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ORIGINAL ARTICLE

Multiplex PCR assay for simultaneous identification of slow rust resistance genes *Lr34*, *Lr46* and *Lr68* in wheat (*Triticum aestivum* L.)

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Abstract

Currently, production of wheat cultivars (*Triticum aestivum* L.) that show durable field resistance against fungal pathogens is a priority of many breeding programs. This type of resistance involves race-nonspecific mechanisms and can be identified at adult-plant stages. Until now, seven genes (*Lr34/Yr18, Lr46/Yr29, Lr67/Yr46, Lr68, Lr75, Lr77* and *Lr78*) conferring durable types of resistance against multiple fungal pathogens have been identified in the wheat gene pool. In this study we showed a multiplex Polymerase Chain Reaction (multiplex PCR) assay, which was developed for detection of slow rusting resistance genes *Lr34*, *Lr46*, *Lr68*, using molecular markers: *csLV34, Xwmc44* and *csGS*, respectively. Identification of molecular markers was performed on 40 selected wheat genotypes which are the sources of slow rusting genes according to literature reports. Multiplex PCR is an important tool to reduce the time and cost of analysis. This multiplex PCR protocol can be applicable for genotyping processes and marker assisted resistance breeding of wheat.

Keywords: leaf rust, *Lr34*, *Lr46*, *Lr68*, multiplex PCR

Introduction

Bread wheat (*Triticum aestivum* L.) is currently one of the most significant cereal foods in the world, not only in terms of food production, but most of all for providing the total amount of food calories and protein in human diet (Gupta *et al*. 2008). Diseases and pests cause at least 10% of global plant production losses (Chakraborty and Newton 2011). The three fungal pathogens: *Puccinia triticina*, *P. striiformis* f. sp. *tritici*, and *P. graminis* f. sp. *tritici*, causing rust diseases of wheat are the most important biotic constraints to wheat production. Yield losses caused by infection of *P. triticina* may reach 40% in susceptible cultivars, and are the result of lower kernel weight and a decreased number of kernels per head (Knott 1989). The aim of modern wheat resistance breeding is to obtain sufficient resistance to all major diseases to reduce the use of plant protection products. Breeding for multiple resistance to disease, which can be achieved by introducing slow rusting genes to plants, is a promising strategy.

Presently, more than 80 leaf rust resistance (*Lr*) genes have been identified and described in wheat and its derivatives (McIntosh *et al*. 2017). Gene pyramiding can increase the durability of plant resistance to pathogens. New breeds of pathogens are rapidly emerging, and resistance conferred by racially specific genes becomes ineffective. Therefore, new solutions in plant breeding are desirable and one of them is the introduction of slow rusting genes into varieties (Singh *et al*. 2000). Up to now, seven genes conferring a partial type of resistance in adult plants have been identified in the wheat gene pool. These genes were named *Lr34*

(=*Yr18/Sr57/Pm18*) (Singh 1992a), *Lr46* (=*Yr29/Sr58/Pm39*) (Singh *et al.* 1998), *Lr67* (=*Yr46/Sr55/Pm46*) (Dyck and Samborski 1979), *Lr68* (Herrera-Foessel *et al.* 2012); *Lr75* (Singla *et al.* 2017), *Lr77* (Kolmer *et al.* 2018a) and *Lr78* (Kolmer *et al.* 2018b). Slow rusting genes provide durable resistance against all races of various pathogens including *Puccinia triticina, P. striformis* f. sp. *tritici, P. graminis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici* that cause leaf rust, stripe rust and powdery mildew, respectively. Slow rusting is a type of resistance characterized by durable resistant of adult plants which is not associated with a mechanism of hypersensitivity reaction as in the case of race specific genes (Bariana *et al.* 2001). The mechanism of slow rusting genes is still not well understood. It has been shown that *Lr34* and *Lr67* encode an ATP-binding cassette transporter and hexose transporter, respectively (Krattinger *et al.* 2009; Moore *et al.* 2015; Dodds and Lagudah 2016). Slow rusting genes ensure plant protection for a long period of growing in an environment favorable to the development of the disease (Johnson and Law 1975). The durable resistance, also known as racially nonspecific immunity, results from an additive effect of minor genes, usually polygenic. Cultivars possessing slow rusting genes show almost the same level of resistance over space and time and the same level of reaction against different races. For example, the 'Frontana' variety, which was registered about half a century ago, still has effective rust resistance in almost all parts of the world (Khan *et al.* 2013). It has been shown that retarded disease progress in plants results from a longer latent period, smaller pustule size, lower receptivity, and slower spore production than a susceptible check (Ohm and Shaner 1976; Wilcoxson 1981; Das *et al.* 1993). Singh *et al.* (2000b) estimated that dozens of slow rusting genes for leaf rust resistance are present in CIMMYT bread wheat germplasm.

