Fine Mapping of Ur-3, a Historically Important Rust Resistance Locus in Common Bean

Oscar P. Hurtado-Gonzalez,* Giseli Valentini,† Thiago A. S. Gilio,‡ Alexandre M. Martins,§ Qijian Song,* and Marcial A. Pastor-Corrales*,†

*Soybean Genomics and Improvement Laboratory, United States Department of Agriculture-Agricultural Research Service, Beltsville Agricultural Research Center-West, Maryland 20705, †Departamento de Agronomia, Universidade Estadual de Maringá, PR 87020900, Brazil, and ‡Coordenação de Tecnologia em Educação a Distancia, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Quadra St. Bancário Norte, Brasilia, DF 70040020, Brazil

ABSTRACT Bean rust, caused by Uromyces appendiculatus, is a devastating disease of common bean (Phaseolus vulgaris) in the Americas and Africa. The historically important Ur-3 gene confers resistance to many races of the highly variable bean rust pathogen that overcome other rust resistance genes. Existing molecular markers tagging Ur-3 for use in marker-assisted selection produce false results. Here, we describe the fine mapping of the Ur-3 locus for the development of highly accurate markers linked to Ur-3. An F2 population from the cross Pinto 114 (susceptible) × Aurora (resistant with Ur-3) was evaluated for its reaction to four different races of U. appendiculatus. A bulked segregant analysis using the SNP chip BARCBEAN6K_3 placed the approximate location of Ur-3 in the lower arm of chromosome Pv11. Specific SSR and SNP markers and haplotype analysis of 18 sequenced bean varieties positioned Ur-3 in a 46.5 kb genomic region from 46.96 to 47.01 Mb on Pv11. We discovered in this region the SS68 KASP marker that was tightly linked to Ur-3. Validation of SS68 on a panel of 130 diverse common bean cultivars containing all known rust resistance genes revealed that SS68 was highly accurate and produced no false results. The SS68 marker will be of great value in pyramiding Ur-3 with other rust resistance genes. It will also significantly reduce time and labor associated with the current phenotypic detection of Ur-3. This is the first utilization of fine mapping to discover markers linked to rust resistance in common bean.

KEYWORDS Phaseolus vulgaris Uromyces appendiculatus fine mapping rust resistance gene KASP marker

The common bean (Phaseolus vulgaris L.) includes dry and snap beans. The dry edible bean is the most important pulse in the diet of humans throughout the world, especially in Latin America and Africa, where dry beans are the main daily source of protein, complex carbohydrates, fiber, and micronutrients, particularly for the poorest populations (Broughton et al. 2003).

A myriad of biotic and abiotic factors constrain common bean production in the world. Among these, bean rust is a devastating disease that results in significant loss of seed yield in dry beans and pod quality in snap beans (Stavely and Pastor-Corrales 1989; Liebenberg and Pretorius 2010).

The bean rust disease is caused by the biotrophic basidiomycete fungus Uromyces appendiculatus, an obligate parasite of common bean. This pathogen has a complex life cycle with five distinct spore stages and three different nuclear conditions, which are indicative of the capacity of this pathogen for genetic recombination (Groth and Mogen 1978; McMillan et al. 2003). Many published reports reveal the rich virulence diversity of U. appendiculatus, with scores of races (virulence phenotypes) identified around the world (Groth and Roelfs 1982; Mmbaga and Stavely 1988; Stavely and Pastor-Corrales 1989; Liebenberg 2003; Araya et al. 2004; Arunga et al. 2012; Acevedo et al. 2012). More than 90 races of U. appendiculatus from the United States, Africa, Asia, and other countries of the Americas have been characterized and maintained by the United States Department of Agriculture-Agricultural Research Service Bean Project at the Beltsville Agricultural Research Center (Stavely 1984; Mmbaga and Stavely 1988; Stavely et al. 1989; Pastor-Corrales 2001).
Genetic resistance is the most cost-effective strategy to manage bean rust disease. Rust resistance in common bean is conditioned by single and dominant genes identified by the Ur- symbol (Kelly et al. 1996). To date, 10 genes have been named and tagged, mostly with RAPD or SCAR molecular markers (Miklas et al. 2002). Five genes (Ur-3, Ur-5, Ur-7, Ur-11, and Ur-14) belong to the Middle American gene pool, while five genes (Ur-4, Ur-6, Ur-9, Ur-12, and Ur-13) belong to the Andean gene pool (Augustin et al. 1972; Ballantyne 1978; Stavely 1984, 1990; Grafton et al. 1985; Finke et al. 1986; Jung et al. 1998; Liebenberg and Pretorius 2004; Souza et al. 2011).

