, showed relatively high resistance with scores of 7–9. Other germplasms with unknown origin showed no resistance (**Table 4**).

Among HC-I-positive germplasms, only three commercial varieties, 'Innovator', 'Furia', and 'G06SC278.004', showed high resistance. 'W792226-1' scored 4–6 by cup test but scored 3 by pot test. Germplasms, including 'Darwina', 'Elles', and 'Irida', all progeny of '$(VT_n)_2$ 62-33-3', which were selected based on information in the database pedigree records, and breeder's evaluations, showed moderate resistance and scored above 4 (**Table 4**).

Effects of MAS by improved C237-I

As the availability of DNA markers largely depends on genetic background, we tested the suitability of the C237-I marker for selection of *G. pallida* resistance in our material. Twenty-seven F_1 progeny derived from 'Eden' (C237-I +) and 'Hokkai 106' (C237-I –) were classified by C237-I genotype and evaluated for resistance to *G. pallida* by pot test. On 'Eden' and 'Hokkai 106', 29.9 and 1357.8 *G. pallida* cysts developed, respectively. On progeny lacking C237-I the cyst count was 85.7–1708.0, while on progeny positive for C237-I the cyst count was 47.8–1074.3 (**Fig. 2A**). Average cyst counts were 672.2 and 317.3 on progeny without and with C237-I, respectively, a significant decrease (Wilcoxon rank-sum test, $p = 0.048$) of 52.8% in the development of cysts was conferred by $GpaIV^{s}_{adg}$ (**Fig. 2B**).

Simultaneous detection of three DNA markers linked to G. pallida and G. rostochiensis resistance

Since *G. rostochiensis* and *G. pallida* are often present in the same fields in Japan, it would be desirable to develop potato varieties possessing resistance against both species. Therefore, the selection of genotypes possessing *H1* and $GpaIV^{s}_{adg}$ or *Gpa5* or both, as indicated by DNA markers, would be an efficient way to develop practical varieties. With this in mind, we developed a method to simultaneously detect DNA markers for *H1*, $GpaIV^{s}_{adg}$, and *Gpa5* to enable breeders to select such genotypes via a single PCR experiment. Usually, two DNA markers, N146 and N195, located on either side of *H1* are used in Japan (Asano *et al.* 2012, Asano and Tamiya 2016, Mori *et al.* 2011, Takeuchi *et al.* 2008). However, since N146 has a Hind III site within its amplicon and is digested by Hind III, only N195 was used for the multiplex PCR. We also designed the BA87d17t3 R2 as a reverse primer for HC (we named it HC-I2).

