New Insights into the Anthracnose Resistance of Common Bean Landrace G 2333

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Abstract: The common bean landrace G 2333 carries a three gene pyramid for anthracnose resistance: *Co-4*^{2}, *Co-5* and *Co-7*. The *Co-4*^{2} gene is well characterized but less information is available on *Co-5* and *Co-7*. The objectives of this study were to determine if a new allele of *Co-5* is present in G 2333; to characterize the spectrum of resistance conditioned by the *Co-7* gene by deriving lines which only possess the *Co-7* gene; and to test the MSU7 lines for allelism to *Co-3*. We propose that G 2333 carries a second allele at the *Co-5* gene, different from that possessed by TU, and that the allele in G 2333 (SEL 1360) be designated *Co-5*^{2}. With the exception of breeding line MSU7-1, the other derived MSU7 lines do not carry the *Co-7* gene and are not allelic to *Co-3* suggesting that G 2333 carries more than three genes for anthracnose resistance.

Key Words: Alleles, *Colletotrichum lindemuthianum*, molecular markers, *Phaseolus vulgaris*, races.

INTRODUCTION

Anthracnose caused by the fungal pathogen *Colletotrichum lindemuthianum*, is a serious seed-borne disease of common beans (*Phaseolus vulgaris* L.). Worldwide, yield losses due to anthracnose are particularly severe in subsistence agricultural systems where beans are a valuable source of plant protein, and income to the producer. Genetic resistance is the most cost effective means to control the disease. Eleven major anthracnose resistance genes (*Co-1 – Co-11*) have been characterized in common bean and are available for use in breeding programs [1, 2]. Achieving durable anthracnose resistance poses a challenge to bean breeders. Due to the high degree of pathogen variability and the continual emergence of new races, single gene deployment is not an effective strategy to control bean anthracnose. The pyramiding of resistance genes which have complementary spectra of resistance has been suggested as a strategy to circumvent the problem of pathogen variability [3]. The most resistant Middle American cultivar in the anthracnose differential series is G 2333 [4]. G 2333 is the landrace cultivar, Colorado de Teopisca from Chiapas, Mexico and a result of a naturally occurring gene pyramid for anthracnose resistance. Due to the high level of resistance in this landrace cultivar, many researchers have sought to isolate and characterize the independent resistance genes responsible. The first report on the nature of resistance in G 2333 suggested that two independent dominant genes were present. A 15:1 (resistant: susceptible) ratio was observed after inoculation of an F_2 population of G 2333/Pijao with race 521 [5]. To properly identify and characterize the individual genes conditioning resistance to anthracnose, the breeding lines, SEL 1360 and SEL 1308, derived from G 2333 through a single backcross to the cultivar, Tacaragua were obtained from CIAT [6]. Neither line was as resistant as the G 2333 parent, nor did either display the same reactions to distinct races of anthracnose, implying that they carried different resistance genes. Young and Kelly [6] demonstrated that SEL 1360 carried a single dominant gene that proved to be the same locus as the *Co-5* gene in the differential cultivar TU. RAPD markers linked to the *Co-5* gene in SEL 1360, confirmed the presence of this locus in TU and G 2333 [3]. In the absence of discriminating races of anthracnose, the gene in SEL 1360 and TU was assumed to be same. Young *et al*. [7] also demonstrated that SEL 1308 carried a single dominant gene that proved to be allelic to the *Co-4* gene in the differential cultivar TO. This allele was named *Co-4*^{2} as it conditioned resistance to a broader array of races than the original allele in TO. RAPD markers linked to the *Co-4*^{2} allele confirmed the presence of *Co-4*^{2} in G 2333 and afforded the opportunity to identify the third gene in G 2333. Therefore, a three gene pyramid was hypothesized to condition resistance in G 2333; *Co-4*^{2}, *Co-5* and since the third gene was not isolated to conduct allelism tests it was tentatively named *Co-7* [7]. Although this hypothesis has been readily accepted by the bean research community, it is interesting to note that all reported crosses with G 2333 have yielded 15:1 (2-gene) segregation ratios (Table **1**) despite the use of diverse races of *C. lindemuthianum*.