The Ur-3 gene present in the Middle American white-seeded common bean, Aurora, was reported by Ballantyne (1978). Since then, this gene has been used extensively as the source of rust resistance in a large number of dry bean cultivars from various market classes of the United States, as well as in fresh market and processing snap beans (Kelly et al. 1994; Stavely et al. 1997; Pastor-Corrales et al. 2007; Urrea et al. 2009; Osorno et al. 2010; Brick et al. 2011; Beaver et al. 2015). Ur-3 has also been used as a source of rust resistance in dry bean cultivars of South Africa (Liebenberg et al. 2005). In addition, Ur-3 has been the subject of different studies, including genetics (Grafton et al. 1985; Kalavacharla et al. 2000), molecular markers, and gene tagging (Haley et al. 1994). The Ur-3 is also present in Middle American cultivars Mexico 235, Ecuador 299, NEP 2, and 51052, in addition to other undefined rust resistance genes (Stavely et al. 1989; Miklas et al. 2000; Hurtado-Gonzalez et al. 2016).

The Ur-3 gene confers resistance to 55 of 94 races of the bean rust pathogen maintained at Beltsville, MD (Pastor-Corrales et al. 2001). More importantly, Ur-3 confers resistance to many races that overcome the resistance of all other named rust resistance genes in common bean. For example, the Ur-3 gene confers resistance to race 22-52 (previously known as race 108), the only race known to overcome the broad-spectrum resistance of the Ur-11 gene present in PI 181996 and PI 190078, and of the Ur-14 gene present in Ouro Negro (Stavely 1998; Alzate-Marín et al. 2004). The name of race 108 and of six other races (41, 47, 49, 53, 67, and 84) used in this study, was changed after these races were phenotyped on a new set of bean rust differential cultivars adopted for the characterization of races of *U. appendiculatus* and a binary system to name these races (Steadman et al. 2002; Pastor-Corrales and Aime 2004). The new and old names (in parentheses) of the races used in this study are: 15-1 (41), 15-3 (47), 22-6 (49), 31-1 (53), 31-22 (67), 37-1 (84), and 22-52 (108).

The Ur-3 gene also complements the broad-spectrum rust resistance in accessions PI 151385, PI 151388, PI 151395, and PI 151396, which are also only susceptible to race 22-52. Similarly, Ur-3 confers resistance to race 37-1, the only known race that overcomes the rust resistance in PI 260418 (Pastor-Corrales 2005). In addition, Ur-3 confers resistance to many races that overcome the Ur-4, Ur-5, Ur-6, Ur-7, Ur-9, Ur-12, and Ur-13 genes. Although Ur-3 is not resistant to all races of Mesoamerican origin, this gene confers resistance to most races of *U. appendiculatus* of Andean origin; that is, races isolated from common beans of the Andean gene pool. Thus, Ur-3 is a critical component of gene pyramiding of common bean cultivars with broad resistance to rust. The information above provides strong evidence of the historical importance and current relevance of Ur-3 for breeding dry and snap beans with broad and durable resistance to rust in the United States and other nations (Stavely 2000; Pastor-Corrales et al. 2001).

The resistant reaction of Ur-3 gene to *U. appendiculatus* is initially characterized by the production of small water-soaked chlorotic spots that subsequently become, in ~48 hr, well-defined necrotic spots without sporulation. This resistant phenotype is classified as grade 2, 2+ and is considered resistant. Reactions 4, 5, and 6 are considered susceptible.