Gene *Lr34* was first described in cultivar 'Frontana' by Dyck and Samborski (1966) on the short arm of chromosome 7D. The level of immunity associated with the presence of the *Lr34* gene in plants is best manifested at low average daily temperatures (0–20°C) under field conditions, which allows a significant reduction in disease progression (McIntosh *et al.* 1995). The *Lr34* gene is the most frequently used disease resistance gene in wheat breeding because of its durability and broadspectrum specificity. The *Lr34res* increase the latency period, the percentage of early aborted colonies not associated with cell necrosis and decrease colony size (Rubiales and Niks 1995). Lagudah *et al.* (2006) developed a molecular genetic marker *csLV34* that maps 0.4 cM from *Lr34*. The sequence-tagged-site (STS) marker is widely used for detection of gene locus in wheat varieties in many countries of the world (Singh *et al.* 2007; Kolmer *et al.* 2008; McCallum *et al.* 2008; Priyamvada *et al.* 2009), but this marker is not diagnostic for some wheat genotypes derived from the Canadian line 'RL4137' (McCallum *et al.* 2008; Lagudah *et al.* 2009).

The second gene involved in slow rusting, *Lr46* was identified in the cultivar 'Pavon' and located on chromosome 1B. To locate the gene, crosses were carried out with a monosomic series of adult plant leaf rust susceptible cultivar 'Lal Bahadur' (Singh *et al.* 1998). The effect of expression of *Lr46* gene is smaller than that of *Lr34* and it also does not provide complete immunity to plants. The presence of the gene is revealed in infected adult plants as a longer disease latency period than the control without this gene (Martinez *et al.* 2001). Lagudah (2011) showed that *Lr46* is more effective in a cooler environment than in higher temperature environments. To date, several markers have been developed to identify the *Lr46* gene in wheat: *Xbarc80* (Lowe *et al.* 2011), *Xgwm259* (Roder 1998), *Xwmc44* (Somers and Isaac 2004) and *csLV46G22* (Lagudah, personal communication 2020). *Lr46* was mapped distal to the microsatellite locus *Xwmc44*, approximately 5–15 cM, and proximal to *Xgwm259*, approximately 20 cM (https://maswheat.ucdavis.edu/protocols/Lr46). Whereas, microsatellite locus *Xbarc80* maps 10–11 cM distal to *Xgwm259* (Lowe *et al.* 2011). The CAPS (Cleaved Amplified Polymorphic Sequence) marker *csLV46G22* is the closest linked to *Lr46* gene (Lillemo *et al.* 2013; Ren *et al.* 2017; Cobo *et al.* 2019), among all the above markers.

Herrera-Foessel *et al.* (2012) identified the following slow rusting adult plant resistance (APR) gene, *Lr68* in wheat cultivar 'Parula'. The gene was mapped to a specific gene-rich area on chromosome 7BL between the locus *Psy1-1* (yellow endosperm) and molecular marker *xgwm146*. The origin of the gene is likely to be Brazilian wheat cultivar 'Frontana', which is known for its APR to leaf rust due to the presence of gene *Lr34* and 2–3 additional unidentified slow rusting genes (Singh and Rajaram 1992). Lillemo *et al.* (2011) revealed that the effect of *Lr68* at sites in Uruguay and Argentina was stronger than *Lr34*. Herrera-Foessel *et al.* (2012) recommended two molecular markers for marker-assisted selection of *Lr68*: co-dominant marker *cs7BLNLRR* positioned at 0.8 cM from the gene and the dominant marker *csGs* at 1.2 cM from the gene. The *csGS* marker was used in a bread wheat breeding program of CIMMYT for diagnosing *L68* in the crossing block.