Table 2. Germplasms positive for C237-I and its possible origin

Germplasm name	Pedigree		Possible origin of C237-I
	Female	Male	
Eden	10899AD	Maris Piper	*S. tuberosum* ssp. *andigena* (CPC2802)
12601ab1	10886 (10)	10899ad (15)	*S. tuberosum* ssp. *andigena* (CPC2802)
12169-37	Eden	Hokkaikogane	*S. tuberosum* ssp. *andigena* (CPC2802)
WB86007-2	*S. tuberosum* ssp. *andigena* 233	Hokkai 51	*S. tuberosum* ssp. *andigena* 233 (Malcachu)
WB86007-80	*S. tuberosum* ssp. *andigena* 233	Hokkai 51	*S. tuberosum* ssp. *andigena* 233 (Malcachu)
W804421-3	*S. tuberosum* ssp. *andigena* 234	R392-13	*S. tuberosum* ssp. *andigena* 234 (Collareja)
WB86015-22	*S. tuberosum* ssp. *andigena* 234	Shimakei554	*S. tuberosum* ssp. *andigena* 234 (Collareja)
85124-12	*S. tuberosum* ssp. *andigena* 553-4	Kita-akari	*S. tuberosum* ssp. *andigena* 553-4
85125-20	*S. tuberosum* ssp. *andigena* 553-4	WB61037-4	*S. tuberosum* ssp. *andigena* 553-4
Flower-4	*S. andigena* 553-4 NP[a]	–	*S. tuberosum* ssp. *andigena* 553-4
S. tuberosum ssp. *andigena* 553-4	*S. andigena*	–	*S. tuberosum* ssp. *andigena* 553-4
WB93009-1	WB88008-1 NP[a]	–	*S. tuberosum* ssp. *andigena* 553-4
PI 473260-1	*S. tuberosum* ssp. *andigena* (PI 473260)	–	*S. tuberosum* ssp. *andigena* (PI 473260)
Flower-3	*S. tuberosum* ssp. *andigena* × *S. tuberosum*	*S. tuberosum*	*S. tuberosum* ssp. *andigena*
Ehud	Panther	Karna 149	*S. tuberosum* ssp. *andigena* (CPC1673)
Juliette	Nicola	Hansa	*S. tuberosum* ssp. *andigena* (CPC1673)
Verdi	Tomensa	Diana	*S. tuberosum* ssp. *andigena* (CPC1673)
Tunika	Lü 56.186-21N	Lü 51.183-2	*S. tuberosum* ssp. *andigena* (CPC1673)
79090-8	Tunika	R392-50	Tunika (CPC1673)
97020-4	Yukirasha	Musamaru	Tunika (CPC1673)
994209-1	Diploid of Musamaru	–	Tunika (CPC1673)
Hokkai 64	Tunika	WB61037-4	Tunika (CPC1673)
Hokkai 96	Musamaru	Hokkaikogane	Tunika (CPC1673)
Kachikei 13	Musamaru	Hokkai 84	Tunika (CPC1673)
Kachikei 21	Yukirasha	Musamaru	Tunika (CPC1673)
Koganemaru	Musamaru	Tokachikogane	Tunika (CPC1673)
Musamaru	Tunika	Kon-iku 20	Tunika (CPC1673)
Natsufubuki	Musamaru	Shimakei544	Tunika (CPC1673)
Shimakei 530	Tunika	WB61037-4	Tunika (CPC1673)
Shimakei 537	Toyosiro	Tunika	Tunika (CPC1673)
Shimakei 572	Shimakei 530	ND860-2	Tunika (CPC1673)
W784227-H.2	Diploid of Tunika	–	Tunika (CPC1673)
W794215-H.20	Diploid of Tunika	–	Tunika (CPC1673)
W794215-H.34	Diploid of Tunika	–	Tunika (CPC1673)
W804220-H.2	Diploid of Hokkai 64	–	Tunika (CPC1673)
Mara	Ehud	22731	Ehud (CPC1673)
W794224-H.4	Diploid of Mara	–	Mara (CPC1673)
W792224-1	W664210-H4	*S. boliviense* (PI 218221)	*S. boliviense* (PI 218221)
98H23-53F1	98H23-53 self	–	*S. chacoense*, *S. phureja*
GLKS 1642/4	*S. vernei*	*S. tuberosum*	*S. vernei*
GLKS 58-1642-4	*S. vernei*	*S. tuberosum*	*S. vernei*
WB80001-1	Irish Cobbler	W784418	*S. vernei* (PI 230468)
WB862209-10	*S. vernei* (PI 230468)	W822229-5	*S. vernei* (PI 230468)
Astarte	SVP RR2-5-43	SVP VT5 62-69-5	unknown
Gladstone	Great Scot	(Arran Chief × Majestic)	unknown

[a] Progeny from natural pollination.

DNA of germplasms positive for all markers are required to establish simultaneous detection. However, there was no such germplasm, we created it mimically by mixing DNAs isolated from 'Eden' (*H1* and $GpaIV^{s}_{adg}$) and 'Innovator' (*Gpa5*) in equal proportions. The multiplex PCR conditions were optimized by varying the primer and Taq DNA polymerase concentrations and annealing temperature. Consequently, simultaneous amplification was achieved using the primers with concentrations shown in **Table 1**: 0.5 units of Taq DNA polymerase and an annealing temperature of 55°C (**Fig. 3**). In 'Eden', both expected PCR products were amplified by N195 and C237-I (**Fig. 3**, Lanes 1, 2). The HC-I2 obtained a 458-bp DNA fragment. After digestion with Hind III, a novel band was observed in 'Innovator' (**Fig. 3**, Lane 4). As with the individual PCR, we observed the Hind III-digested HC-I2 products (238 bp and 225 bp) as a single band around 230 bp. For the mixed DNA sample ('Eden' + 'Innovator'), all the expected bands were visually confirmed by electrophoresis on 2.0% agarose gels (**Fig. 3**, Lane 6).