### Table 1 Reaction of the common bean cultivars used in this study to races 15-1 (41), 31-1 (53), 37-1 (84), and 22-52 (108) of *Uromyces appendiculatus*, the causal agent of the bean rust disease

| Cultivar                  | Ur Gene | Reaction to Races of *Uromyces appendiculatus* |
|--------------------------|---------|------------------------------------------------|
|                          |         | 15-1  | 31-1  | 37-1  | 22-52 |
| Pinto 114                | –       | 5, 4  | 5, 4  | 5, 4  | 5, 4  |
| Aurora                   | Ur-3    | 2     | 2+    | 2+    | 2+    |
| Early Gallatin           | Ur-4    | 4, 5  | 4, 5  | 4, 5  | 4, 5  |
| Golden Gate Wax          | Ur-6    | 3, 4  | 4, 5  | 3, 4  | 4, 5  |
| PI 181996                | Ur-11   | f2    | f2    | f2    | f2    |

Standard bean rust grading scale: 1 = no visible symptoms; 2, 2+ = necrotic spots without sporulation; f2 = faint and tiny chlorotic spots; 3 = tiny uredinia (sporulating pustules) <0.3 mm in diameter; 4 = uredinia, 0.3–0.5 mm in diameter (large sporulating pustules); 5 = large uredinia, 0.5–0.8 mm in diameter; 6 = very large uredinia, >0.8 mm in diameter. Reactions 2, 3, and f2 are considered resistant. Reactions 4, 5, and 6 are considered susceptible.
but highly laborious and time consuming. The objective of this study is to identify and pyramiding of resistance genes using marker-assisted selection. As indicated earlier, at least 129 F2 plants were derived from the cross Pinto 114 × Aurora, the bean rust disease resistance parent containing the resistance gene, Ur-3. Thus, to improve the durability of common bean cultivars to the highly variable bean rust pathogen, U. appendiculatus, tightly linked, effective molecular markers are making possible the identification of more effective molecular markers.

Although the Ur-3 is a very important rust resistance gene in common bean, to date there is not a reliable molecular marker tagging Ur-3. Thus, to improve the durability of common bean cultivars to the highly variable bean rust pathogen, Ur-3 cannot be combined with other rust resistance genes using marker-assisted selection. As indicated earlier, at present, pyramiding Ur-3 with other rust resistance genes is only feasible using specific races of the rust pathogen, an activity that is reliable but highly laborious and time consuming. The objective of this study was to develop highly specific, tightly linked, effective molecular markers for the detection of the historically important and widely used Ur-3 rust resistance gene, either alone or in combination with other rust resistance genes of common bean.

### MATERIALS AND METHODS

#### Population development and phenotypic evaluation of the bean rust disease

A total of 129 F2 plants were derived from the cross Pinto 114 × Aurora. Both are dry beans of the Middle American pool of common bean, where Pinto 114 was the susceptible parent and Aurora was the resistant parent containing the Ur-3 gene. The following cultivars with known rust resistance genes were included in the inoculation as internal controls of successful rust inoculation: Early Gallatin (Ur-4), Golden Gate Wax (Ur-6), and PI 181996 (Ur-11) (Table 1). All F2 plants, parents, and control cultivars were grown in 12.7-cm diameter pots containing two plants per pot. The primary (unifoliate) leaves of bean plants were inoculated ~7 d after seeding, when the primary leaves were ~35–65% expanded (Stavely 1984). To prepare the rust inocula, suspensions of frozen urediniospores of various races of U. appendiculatus were placed in a 25-ml solution of cold tap water and 0.01% Tween 20 in a 250-ml Erlenmeyer flask. The spore solutions were prepared with a concentration of 2 × 10^4 urediniospores per ml. All 129 F2 plants and the control cultivars were inoculated with races 15-1, 31-1, 37-1, and 22-52 of U. appendiculatus. Races 15-1, 31-1, 37-1, and 22-52 elicited the same resistance (HR) reaction on plants with Ur-11, as shown in Supplemental Material, Table S1. However, these races elicited a different type of reaction on PI 181996 (the control cultivar with Ur-11) and on cultivars with other rust resistance genes. Thus, one important reason for using four races to phenotype the F2 population was to unequivocally ensure the phenotype of each F2 plant, the parents, and of the control cultivars, which included plants with Ur-4, Ur-6, Ur-11, and other rust resistance genes. The F2 plants were inoculated using a cotton swab to apply the spore solution of each of the races on the abaxial side of the primary leaves. After inoculation, the plants were transferred to a mist chamber (20 ± 1° and relative humidity >95%) for 18 hr, under darkness. After this period, the plants were transferred to the greenhouse. Visible rust symptoms were observed on susceptible plants ~10–12 d after inoculation (dai).