Pinto da Silva *et al.* (2018) reported that pyramiding of slow rusting genes in different combinations in one genotype confers a high or sustainable level of durable resistance to *P. triticina*. Singh *et al.* (2014)

observed that the presence of single APR genes do not confer adequate resistance under high disease pressure, but combinations of four or five such genes usually result in "near immunity". For this reason, the task of resistance breeding wheat should be to look for sources (genotypes) having more than one slow rust gene. The traditional PCR method which can detect one gene in one reaction is time consuming and expensive. An alternative method may be to use the multiplex Polymerase Chain Reaction (multiplex PCR). Multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in one reaction. Multiplex PCR is used to increase the amount of information generated in one assay, and to reduce consumables and labor costs (Henegariu *et al.* 1997). The method was first used in 1988 (Chamberlain *et al.* 1988) and since then has been successfully applied in many areas of DNA testing, including analyses of deletions (Henegariu *et al.* 1994), mutations (Shuber *et al.* 1993) and polymorphisms (Mutirangura *et al.* 1993). The studies reported that the result of multiplex analysis is influenced by some factors (e.g., primer concentration, cycling profile) (Chamberlain *et al.* 1990; Vandenvelde *et al.* 1990). Also, there are specific problems associated with multiplex PCR, including uneven or lack of amplification of some loci and difficulties in reproducing some results (Henegariu *et al.* 1997). Moreover, the development of multiplex PCR assays on plants is difficult due to the large genome sizes and polyploidy. Bread wheat, one of the world's most important cereal crops (Donini *et al.* 1998), is an allohexaploid with a large and complex genome, comprised of paralogous gene families and about 75% repetitive DNA (Bennett and Smith 1975). For this plant, extensive optimization is required for the multiplex PCR reaction. Therefore, the aim of this study was to develop and optimize a multiplex PCR assay for the simultaneous identification of three slow rust genes (*Lr34, Lr46* and *Lr68*) and use a method to identify these genes in 40 genotypes which according to the literature are carriers of various APR genes.

Materials and Methods

Plant material

Plant material consisted of 40 spring wheat *T. aestivum* L. cultivars (Table 1) which had been reported as sources of slow-rusting genes and three reference materials for *Lr34*, *Lr46* and *Lr68* genes ['Lr34' (GSTR 433), 'Pavon F76' (PI 520003) and 'Parula', respectively], derived from the National Small Grains Collection, the Agriculture Research Station in Aberdeen. Seeds were germinated on Petri dishes and DNA was extracted from the leaf tissue of 10-day-old seedlings with the use of GeneMATRIX Plant & Fungi DNA Purification Kit (EURx Ltd., Poland). DNA concentration and quality were checked using a DeNovix spectrophotometer (DeNovix Inc., USA) and the samples were diluted with Tris buffer (EURx Ltd., Poland) to a concentration of 50 ng · μl^{-1}.

Development of multiplex PCR and identification of *Lr34*, *Lr46* and *Lr68* genes

The following molecular markers were used to simultaneously identify the *Lr34, Lr46* and *Lr68* genes: *csLV34*, *Xwmc44* and *csGs*, respectively. In this experiment, we could not use the closer linked *csLV46G22* marker for the *Lr46* gene, because the methodology of using the marker makes it impossible to combine it with the other selected markers (Lagudah, personal communication). Primer sequences, size of expected product and recommended annealing temperature for each molecular marker are presented in Table 2.

In this study, we attempted to create three different muliplex PCR variants for the simultaneous identification of the *Lr34* + *Lr46* + *Lr68* (I variant), *Lr46* + + *Lr68* (II variant) and *Lr34* + *Lr68* genes (III variant). Skowrońska *et al.* (2019) developed the multiplex PCR reaction for the *Lr34* + *Lr46* variant. Various options of mix composition and PCR profile of multiplex PCR were tested. The final 27 µl mix composition of

Table 1. Primer sequences, size of expected product and recommended annealing temperature for each molecular marker which was used for multiplex PCR

Markers	Primer sequences	Size of products	Annealing temperatures*	Sources
csLV34	csLV34F 5'-GTTGGTTAAGACTGGTGATGG-3'; csLV34R 5'- TGCTTGCTATTGCTGAATAGT-3'	150 bp (+) 229 bp (–)	55°C	Lagudah *et al.* 2006
Xwmc44	WMC44F 5'-GGTCTTCTGGGCTTTGATCCTG-3'; WMC44R 5'-GTTGCTAGGGACCCGTAGTGG-3'	242 bp (+)	61°C	Suenaga *et al.* 2003
csGs	csGS-F 5'-AAGATTGTTCACAGATCCATGTCA-3;' csGS-R 5-'GAGTATTCCGGCTCAAAAAGG-3'	385 bp (+)	60°C	Herrera-Foessel *et al.* 2012