For example, Pathania *et al.* [8] reported separate two gene ratios in crosses of G 2333 with two susceptible Indian cultivars inoculated with races 3 and 515. Since race 515 defeats the *Co-5* gene, a 2-gene segregation ratio would imply presence of *Co-4*^{2} and *Co-7* genes, where *Co-7* is resistant to race 515. In the case of race 3 to which *Co-4*^{2} and *Co-5* genes condition resistance, the *Co-7* gene must be susceptible to race 3. Alzate-Marin *et al*. [9], in Brazil, reported another15:1 segregation ratio in a F_2 population from the cross Ruda/G 2333 inoculated with race 73, again suggesting that G 2333 possesses only two independent genes *Co-4*^{2} and *Co-5*, based on the published work. The third gene, named

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Table 1. Inheritance Studies of Anthracnose Resistance in G 2333

Population	Race	Ratio	Reference
Pijao/G 2333	521	15:1	[5]
Ruda/G 2333	73	15:1	[9]
Jawala/G 2333	3	15:1	[8]
Kanchan/G 2333	3	15:1	[8]
Jawala/G 2333	515	15:1	[8]
Kanchan/G 2333	515	15:1	[8]

Co-7, should be defeated by race 73 but resistant to races 521 and 1545. Since no further characterization was performed on the third gene, little is known about the spectrum of resistance conferred by *Co-7*. By implication, the third gene in G 2333, named *Co-7*, must condition resistance to races 521 or 515 and 1545 (carry the *Co-5* virulence gene) yet is susceptible to races 3 and 73.

To begin to isolate the *Co-7* gene from the other anthracnose resistance genes in the G 2333 pyramid, Young *et al*. [7] used a combination of marker-assisted selection (MAS) and inoculation with different races of *C. lindemuthianum*. An F_2 population derived from the cross, SEL 1360/G 2333, in which the *Co-5* gene was fixed was used. The first selection was carried out to remove individuals from the population which carry the *Co-4*^{2} gene by selecting against the linked SCAR marker SAS13 (derived from original RAPD-OS13 marker). Markers were used as the broad resistance of *Co-4*^{2} in G 2333 precluded the use of discriminating races of anthracnose. Following the MAS, remaining individuals were screened with race 521 (Hd 16.1 from Honduras) to identify resistant lines that should possess the *Co-7* gene since race 521 defeats the *Co-5* gene fixed in this population. Up to 15 plants of 14 lines lacking the SAS 13 marker were inoculated with race 521 and five lines with complete resistance were identified. Through this process the single F_4 breeding line SEL 111 was identified as resistant and was assumed to carry *Co-7* along with the *Co-5* gene. The goals of the current study were to (1) derive a line which only carried the *Co-7* gene for anthracnose resistance (2) characterize the resistance spectrum conditioned by the *Co-7* gene, (3) test for allelism to *Co-3* gene and (4) further investigate the *Co-5* allele derived from G 2333 in SEL 1360.

MATERIALS AND METHODS

Plant Material and *C. lindemuthianum* Races

Populations developed in this study are shown in Table **2**. To confirm that the original breeding line SEL 111 carries only two major genes for anthracnose resistance, a segregating F_2 population was derived from the cross between the black bean cultivar Black Magic (BM) and SEL 111. BM is considered universally susceptible to anthracnose. This F_2 population of 72 individuals was inoculated with race 7 which produced an S/R reaction in the parents (BM and SEL 111 respectively). During the course of this study, race 521 lost virulence so in the absence of a discriminating race of *C. lindemuthianum* in our collection, we utilized a MAS strategy to identify and remove individuals carrying the *Co-5* gene. The molecular marker (SAB3, [10] linked to the *Co-5* locus was used to select against the presence of the *Co-5* gene among the progeny segregating for the *Co-5* and *Co-7* loci within the BM/SEL 111 population. A F_3 progeny test was conducted to determine which of the F_2 individuals, showing the absence of the *Co-5* locus based on MAS, were homozygous for *Co-7* by inoculation of F_3 families of 15 individuals each, with race 7. The derived lines were named MSU7-1 through 6 and were advanced through five generations of self-pollination. Line MSU7-5 was not used in this study because of insufficient seed. These $F_{2:5}$ MSU7 lines were inoculated with a series of *C. lindemuthianum* races (7, 31, 47, 73, 357, 448 and 1545) to determine the resistance spectrum conferred by the *Co-7* gene. Additionally, specific $F_{2:5}$ MSU7 lines were crossed with the cultivar Michigan Dark Red Kidney (MDRK) and the resulting F_2 population was inoculated with race 7 which yields an S/R reaction in the parents (MDRK and MSU7 respectively) to determine the number of genes segregating for anthracnose resistance. A new isolate of race 521 (Gu 9.0 from Guatemala) was isolated in 2006 and was used to inoculate all test material. Prior work had confirmed that the *Co-2* gene is not present in G 2333 based on allelism tests and the absence of markers linked to the *Co-2* gene [7]. The only major gene, other than the *Co-2* gene, defeated by race 73 is the *Co-3* gene in the differential cultivar Mexico 222. To determine if *Co-7* was allelic to *Co-3* the differential cultivar Mexico222 was crossed with MSU7.3 and inoculated with race 7 which produces an R/R reaction in the parents.