The F2 population and parents were evaluated for their rust phenotype ~12–14 dai using a 1–6 scale (Stavely and Pastor-Corrales 1989), scored as follows: 1 = no visible rust symptoms; 2 = necrotic or chlorotic spots without sporulation, <0.3 mm in diameter (HR);...
susceptible. Thereafter, the F2 plants were maintained in the greenhouse (sporulating pustules). Plants with grades 2 and 3 were classified similarly to the F2 plants, as described above. The 31-1 on the abaxial side of the leaves. After spraying, plants were prepared. Each bulk consisted of DNA from eight different F2 plants. 10 ng of genomic DNA, 0.25 μM of forward and reverse primers, 1× PCR buffer [200 mM Tris-HCl (pH 8.0), 500 mM KCl, 2 mM each dNTP, 10% glycerol, 15 mM MgCl2, 20 ng/μl of single-stranded binding protein], and 0.1 unit of Taq DNA polymerase. The PCR thermocycling parameters were 3 min at 92°C and 38 cycles of 50 sec at 90°C, 45 sec at 58°C, and 45 sec at 72°C, followed by a 5 min extension at 72°C and hold at 10°C. PCR products were resolved on 2–3% agarose gels (Agarose SFR; Amresco, Dallas, TX) prepared with TBE 1× buffer (Tris-borate-EDTA) and stained with 1 μg/ml ethidium bromide. Developing and testing Kompetitive Allele Specific PCR markers A subset of SNPs positively associated with the Ur-3 locus found using BSA were used to map the Ur-3 rust resistance locus. The polymorphisms and quality of the SSR markers were polymorphic between the Pinto 114 (susceptible) and Aurora (resistant with Ur-3) and were used to map the Ur-3 rust resistance locus. The SSRs were polymorphic between the parent Pinto 114 (susceptible) and Aurora (resistant) parents and the three susceptible bulks were homozygous and clustered tightly with the susceptible parent (Pinto 114). Developing and evaluating simple sequence repeat markers linked to Ur-3 The sequence fragments containing SNPs associated with the Ur-3 locus were aligned to the common bean reference genome using Standalone Megablast (Margulis et al. 2008) to identify the scaffolds in the reference genome. Scaffolds were downloaded from Phytozone (https://phytozone.jgi.doe.gov/pz/portal.html), DOE, JGI (Department of Energy, Joint Genome Institute). The scaffolds were screened for the presence of simple sequence repeat (SSR) markers. Procedures for SSR identification, SSR screening, and primer design were previously described by Song et al. (2010).

The polymorphism and quality of the SSR markers were first tested using DNA from the Pinto 114 (susceptible) and Aurora (resistant) parents. Polymorphic SSR markers between Pinto and Aurora were then used to analyze the DNA of the F2 population from the Pinto 114 × Aurora cross. Polymerase chain reaction (PCR) was performed with 5 ng of genomic DNA, 0.25 μM of forward and reverse primers, 1× PCR buffer [200 mM Tris-HCl (pH 8.0), 500 mM KCl, 2 mM each dNTP, 10% glycerol, 15 mM MgCl2, 20 ng/μl of single-stranded binding protein], and 0.1 unit of Taq DNA polymerase. The PCR thermocycling parameters were 3 min at 92°C and 38 cycles of 50 sec at 90°C, 45 sec at 58°C, and 45 sec at 72°C, followed by a 5 min extension at 72°C and hold at 10°C. PCR products were resolved on 2–3% agarose gels (Agarose SFR; Amresco, Dallas, TX) prepared with TBE 1× buffer (Tris-borate-EDTA) and stained with 1 μg/ml ethidium bromide.