(+) – size of product indicative of the presence of the gene, (–) – size of band in susceptible genotypes
*recommended primer annealing temperature according to literature references

Table 2. Presence of *Lr34*, *Lr46* and *Lr68* gene in tested wheat varieties

No.	Cultivar/Genotype	Plant ID	Origin	Pedigree (https://npgsweb.ars-grin.gov/gringlobal/search.aspx)	*csLv34* linked to *Lr34*	*Xwmc44* linked to *Lr46*	*csGS* linked to *Lr68*
1	2	3	4	5	6	7	8
1	Ceruga-4	PI 560118	United States, Georgia	Coker 916//Tyler/Buck Manantial	–	+	–
2	San Martin	PI 116314	Argentina, Buenos Aires	Americano 25E/Pelon 33C	–	–	–
3	H 51	PI 191925	Argentina, Buenos Aires	Americano 25e/Favorito//Universal	–	–	–
4	Artigas	PI 192535	Uruguay	Americano 25E/Americano 26N	–	–	+
5	Larranaga	PI 191713	Uruguay	Americano 25e/Pelon 33c	–	–	+
6	ProINTA Imperial NIL Glu-B3i_BuckManant.	PI 674008	United States, California	Buck Manantial/ProINTA Imperial//*6 ProINTA Imperial	+	–	–
7	NP 818	PI 422294	India, Delhi	Democrat/C 518//Spaldings Prolific/NP 114/3/E 220 (Kenya C10854)	–	–	–
8	Buck Manantial	PI 344455	Argentina, Buenos Aires	Rafaela MAG/Buck Quequen	–	–	–
9	Janz	PI 591910	Australia, Queensland	3AG3/4*Condor//Cook	+	+	–
10	7536K-51A4	PI 553001	Canada, Saskatchewan	Willet/McMurachy//Glenlea	+	–	–
11	7531-V3D	PI 552994	Canada, Saskatchewan	Glenlea/NB313	+	–	+
12	Jacui	PI 520498	Brazil, Rio Grande do Su	S 8/Toropi	+	+	–
13	P8802-C1*3A2C16	PI 596351	Canada, Saskatchewan	Benito*6/Glenlea//Benito	–	+	–
14	P8802-C1*3A2A2U	PI 596350	Canada, Saskatchewan	Benito*6/Glenlea//Benito	–	+	–
15	HD 2329	PI 648391	India, Delhi	HD 1962/E 4870//K 65/3/HD 1553/UP 262	–	+	–
16	K494	PI 250413	Pakistan	–	–	–	–
17	Glenlea	CItr 17272	Canada, Manitoba	Pembina*2/Bage//CB 100 CB 100 = Sonora 64/Tezanos Pintos Precoz//Nainari 60	+	+	+
18	Artigas	PI 73046	Uruguay	Americano 25E/Americano 26N	–	–	–
19	Amurskaya 90	PI 592036	Russian Federation, Amurskaja oblast’	Glenlea/Altair 12	–	–	–
20	Lerma Rojo	CItr 13651	Ciudad de México Mexico	Lerma 50/Yaqui 48//Mario Escobar*2/Supremo 211	+	+	–

Table 2. Presence of *Lr34*, *Lr46* and *Lr68* gene in tested wheat varieties – continuation