Table 3. Germplasms positive for HC-I and their possible origin

Germplasm name	Pedigree		Possible origin of HC-I
	Female	Male	
Innovator	Shepody	RZ-84-2580	*S. vernei*
Furia	G88TT270004	Florijn	*S. vernei*
G06SC278.004	Stobrawa	Florijn	*S. vernei*
W794405-1	Irish Cobbler	*S. vernei* (PI 230468)	*S. vernei* (PI 230468)
WB80001-1	Irish Cobbler	W784418	*S. vernei* (PI 230468)
WB862209-8	*S. vernei* (PI 230468)	W822229-5	*S. vernei* (PI 230468)
WB862209-10	*S. vernei* (PI 230468)	W822229-5	*S. vernei* (PI 230468)
W784419-2	*S. vernei* (PI 230468)	Norin 1	*S. vernei* (PI 230468)
W792226-1	W664210-H4	*S. vernei* (PI 230468)	*S. vernei* (PI 230468)
W792224-1	W664210-H4	*S. boliviense* (PI 218221)	*S. boliviense* (PI 218221)
Flower-1	*S. tuberosum* ssp. *andigena* × *S. tuberosum*	*S. tuberosum*	*S. tuberosum* ssp. *andigena*
Esperanza violeta	*Solanum* sp.	WB59177-4	unknown

Table 4. Inoculation for candidate germplasms

Germplasm name	Marker	Possible origin of marker	2016-1		2017-1		2017-2		Resistant/ Susceptible[b]
			Cup	Pot	Cup	Pot	Cup	Pot	
			Kitaakari[a]	Kitaakari	Sanjumaru	Kitaakari	Pearl Starch	Kitaakari	
Eden	C237-I	*S. tuberosum* ssp. *andigena* (CPC2802)	–	–	9	7	8	–	R
12601ab1	C237-I	*S. tuberosum* ssp. *andigena* (CPC2802)	–	–	–	–	–	9	R
12169-37	C237-I	*S. tuberosum* ssp. *andigena* (CPC2802)	–	–	9	6	6	7	R
WB86007-2	C237-I	*S. tuberosum* ssp. *andigena* 233 (Malcachu)	3	3	4	5	5	–	r
WB86007-80	C237-I	*S. tuberosum* ssp. *andigena* 233 (Malcachu)	3	6	4	5	5	–	R
W804421-3	C237-I	*S. tuberosum* ssp. *andigena* 234 (Collareja)	4	4	5	6	4	–	R
WB86015-22	C237-I	*S. tuberosum* ssp. *andigena* 234 (Collareja)	1	4	–	–	–	–	r
S. tuberosum ssp. *andigena* 553-4	C237-I	*S. tuberosum* ssp. *andigena* 553-4	–	–	6	–	6	–	R
S. tuberosum ssp. *andigena* (PI 473260)	C237-I	*S. tuberosum* ssp. *andigena* (PI 473260)	–	1	–	–	–	–	S
Ehud	C237-I	*S. tuberosum* ssp. *andigena* (CPC1673)	–	2	–	–	–	–	S
Juliette	C237-I	*S. tuberosum* ssp. *andigena* (CPC1673)	–	1	–	–	–	–	S
Verdi	C237-I	*S. tuberosum* ssp. *andigena* (CPC1673)	–	1	–	–	–	–	S
Tunika	C237-I	*S. tuberosum* ssp. *andigena* (CPC1673)	–	1	–	–	–	–	S
Koganemaru	C237-I	Tunika (CPC1673)	1	1	–	–	–	–	S
Musamaru	C237-I	Tunika (CPC1673)	1	1	–	–	–	–	S
Natsufubuki	C237-I	Tunika (CPC1673)	–	1	–	–	–	–	S
W792224-1	C237-I/HC	*S. boliviense* (PI 218221)	–	–	5	3	–	–	r
98H23-53F1	C237-I	*S. chacoense* or *S. phureja*	–	1	–	–	–	–	S
WB80001-1	C237-I	*S. vernei* (PI 230468)	–	1	–	–	–	–	S
WB862209-10	C237-I	*S. vernei* (PI 230468)	–	1	–	–	–	–	S
Astarte	C237-I	unknown	–	1	–	–	–	–	S
Gladstone	C237-I	unknown	–	1	–	–	–	–	S
Innovator	HC	*S. vernei*	–	–	–	–	–	9	R
Furia	HC	*S. vernei*	4	6	9	6	9	5	R
G06SC278.004	HC	*S. vernei*	–	–	6	5	6	–	R
WB80001-1	HC	*S. vernei* (PI 230468)	–	1	–	–	–	–	S
WB862209-8	HC	*S. vernei* (PI 230468)	–	2	–	–	–	–	S
WB862209-10	HC	*S. vernei* (PI 230468)	–	1	–	–	–	–	S
W784419-2	HC	*S. vernei* (PI 230468)	–	–	3	3	–	–	S
W792226-1	HC	*S. vernei* (PI 230468)	–	–	4	3	6	–	r
Flower-1	HC	*S. tuberosum* ssp. *andigena*	–	1	–	–	–	–	S
Esperanza violeta	HC	unknown	–	1	–	–	–	–	S
Elles	none	(VTn)2 62-33-3	–	–	–	4	3	–	r
Irida	none	(VTn)2 62-33-3	–	–	–	4	4	2	r
Starter	none	(VTn)2 62-33-3	–	–	4	4	4	2	r
Darwina	none	(VTn)2 62-33-3	–	–	7	3	5	–	r