| SSR BARC ID | Motif | Product Size | Forward Primer Position (bp) | Reverse Primer Position (bp) | Forward Primer Sequence (5’–3’) | Reverse Primer Sequence (5’–3’) |
|-------------|-------|--------------|-----------------------------|-----------------------------|---------------------------------|---------------------------------|
| BARCPVSSR13992 (AT)10 | 293 | 46,266,888 | 46,267,182 | CAATCTAAGTGTCATCGCAAG | TTCCATCCATCATATTTTCAGGCAGGAACAGCTGAGTGATC | GCCTTTTTCCTTGTGTCCTGAGTATG |
| BARCPVSSR13998 (TA)16 | 280 | 46,402,850 | 46,403,129 | TTGGTCAAGGAAAGTCATCGAC | AAGCTGAATTTTATTTCAGTTGCTCC TGTCTTGGGTTGAGATGTATT | AAGCTGAATTTTATTTCAGTTGCTCC TGTCTTGGGTTGAGATGTATT |
| BARCPVSSR14001 (TA)16 | 234 | 46,535,562 | 46,535,795 | TCTGCAATTTTCTGCTTATCGAC | GGCCTCAGACTGGTGAGTGT ACCATCCGAAAAGGGTTTCTGCT | GGCCTCAGACTGGTGAGTGT ACCATCCGAAAAGGGTTTCTGCT |
| BARCPVSSR14007 (TC)12 | 276 | 46,684,154 | 46,685,469 | TGATCATTTTCTGCTATCATCGAC | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14082 (AT)17 | 206 | 47,291,401 | 47,291,606 | TGGATGACGTTCCACTCGTA | CGCCTCAGACTGGTGAGTGT ACCATCCGAAAAGGGTTTCTGCT | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14083 (AT)10 | 282 | 47,336,615 | 47,336,896 | TGGATGACGTTCCACTCGTA | TGCCTACTTTTCTGCTTATCGAC TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14084 (AT)10 | 225 | 47,398,825 | 47,399,049 | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14085 (TTA)29 | 243 | 47,718,795 | 47,719,037 | TTGGATGACGTTCCACTCGTA | TGCCTACTTTTCTGCTTATCGAC TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14086 (AT)12 | 155 | 47,967,465 | 47,967,619 | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14088 (ATA)21 | 169 | 48,416,592 | 48,416,760 | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14079 (TAT)10 | 171 | 48,441,279 | 48,441,449 | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14080 (ATT)13(TAT)10 | 279 | 48,565,882 | 48,566,160 | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |
| BARCPVSSR14081 (TG)10 | 298 | 48,664,607 | 48,664,905 | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC | TGCCTACTTTTCTGCTTATCGAC |

The SSRs were polymorphic between the parent Pinto 114 (susceptible) and Aurora (resistant with Ur-3) and were used to map the Ur-3 rust resistance locus. Newly emerged trifoliate leaves from each of the F2 plants were collected and total genomic DNA was isolated using DNeasy 96 Plant Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. Based on the rust reaction of each of the F2 plants, three susceptible (r) bulks were prepared. Each bulk consisted of DNA from eight different F2 susceptible plants. Bulks of resistant F2 plants were not prepared to avoid the inclusion of heterozygous-resistant (Rr) plants. These bulks were used for bulk segregant analysis (BSA) for identification of markers potentially associated with the Ur-3 gene (Michelmore et al. 1991). The DNA from susceptible bulks and two samples from each parent were used for bulk segregant analysis (BSA) for identification of markers. Procedures for SSR identification, SSR screening, and primer design were previously described by Song et al. (2010).
machines, using white semiskirted polypropylene 0.2 ml 96-well PCR plates (USA Scientific), and sealed with MicrosealB (Bio-Rad, Hercules, CA). After PCR amplification was completed, PCR plates were scanned using the Mx3000P qPCR machine (Agilent, St. Clara, CA) and allele calls for each genotype were obtained using the MxPro software (Agilent) or the Klustercaller software (LGC).