Inoculum Preparation and Inoculation Procedures

All races utilized in this study were grown from monosporic cultures maintained on filter papers and stored at -20°

Table 2. F_2 Populations Inoculated with *C. lindemuthianum* Race 7

Population	Parental Reaction	Pop. Size	Observed Ratio	Expected Ratio	P-Value
Black Magic/SEL 111	S/R	155	147:8	15:1	0.57
Mexico222/MSU7-2	R/R	129	127:2	63:1	1.0
Mexico222/MSU7-3	R/R	129	127:2	63:1	1.0

C. *C. lindemuthianum* races were grown from a filter paper on either potato dextrose agar (PDA) or Mathur's agar (dextrose (8 g/L), $MgSO_4$. 7 H_2O (2.5 g/L), neopeptone (2.4 g/L), yeast extract (2 g/L), and agar (16 g/L)) at 22°C in the dark. After the onset of sporulation, spores were re-plated in replicate to generate sufficient inoculum. Spores were scraped from the media into a solution of water and 0.01% Tween 20. An estimated spore concentration was determined using a hemacytometer and the concentration was adjusted to 1.2 x 10^6 spores/ml. The prepared inoculum was then sprayed onto the abaxial and adaxial surfaces of the leaves. Inoculated seedlings were incubated for 48 hours at approximately 100% relative humidity. Seedlings were rated for disease 5 days post inoculation on a scale described by [4]. All races used in this study were first inoculated on the anthracnose differential series to confirm its identity.

MAS Analysis and Derivation of a SCAR Marker

To dissect the *Co-5*, *Co-7* gene pyramid in SEL 111, the BM/SEL 111 segregating population was inoculated with race 7, and a molecular marker linked to the *Co-5* locus was used in a MAS strategy. The RAPD marker, $AB3_{450}$, is linked in coupling-phase to *Co-5* (5.9 ± 1.9 cM) [3]. This marker was converted into a sequence characterized amplified region (SCAR) marker, using the protocol described by [11], to facilitate its use in a MAS approach. Primer sequences for $SAB3_{450}$ are shown in Table **3**. The thermal cycler profile used to amplify the SAB3 marker consisted of 34 cycles of 10 s at 94ºC, 40 s at 67ºC, and 2 min at 72ºC; one cycle of 5 min at 72ºC.

Table 3. Primer Sequences for the SCAR Marker $SAB3_{400}$ Linked to the *Co-5* Locus

Forward[a]	**5'-TGGCGCACACATAAGTTCTCACGG-3'**
Reverse[a]	**5'-TGGCGCACACCATCAAAAAAGGTT-3**

[a]The underlined sequences are the original RAPD primer.

RESULTS AND DISCUSSION

Derivation and Genetic Characterization of MSU7 Lines

To confirm that SEL 111 carried two major genes for resistance to anthracnose, SEL 111 was crossed with BM and the resulting F_2 population was inoculated with race 7, which yields and S/R reaction in the parents (BM and SEL 111 respectively). The segregation of resistant to susceptible individuals fit a 15:1 ratio (p = 0.57) indicating that two dominant anthracnose resistance genes segregating for resistance to race 7 came from SEL 111 (Table **2**).

To isolate *Co-7* from *Co-5*, the SCAR marker $SAB3_{400}$ was used to indirectly select against the *Co-5* locus. All individuals from the BM/SEL 111 F_2 population with resistant to race 7 were screened. Those individuals which did not carry $SAB3_{400}$ were used for further study. F_3 families of these individuals were subjected to a progeny test in which 15 $F_{2:3}$ seedlings were inoculated with race 7 to determine which F_2 individuals were homozygous for *Co-7*. Those F_2 individuals whose F_3 family did not segregate for resistance when inoculated with race7 (data not shown) were self-pollinated for five generations and designated MSU7-1 through MSU7-6. To determine if the *Co-7* gene is allelic to *Co-3*, MSU7-2 and MSU7-3 were crossed with Mexico222 and the resulting segregating populations were inoculated with race 7 (R/R). Segregation of resistance was observed indicating that gene(s) conditioning resistance to race 7 in the MSU7 lines were not allelic to the *Co-3* gene. Both populations exhibited a 63:1 segregation ratio of resistant to susceptible individuals (p=0.99) suggesting that there are three independent genes conditioning resistance. In the absence of an SxR analysis we cannot determine the number of genes in the MSU7 lines which confer resistance to race 7, but [12] reported that Mexico222 carried two independent genes conditioning resistance to race 7.