Resistance scores are shown on a scale of 1–9 based on relative susceptibility of tested germplasm to the standard variety. – indicates not tested. [a] Standard variety used to estimate relative susceptibility. [b] Evaluation of resistance of the germplasm. RS = 1–3; susceptible (S), RS = 4–6; moderately resistant (r), RS = 7–9; resistant (R).

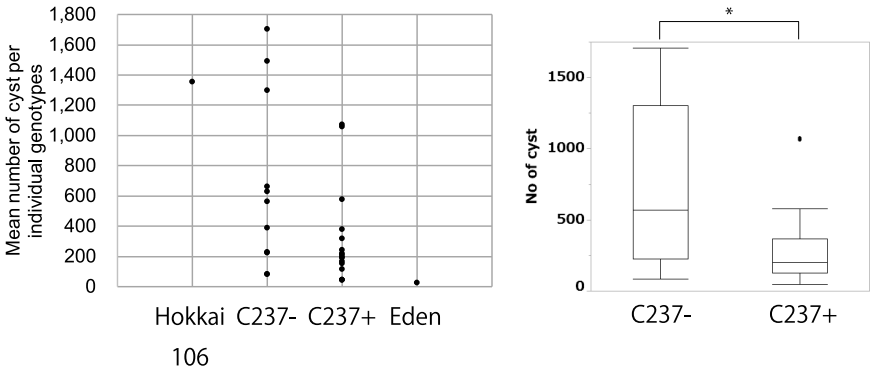

Fig. 2. The Number of cysts developed on genotypes with different marker alleles for C237-I. (A) Dot histogram of the number of newly formed cysts on parents ('Hokkai 106' and 'Eden') and progeny (C237-I +/C237-I –). (B) Boxplot comparing the cysts newly formed on each marker class. Each box's bottom and top represent the lower and upper quartiles, respectively, and the line inside each box represents the median. The bottom and top bars represent the minimum and maximum number, respectively. Data falling outside the bottom and top bars are plotted as outliers of the data. * indicates the significant difference between genotypes.

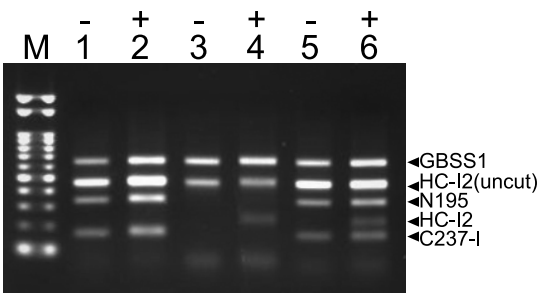

Fig. 3. Simultaneous detection of three PCN-resistance markers. M: 100 bp marker, 1,2: 'Eden', 3,4: 'Innovator', 5,6: DNA mixture ('Eden' + 'Innovator'). Lanes 1, 3, 5 and lanes 2, 4, 6 are samples before (–) and after (+) digestion with Hind III, respectively.