Construction of genetic linkage map around the Ur-3 locus

The genetic distance between the SSRs, KASPs, and the Ur-3 locus in the F2 population (129 plants) was estimated using the software JoinMap 4.0 (Van Ooijen 2006). Default settings of a Regression Mapping algorithm based on Kosambi map function were attributed to define linkage order and distances in centimorgans (cM). A minimum likelihood of odds (LOD) ≥3.0 and a maximum distance of ≤50 cM were used to test linkages among markers.

Fine mapping of the Ur-3 locus in F3 plants using KASP markers

F3 families were selected based on the recombination between Ur-3 and the SSRs and KASPs molecular markers found in the F2 population. A total of 10 F3 families were selected for screening with KASP markers.

### Table 4 Physical position and primer sequences of KASP markers associated with Ur-3 rust resistance gene in common bean

| Short Marker Name | Physical Position Pvul V1.0 (bp) | KASP Assay Primer Sequences (5’–3’) |
|-------------------|---------------------------------|-------------------------------------|
| SS1               | 46,437,627                      | GAAGTGCAAAGTTTCTCTACATTCACATATTAT   |
|                   |                                 | AGCTGGCTTTTGATCATCACAA             |
| SS3               | 46,494,532                      | GAAGTGCAAGTTTCTCTACATTCACATATTAT   |
|                   |                                 | GTTCTCAGATTTCTCAACCATTATGAAAT      |
| SS4               | 46,613,419                      | GAAGTGCAAGTTTCTCTACATTCACATATTAT   |
|                   |                                 | GTTCTCAGATTTCTCAACCATTATGAAAT      |
| SS5               | 46,667,862                      | GAAGTGCAAGTTTCTCTACATTCACATATTAT   |
|                   |                                 | GTTCTCAGATTTCTCAACCATTATGAAAT      |
| SS6               | 47,083,906                      | GAAGTGCAAGTTTCTCTACATTCACATATTAT   |
|                   |                                 | GTTCTCAGATTTCTCAACCATTATGAAAT      |

KASP markers were used to genotype the F2 mapping population and F3 families for fine mapping from the cross Pinto 114 (susceptible) × Aurora (resistant with Ur-3).
SS4 and SS6 flanking the Ur-3 locus. One homozygous-resistant family and one susceptible family were evaluated as internal controls. The number of plants per family varied from 22 to 32, according to the availability of seeds. A total of 281 F3 plants were inoculated with race 31-1 of U. appendiculatus, as described in Materials and Methods. DNA from the F3 plants was isolated according to Lamour and Finley (2006) and were genotyped with KASP markers SS4 and SS6. F3 plants showing recombination between markers SS4 and SS6 were selected for additional genotyping with newly designed KASP markers in order to narrow the genomic region containing the Ur-3 locus.

Haplotype analysis of the Ur-3 locus

Haplotype analysis was performed in the genomic region flanked by the SS4 and SS6 KASP markers. These two markers flanked a region of 470,487 bp on Pv11, from 46,613,419 to 47,083,906 bp. Eighteen diverse bean varieties, including C20, Matterhorn, Stampe, T-39, Sierra, Red Hawk, Jalo EEP 558, Michelite, UC White, Kardinal, Laker, Cornell 49242, BAT 93, Buckskin, Fiero, Lark, UI 906, and CELRK, were sequenced by Song et al. (2015) and used for the haplotype analysis. These lines were also inoculated with races 22-6, 31-1, 31-22, and 22-52 of U. appendiculatus. The sequence variants in the targeted genomic region of the 18 varieties and their phenotypes were used to identify haplotypes associated with resistance and susceptibility to race 31-1. All SNPs identified between KASP markers SS4 and SS6 were handled using Microsoft Excel and haplotypes were identified by visual inspection. At least two KASP markers were designed for each of the observed haplotypes. Whenever feasible, SNP markers were located every 10 kb across the 470,487 bp genomic region. When KASP markers were polymorphic between the Pinto 114 (ur-3) and Aurora (Ur-3) parents, they were used to genotype F3 plants with recombination between the markers SS4 and SS6.