1	2	3	4	5	6	78	
21	363-11	PI 527696	Canada, Saskatchewan	PI 191320/Glenlea	+	+	+
22	256-3	PI 527695	Canada, Saskatchewan	PI 191320/Glenlea	–	–	–
23	NP 846	PI 322263	Delhi India	Rio Negro/NP 760	+	+	+
24	75M-505-001-001	PI 556464	Canada, Alberta	Bluebird sib/Tobari 66/4/Toropi//Ciano 67/Inia 66 sib/3/Ciano 67/Inia 66 sib	–	–	–
25	7531-AP5A	PI 552997	Canada, Saskatchewan	Glenlea/NB313	–	+	–
26	7531-AG5B	PI 552996	Canada, Saskatchewan	Glenlea/NB313	–	–	–
27	7531-AG5A	PI 552995	Canada, Saskatchewan	Glenlea/NB313	+	+	–
28	CM 46725-3P-1P-3P-2P	PI 520562	Mexico, Ciudad de México	Zopilote sib/4/Toropi//Ciano/Noroeste 66/3/Bluebird/Ciano/5/Ciano sib/3/ Penjamo 62//Gallo/Bluetit/6/Pavon sib	–	+	–
29	Cook	PI 442900	Australia, Queensland	Timgalen/Condor sib//Condor	+	+	–
30	PAT 7219	PI 422416	Brazil, Rio Grande do Sul	S12/J9280–67//Nobre/Toropi	–	+	+
31	HI 617	PI 422283	India	selection from C306	–	–	–
32	Oxley	PI 386167	Australia, Queensland	Penjamo 62/4*Gabo 56//Tezanos Pintos Precoz/Nainari 60/4/2* Lerma Rojo// Norin 10/Brevor 14/3/3*Andes; sister line of Condor ; "Norin 10/Brevor 14" = CItr 13253, Norin 10/Brevor, Sel. 14	+	+	–
33	NP 718	PI 322236	India, Delhi	NP 52/NP 165	–	–	–
34	San Martin	PI 104137	Argentina, Buenos Aires	Americano 25E/Pelon 33C	–	–	+
35	A99AR	PI 600923	United States, Minnesota	Glenlea/Zaragoza	+	+	+
36	Klein San Martin	PI 191884	Argentina, Buenos Aires	Americano 25E/Pelon 33C	–	–	+
37	H 51	PI 184512	Argentina, Buenos Aires	Americano 25e/Favorito//Universal	–	–	–
38	San Martin	PI 117500	Argentina, Buenos Aires	Americano 25E/Pelon 33C	–	–	–
39	San Martin	Cltr 8437	Argentina, Buenos Aires	Americano 25E/Pelon 33C	–	–	+
40	Record	Cltr 8399	Argentina, Buenos Aires	Americano 26N/Americano 25E	–	–	+

multiplex PCR volume in variant I consisted of the following: 12.5 µl 2 × PCR TaqNovaHs PCR Master Mix (Blirt), which included 2 × concentrated PCR reaction buffer, 4 mM $MgCl_2$; 1.6 mM dNTPs mix (0.4 mM of each dNTP); 0.8 $µ_L$ *csLv34* forward primer; 0.8 µl *csLv34* reverse primer; 1.2 µl *Xwmc44* forward primer; 1.2 $µ_L$ *Xwmc44* reverse primer, 1 µl *csGs* forward primer; 1 µl *csGs* reverse primer (the concentration for each primer was 100 µM); 2 µl DNA template (50 ng · $µl^{-1}$) and 6.5 µl PCR grade water. For the II variant, the total volume of the multiplex PCR mix composition was 25,4 µl and consisted of 12.5 µl 2 × PCR TaqNovaHs PCR Master Mix (Blirt), 1.2 µl *Xwmc44* forward primer; 1.2 µl *Xwmc44* reverse primer, 1 µl *csGs* forward primer; 1 µl *csGs* reverse primer, 2 µl DNA template (50 ng · $µl^{-1}$) and 6.5 µl PCR grade water. For the last, the third variant the PCR multiplex mixture with a total volume of 24,3 µl contained 12.5 µl 2 × PCR TaqNovaHs PCR Master Mix (Blirt), 0.8 µl *csLV34* forward primer; 0.8 µl *csLV34* reverse primer, 1 µl *csGs* forward primer; 1 µl *csGs* reverse primer, 2 µl DNA template (50 ng · $µl^{-1}$) and 6.5 µl PCR grade water. The PCR profile was modified with reference to standard protocol and various temperatures recommended for primer annealing were tested (Table 2). The final PCR reaction consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles (denaturation, 94°C for 45 s; primer annealing, 60°C for 30 s; elongation, 72°C for 1 min), followed by the final extension for 7 min at 72°C and and storage at 4°C. The multiplex PCR was carried out using Labcycler thermal cyclers (SensoQuest GmbH). Amplifications were prepared by adding 1 µl Midori Green Direct (NIPPON Genetics EUROPE) to each tube. The reaction products were separated using 2% agarose (SIGMA) gel in 1 × TBE buffer (BioShop) at 100 V for 2 h.

In order to simultaneously identify all genes in 40 wheat varieties originating from the National Small Grain Collection, the first variant and the methodology described above were used. Cooling during electrophoretic separation of products was used to obtain clearer results.