Discussion

We improved the markers specificity of two DNA markers for *G. pallida* resistance. The original versions could not detect a specific band considered to be linked to the resistances, while specific bands were detected by improved markers, C237-I, HC-I, and HC-I2. To improve these markers, we used PCR-based markers. With the recent progress in SNPs genotyping and next-generation sequencing (NGS), there are several technics for obtaining SNPs genotyping, including amplicon sequencing by NGS, Kompetitive Allele-Specific PCR (KASP), High resolution melting analysis, and Pyrosequencing (Thomson 2014). Compared to these technologies, our markers' advantage is high versatility; to detect markers, special equipment is not required. Also, our markers can be used even if DNAs were roughly extracted. Thus, our markers are useful for practical breeding of *G. pallida*-resistant varieties.

Although the two DNA markers proved to be insufficient for selecting resistant germplasms (because many germplasms positive for both markers did not show resistance), they were useful for narrowing down candidate germplasms for inoculation testing. By combining DNA marker selection with inoculation testing, we efficiently selected several resistant germplasms from the 1,143 germplasms available to us. In addition to increased efficiency, the DNA evaluations enabled us to select germplasms that would not have been expected to be resistant merely by examining their pedigree or breeder's information. Among germplasms we evaluated in this study, *S. tuberosum* ssp. *andigena* '553-4' and progeny of *S. tuberosum* ssp. *andigena* '233' (var. 'Malcachu') and '234' (var. 'Collareja') were positive for C237-I and showed moderate resistance to *G. pallida*. They originated from Colombia, Bolivia, and Argentina, respectively, and have been used to introduce not *G. pallida* resistance but resistance to late blight (*S. tuberosum* ssp. *andigena* '553-4') and tolerance to low-temperature sweetening (*S. tuberosum* ssp. *andigena* '233' and '234') in Japan. The resistance of these germplasms could not have been predicted from pedigree information only. Although germplasms with large genetic diversity are crucial for developing superior varieties with *G. pallida* resistance and other favorable characteristics, Rigney *et al.* (2017) pointed out the limited sources of $GpaIV^{s}_{adg}$ in breeding programs. Our materials, including 'WB86007-2', 'WB86007-80', 'W804421-3', 'WB86015-22', and *S. tuberosum* ssp. *andigena* '553-4', will contribute to expanding the sources of $GpaIV^{s}_{adg}$ in breeding programs for *G. pallida*-resistant varieties because the origin of their resistance differs from that of *S. tuberosum* ssp. *andigena* 'CPC 2802' which originated in Bolivia. Also, they possess favorable characteristics, such as late blight resistance and low-temperature sweetening tolerance along with *G. pallida* resistance.

In some cases, DNA markers are not fully diagnostic for the target traits because the particular alleles of the marker linked to the QTL in the original mapping populations are not common in a broader set of germplasms due to recombination, the complex genetic background of potato, or because that particular marker allele is present at a high frequency. For one or more of these reasons, we witnessed the inconsistency between resistance phenotype and marker

genotype observed in this study. Our results imply that SNPs used to develop C237 and HC may be present at high frequency in *S. tuberosum* ssp. *andigena* and *S. vernei* not possessing *G. pallida* resistance because many germplasms derived from *S. tuberosum* ssp. *andigena* and *S. vernei* were positive for these markers but did not show *G. pallida* resistance. Our results are consistent with those of a study by Milczarek *et al.* (2011) whereby low diagnosticity of the HC marker was indicated.

Alternatively, the availability of MAS by HC and C237 has been shown in populations derived from parents in which marker presence and resistance are confirmed (Dalton *et al.* 2013, Rigney *et al.* 2017). Here, the number of PCN cysts forming on C237-I + genotypes were significantly lower than that on C237-I – genotypes, but the difference was close. The relatively low numbers of cysts were observed on some C237-I – genotypes and *vice versa* (**Fig. 2**). The small difference in average cyst number and inconsistency between marker genotype and resistance phenotype may raise question of whether C237-I is robust enough to select resistant genotype by itself. Moloney *et al.* (2010) suggested the use of C237 for negative selection in breeding programs. That is, there would be very few instances in which the most resistant genotypes are incorrectly discarded if all genotypes that lack the marker are discarded at an early stage in a breeding program (Moloney *et al.* 2010).