Resistance Spectrum of MSU7 Lines

Initial characterization of the MSU7 lines was performed by direct inoculation with *C. lindemuthianum* races 7, 73 and 448. The disease reaction profile of the MSU7 lines was compared with SEL 111 and SEL 1360 (Table **4**). Inoculation of the MSU7 lines with these three races revealed that MSU7-1 had the same disease reaction as SEL 111 and SEL 1360. Lines MSU7-3, 7-4, and 7-6, however, were susceptible to races 73 and 448. These results suggest that MSU 7-1 is more resistant than lines MSU7-3, MSU7-4, and MSU7-6. The fact that the resistance spectrum of MSU7-1 is identical to that of SEL 111 (*Co-5* and *Co-7*) and SEL 1360 (*Co-5*) suggests that MSU7-1 carries *Co-5* or both *Co-5* and *Co-7* and is a result of a recombination event between $SAB3_{400}$ and the *Co-5* locus. The other gene(s) in MSU7-1 may contribute to the allelism reported by Gonçalves-Vidiga1 *et al*. [12] in crosses with Mexico 222. Likewise MSU7-2 does not have the profile of the *Co-7* or *Co-5* genes as it is resistant to races 7 and 73 but susceptible to race 448.

Further characterization of the anthracnose resistance conditioned by the *Co-7* gene was conducted by inoculating MSU7-3, MSU7-4 and MSU7-6 with additional *C. lindemuthianum* races: 31, 47, 357, and 1545 (Table **4**). In 2006 we obtained a Guatemalan isolate of race 521 which we then used to inoculate all test materials. Surprisingly, all MSU7 lines tested including the parental material, SEL 111, inoculated with races 521 and 1545 were susceptible. Race 357 overcame resistance in all three MSU7 lines tested but not the resistance in SEL 111 and SEL 1360, indicating that the resistance in SEL 111 to race 357 is conferred by the *Co-5* gene and not *Co-7*. Inoculation with races 31 and 47 showed that MSU7-3 possessed partial resistance when compared to MSU7-4 and MSU7-6 which were susceptible. The authors define partial resistance as the appearance of very small constricted lesions that do not lead to plant death.

Based on inferences from previous studies, the *Co-7* gene is narrowly defined as conditioning resistance to races 515, 521 and 1545 but susceptibility to races 3 and 73. Pereira and Santos [13] reported the isolation of the *Co-7* gene from a 3-way cross originating from G 2333. In the absence of genetic studies they concluded that the breeding line H1 [14] carried the *Co-7* gene based on the result of inoculations with race 73 (S), 1545 (R) and 2047 (S). In our inoculations with the Guatemalan isolate of race 521, H1 was susceptible which by definition would suggest that it does not carry *Co-*

7. In the present study, MSU7 lines were susceptible to race 73 and race 521. SEL 111, the starting genetic stock material used to derive the *Co-7* lines, was also susceptible to the Guatemalan isolate of race 521 and race 1545. The conflicting data may result from the use of different isolates of race 521. Although all isolates used in this study were tested on the differential series to identify their race designations, the differential series does not adequately represent the high level of avr gene diversity present in *C. lindemuthianum*. It is possible that the isolates of races 521 and 1545 used later in the study and those used earlier to develop the SEL 111 lines are different in their Avr gene constitution. Different isolates of race 65 have shown contrasting virulence patterns in other studies [1]. It is possible that SEL 111 did not possess the *Co-7* gene from the outset. Since SEL 111 was confirmed to carry two independent genes for anthracnose resistance, and one of those is *Co-5* and if the other is not *Co-7* by definition, this suggests that G 2333 may possess other genes for resistance.