Results and Discussion

Breeding programs have successfully used molecular markers to assist in the development of varieties with leaf, yellow and stripe rust resistance genes (Alemu 2019). Numerous genes conferring disease resistance to wheat have been identified and used in breeding, but many of these genes have lost their effectiveness due to the emergence of new virulent breeds (Singh *et al.* 2000). Unfortunately, it takes several years to introduce new resistance genes that are effective for new breeds of the pathogen, mainly due to the long process involved in the establishment of pure breeding wheat lines (Alemu 2019). The solution may be to introduce genes that give durable tolerance to many pathogens to varieties, which, in combination with racial-specific genes, can help minimize the use of fungicides in wheat cultivation.

In this experiment, we developed a multiplex PCR method for the simultaneous identification of various combinations of slow rust genes: *Lr34* + *Lr46* + *Lr68* (variant I), *Lr46* + *Lr68* (variant II) and *Lr34* + *Lr68* (variant III). Optimization of the method consisted of selecting the appropriate primer volume for all genes in each variant and adjusting the appropriate primer annealing temperature in order to obtain uniformly intense bands on the gel. For this purpose, a smaller volume of the *csLV34* primer was used for each sample than the others, because the primer showed very distinct bands and dominated the others. In addition, the volume of the *Xwmc44* primer in each sample and variant was increased because the bands on the gel were not sufficiently visible when the same volume of all primers was used. There was no effect of changes in the *csGS* primer volume for the *Lr68* gene, at different volumes. The products of all markers were equally visible and readable.

In variant I, in the reference variety 'Lr34', a 145 bp product specific for the *Lr34* gene and a 242 bp product associated with the *Lr46* gene were obtained. In our study, the size of the *Lr34* gene-specific product differed from the size of 150 bp reported by Lagudah *et al.* (2006). Differences in the size of products may result from the size of the DNA ladder used, which was also noted by Skowronska *et al.* (2019). In 'Pavon 76', two products were identified: a 242 bp band, indicating the presence of the *Lr46* gene, and 229 bp band indicating the absence of the *Lr34* gene. In the 'Parula' variety, which is the reference material for the *Lr68* gene, a specific product of the 385 bp *csGS* marker and a 145 bp product indicating the presence of the *Lr34* gene were identified. In variant II (*Lr46* + *Lr68*) and variant III (*Lr34* + *Lr68*) the above-described results have been confirmed, which indicates that all developed multiplex PCR variants can be used in the selection of materials in the wheat breeding process.

Variant I of the multiplex PCR method that we developed allowed for the identification of the *Lr34, Lr46* and *Lr68* genes in wheat materials that are reported as having slow rust genes. They are characterized by durable resistance, indicating the presence of slow rust genes. For example, the 'Glenlea' variety was registered in 1972 and was the first major variety bearing the *Lr34* gene in western Canada (Evans *et al.* 1972; Dyck *et al.* 1985). In this work, all three slow rust genes (*Lr34* + + *Lr46* + *Lr68*) were identified in the 'Glenlea' variety. In addition, some varieties possessing 'Glenlea' in their

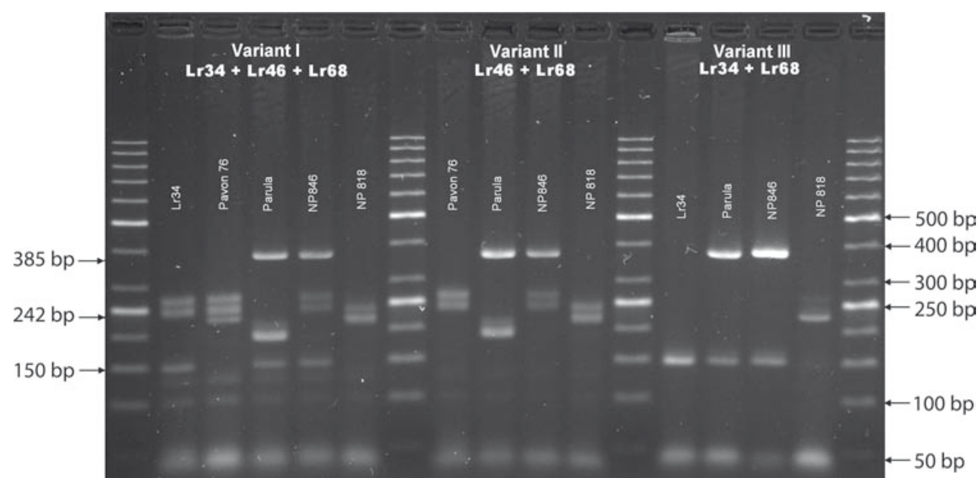

Fig. 1. Electrophorogram showing the presence of molecular markers *csLV34* (for *Lr34*), *Xwmc44* (for *Lr46*) and *csGS* (for *Lr68*) in wheat varieties. M – GeneRuler 50 bp DNA ladder (Nippon Genetic Europe, Germany)