Alternatively, our data indicate the possibility that some resistant individuals are incorrectly discarded if selection depends on C237-I only. Although the reliability of C237-I is not enough, considering breeding efficiency and labor for inoculation testing, it is better to use the marker because we can reduce the number of individuals during the breeding process. To avoid incorrectly discarding genotypes with both good agricultural characteristics and resistance, individuals with particularly excellent agricultural characteristics should be selected even if the marker is negative. We have recently applied MAS by C237-I for our breeding materials and selected resistant lines positive for C237-I. Besides, combined with the evaluation of agricultural characteristics, resistant lines negative for C237-I (13041-49 and 17114-8) could also be selected (**Supplementary Table 1**). For more accurate and efficient MAS for *G. pallida* resistance, improvement in marker accuracy is required. SNP used to develop C237-I is the same as that used in the development of the original C237 marker. Thus, identification of SNPs more closely located to $GpaIV^{s}_{adg}$ is required to develop more accurate markers. We are currently working on the identification of these SNPs and the development of robust markers for $GpaIV^{s}_{adg}$.

Here, we conducted inoculation testing for some germplasms several times in different seasons. Some germplasms showed different scores in different seasons or different methods. Compared with resistance tests against *G. rostochiensis*, resistance tests for *G. pallida* are relatively unstable. The resistance to *G. rostochiensis* conferred by the *H1* gene is qualitative and confers nearly complete resistance (Bakker *et al.* 2004, Gebhardt *et al.* 1993), making resistance evaluation relatively easy and stable. In resistant potato varieties possessing the *H1* gene, *G. rostochiensis* rarely develops new cysts; thus, resistance can be judged by whether many cysts develop or not.

In contrast, due to its quantitative nature, the phenotypic evaluation of resistance to *G. pallida* requires quantitative evaluation. In European countries, resistance to PCNs is scored on a 1–9 scale based on the tested germplasm's susceptibility relative to the standard variety, 'Desiree' (OEPP/EPPO 2006). Here, several varieties were used as standards, but we found that the development of cysts varied even among varieties susceptible to *G. pallida*. In our experiments, the number of cysts that developed on 'Kitaakari' was lower than on 'Sanju-maru' and 'Pearl Starch' (data not shown). Since resistance is quantified by comparison with the standard varieties, resistance may be underestimated if the number of cysts formed on the standard varieties is low. The standard European variety, 'Desiree', is suitable for that purpose because many cysts develop on it; however, since this variety is not commercially cultivated in Japan, it is difficult to prepare a reliable supply of disease-free healthy seed. Thus, we recently compared the multiplication of *G. pallida* on several Japanese varieties and selected 'Sanju-maru', and 'Pearl Starch' as standard varieties on which *G. pallida* can develop cyst as many as Desiree (naro.affrc.go.jp/publicity_report/publication/pamphlet/tech-pamph/134414.html).

Resistant germplasms selected in this study could be used in breeding programs to develop *G. pallida*-resistant varieties in Japan. For resistant germplasm positive for markers, MAS can be conducted by simultaneous detection of three DNA markers. Resistant germplasm negative for both markers, 'Elles', 'Irida', 'Starter', and 'Darwina', derived from '$(VTn)_2$ 62-33-3' can also be used as resistant sources, although they are moderately resistant, and MAS cannot be applied. Since sources of these germplasms' resistance are considered to be different from $GpaIV^{s}_{adg}$ and *Gpa5*, they will contribute to broadening the genetic background of *G. pallida* resistance varieties. Some commercial varieties selected as resistant in this study could be candidates for practical cultivation in *G. pallida* infested regions if their superior agricultural characteristics are confirmed. In fact, 'Furia', which was bred in France, was selected as a suitable starch variety for Japan and its commercial cultivation has begun very recently. This shows that our strategy to select candidate germplasms by combining DNA marker evaluation and inoculation testing effectively takes quick response to new pests and disease infestations.

Author Contribution Statement

K. Asano, TN and ST designed the research. ES and YY developed DNA markers and simultaneous detection method. K. Asano, ES, YY, K. Akai, SO and ST evaluated

germplasms by DNA markers. TN, SA and IS conducted inoculating testing. K. Asano wrote the manuscript. All authors read and approved the manuscript.

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