Allelic Differences at the *Co-5* Locus

The availability of differentiating races of anthracnose is critical for the separation of resistance alleles at a locus. An absence of discriminating races during the initial characterization of the *Co-5* gene in G 2333 led to the assumption that G 2333 carried the same allele present in the differential cultivar, TU. In the initial studies with G 2333, Young and Kelly [6] assumed that the *Co-5* alleles in G 2333 and TU were the same. More recently, TU (*Co-5*) was reported to be resistant to races 3481 and 3545 whereas G 2333 was susceptible [15]. Since Young and Kelly [6] showed that G 2333 has an allele at the *Co-5* locus (SEL 1360), then data from [15] would suggest that different alleles of *Co-5* gene reside in SEL 1360 (G 2333) and TU. The allele in TU appears to be more effective as it conditions resistance to races 3481 and 3545. We propose that G 2333 carries a second allele of the *Co-5* gene, different from that possessed by the differential cultivar TU and the allele in SEL 1360 (G 2333) be designated *Co-5*^{2}. Additionally, Mahuku *et al*. [16] reported that G 2333 is susceptible to race 3481 while a related line G 2338 is resistant (Table **4**). Initial characterization of G 2338 showed that it carries three genes for anthracnose resistance [17] similar to G 2333. Based on molecular marker data (not shown) G 2338 appears to carry alleles at the *Co-4* and *Co-5* loci as both markers tag the locus not the allele. We hypothesize that since TU is also resistant to race 3481, the difference in resistance between G 2333 and G 2338 lies at the *Co-5* locus and that G 2338 may carry the same allele as TU.

CONCLUSIONS

In an attempt to isolate and characterize the *Co-7* gene in G 2333, the MSU7 breeding lines were derived through a series of crosses and selection that combined molecular markers and direct inoculations with different anthracnose races. The final result produced MSU7 lines that do not demonstrate the expected resistance reaction to races 515, 521 and 1545. Therefore, the anthracnose resistance of the MSU7 lines is not conditioned by the *Co*-7 gene described in the literature. The genes isolated in this study conditions resistance to race 7, and in the case of MSU7-2, also to race 73 but susceptibility to all other races of *C. lindemuthianum* tested. This indicates that these genes do not contribute significantly to the resistance spectrum of G 2333, although epistatic interactions have not been tested. In this study, the dissection of a gene pyramid was accomplished through a MAS and inoculation strategy. A caveat of working with molecular markers for the dissection of gene pyramids is the potential for selecting recombinant individuals, that is, individuals in which the molecular marker and the resistance

Table 4. Resistance Spectra of Different Genotypes in the Study

Genotypes	Races of *Colletotrichum lindemuthianum*									
	7	**31**	**47**	**73**	**357**	**448**	**521**	**1545**	**3481**	**3545**[a]
G 2333	R	R	R	R	R	R	R	R	S	S
SEL 1308	R	R	R	R	R	R	R	R	P[b]	NT[c]
SEL 1360	R	R	R	R	R	R	S	S	S	NT
TU	R	R	R	R	R	R	S	S	R	R
SEL 111	R	R	R	R	R	R	S	S	NT	NT
MSU7-1	R	NT	NT	R	NT	R	NT	NT	NT	NT
MSU7-2	R	NT	NT	R	NT	S	NT	NT	NT	NT
MSU7-3	R	P	P	S	S	S	S	S	NT	NT
MSU7-4	R	S	S	S	S	S	S	S	NT	NT
MSU7-6	R	S	S	S	S	S	S	S	NT	NT
G 2338	R	R	R	R	R	R	R	R	R	R

[a] [16] [b] P: Partial resistance [c] NT: not tested

allele have been uncoupled as a result of a recombination event. In the present study, we identified the line MSU7-1 which has the same disease reaction as SEL 111 and SEL 1360 indicating that it still carries the *Co-5* gene and possibly the *Co-7* gene, but does not have the $SAB3_{400}$ marker linked to the *Co-5* locus. MSU7-3 appears to be more resistant than MSU7-4 and MSU7-6, conferring partial resistance to races 31 and 47, both races of Andean origin. The partial resistance may be due to different background genetic effects between the different MSU7 lines. Finally we report that a second allele at the *Co-5* locus is present in G 2333 and differs in resistance spectrum to that in the differential cultivar TU. The allele, proposed as $Co\text{-}5^2$, does not offer the broad resistance spectrum of the original *Co-5* allele.

ABBREVIATIONS

MAS = Marker-assisted selection

RAPD = Random amplified polymorphic DNA

SCAR = Sequence characterized amplified region

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