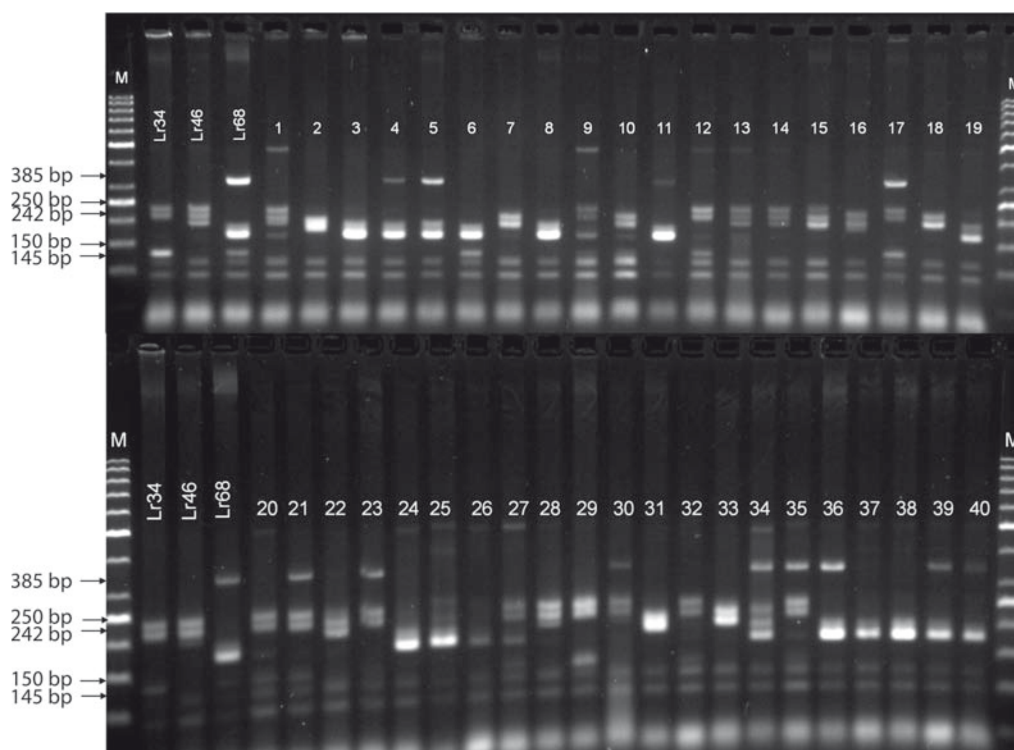

Fig. 2. Electrophorogram showing the presence of molecular markers *csLV34* (for *Lr34*), *Xwmc44* (for *Lr46*) and *csGS* (for *Lr68*) in wheat varieties. M – GeneRuler 50 bp DNA ladder (Nippon Genetic Europe, Germany), 1–40 – wheat varieties

pedigree also carry various combinations of genes: 'A99AR' and '363-11' (*Lr34* + *L46* + *Lr68*), '7531--V3D' (*Lr34* + *Lr68*), '7531-AG5A' (*Lr34* + *Lr46*), '7536K-51A4' (*Lr34*), 'P8802-C1*3A2C16', 'P8802--C1*3A2A2U' and '7531-AP5A' (*Lr46*) (Table 2, Fig. 2). 'Lerma Rojo' is one of the semi dwarf varieties developed at CIMMYT during the green revolution and contributed to yield breakthroughs in India, Pakistan, Turkey and other parts of the world. 'Lerma Rojo' is characterized by a long life span due to its resistance to pathogens (Borlauge 1968). We identified two slow rust genes in the 'Lerma Rojo' variety: *Lr34* and *Lr46* (Table 2, Fig. 2). The Oxley variety, whose genealogy includes 'Lerma Rojo', also has the *Lr34* and *Lr46* genes (Table 2, Fig. 2). The next variety in which the three slow rust genes have been

identified is the Indian variety 'NP 846', also known as 'New Pusa 846'. So far, NP 846 was known to have gene *Lr34* (Kaur *et al.* 2000; Kolmer *et al.* 2008). In summary, the *Lr34* gene was identified in 13 of the 40 varieties tested, the *Lr46* gene in 17 varieties, and the *Lr68* gene in 12 varieties. In four varieties all three tested genes were identified, and in eight varieties the presence of two tested genes in one variety was detected. In 14 varieties none of the slow rust genes were identified (Table 2, Fig. 2). Altieri *et al.* (2008) determined the number and characterization of resistance genes to wheat leaf rust present in 'Buck Manantial', an Argentinian cultivar that shows durable resistance. They also used closely linked marker *csLV34* to *Lr34* to detect the presence of this resistance gene in 'Buck Manantial' as hypothesized by Dyck (1989). As a result of the study conducted by the authors, the presence of the gene could not be confirmed in Buck Manantial based on the allele detected by this molecular marker. In our study, we also did not identify any slow rust gene in the 'Buck Manantial' variety, although, according to other sources, the cultivar has the *Lr34* gene (McIntosh *et al.* 1995; McIntosh *et al.* 2008; McCallum 2012) (Table 2, Fig. 2).

In the literature one can find many examples of the development the multiplex PCR method to identify resistance genes. Leśniowska-Nowak *et al.* (2013) developed a multiplex PCR method to identify two resistance genes for leaf rust *Lr9* and *Lr19*. Other race specific resistance genes for *P. triticina, Lr29* and *Lr37* were identified simultaneously by Sumikova and Hanzalova (2010). Gogół *et al.* (2015) used the multiplex PCR method to simultaneously identify genes of resistance to two different diseases: *Lr21* (leaf rust) and *Pm4b* (powdery mildew). Tomkowiak *et al.* (2019a) identified the *Pm2, Pm3a, Pm4b*, and *Pm6* genes and developed multiplex PCR reaction conditions for simultaneous identification of *Pm2* and *Pm4b* genes. The multiplex PCR conditions have been developed for the simultaneous identification of the *Lr11* + *Lr16* and *Lr11* + *Lr26* gene pairs by Tomkowiak *et al.* (2019b). The method of simultaneous identification for slow rusting genes *Lr34* and *Lr46* was developed by Skowrońska *et al.* (2019). The authors also used a smaller volume of the *csLv34* primer and a larger volume of the *Xwmc44* primer, and their method may complement the results of the above work.

Moreover, there are many examples of the use of multiplex to identify other genes in wheat. Zhang *et al.* (2008) developed two multiplex PCR assays targeting improvement of bread-making and noodle qualities in common wheat that validated using 70 cultivars and advanced lines from Chinese autumn-sown wheat regions. Wang *et al.* (2010) identified genes at Glu-A3 locus, developed the STS markers, and established multiplex PCR with the STS markers for Glu-A3 alleles. The multiplex PCR system was validated on 141 CIMMYT wheat varieties and advanced lines with different Glu-A3 alleles, confirming that they can be efficiently used in marker-assisted breeding. Moczulski and Salmanowicz (2003) used the multiplex PCR method to identify the allele composition of HMW glutenin complex Glu-1 loci (Glu-A1, Glu-B1 and Glu-D1) in common wheat genotypes. Compared to conventional PCR techniques that allow the identification of single resistance genes, multiplex PCR is a method that can simultaneously detect multiple resistance genes in one system with high sensitivity and specificity. Consequently, multiplex PCR is a more appropriate and less time-consuming method of detecting several resistance genes (Ballabio *et al.* 1990). In addition, the multiplex PCR method provides the basis for the future development of a quantitative and more sensitive PCR method using real-time PCR technology (Côté *et al.* 2004). The multiplex PCR method is a convenient tool for selecting materials in plant breeding. The method presented in this article can be successfully used to simultaneously identify the *L34*, *Lr46* and *Lr68* genes. Moreover, additional variants of the method allowing for the simultaneous identification of the *Lr46* and *Lr68* genes as well as the *Lr34* and *Lr68* genes can also be a helpful tool in plant breeding. Varieties containing slow rust type genes, identified in the test using the multiplex PCR method can provide APR resistance genes for breeding wheat or other cereal species.

Conclusions

In summary, a multiplex PCR method was developed for the simultaneous identification of different combinations of slow rusting genes: *Lr34* + *L46* + *Lr68*, *Lr34* + *Lr68* and *Lr46* + *Lr68*. This method can be applicable for the genotyping process and marker assisted selection for breeding programs of wheat.

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