Validation of the markers linked to the Ur-3 locus

A panel of 130 diverse bean cultivars that included all rust resistance genes in common bean were genotyped using KASP markers tightly linked with Ur-3. This was performed with the purpose of generating accurate Ur-3 markers useful in marker-assisted selection. In this panel, some cultivars had the Ur-3 gene alone, other cultivars had Ur-3 combined with other rust resistance genes, while others did not have any reported rust resistance genes. The cultivars in the panel were phenotyped before or during this study with multiple races of the bean rust pathogen, including race 31-1, the phenotypic marker for the Ur-3 gene.
RESULTS

Inheritance of rust resistance in common bean Aurora

A total of 129 F2 plants from the Pinto 114 × Aurora cross were evaluated for their reaction to races 15-1, 31-1, 37-1, and 22-52 of **U. appendiculatus** (Table S2). Aurora was resistant to all four races and exhibited the same type of reaction that was characterized by necrotic spots without sporulation (grades 2, 2+). Pinto 114 was susceptible to the same four races, with a reaction characterized by large uredinia (grades 4, 5, and 6). Based on the reaction to all four races, the inheritance of rust resistance study of the 129 F2 plants exhibited a segregation equal to 101 resistant and 28 susceptible plants, fitting a ratio of 3 resistant to 1 susceptible (χ² = 0.747, *P* value = 0.38), confirming that the rust resistance in Aurora was conferred by the single and dominant Ur-3 gene (Table S2).

BSA and SNP genotyping using BARCEBAN6K-3 BeadChip

Based on the BSA, 28 SNPs were associated with Ur-3 (Table 2). The alleles of these SNPs could separate the susceptible Pinto 114 and the three susceptible bulks from the resistant Aurora parent. According to the genetic linkage map created by Song et al. (2015), these 28 SNPs were distributed from 72.3 to 76.2 cM on the lower end of the common bean chromosome Pv11. The physical location of the associated 28 SNPs was between 46,437,627 bp (ss715647455) and 48,784,158 bp (ss715641910), a region spanning a total of 2.1 Mbp (Table 2).

Mapping of the Ur-3 gene

The large portion of the genomic region containing the 28 SNPs associated with the Ur-3 rust resistance gene was targeted for SSR development. A total of 48 SSR markers located between 46,266,888 and 48,664,905 bp on Pv11 were developed. Thirteen of the 48 SSRs markers were polymorphic between the parents Pinto 114 (susceptible) and Aurora (resistant) parents (Table 3). These markers, which showed unequivocal allele separation in agarose gel, were used to map the Ur-3 locus in the F2 population Pinto 114 × Aurora. Linkage analysis positioned the Ur-3 locus between markers BARCPVSSR14001 (46,535,562 bp) and BARCPVSSR14082 (47,291,606 bp), a 756,044 bp genomic region (data not shown). In addition, four positively associated SNPs from the BSA and two SNPs [retrieved from Song et al. (2015)] near the SSRs flanking the Ur-3 locus were selected and converted into KASP markers (Table 4).

Five KASP markers (SS1, SS3, SS4, SS5, and SS6) showed clear separation of the three clusters (two homozygous and one heterozygous) and were polymorphic between the Pinto 114 and Aurora parents. These KASP markers were used to refine the Ur-3 gene map. Linkage analysis in the F2 population genotyped with 13 SSRs and the 28 SNPs [retrieved from Song et al. (2015)] near the SSRs flanking the Ur-3 locus were selected and converted into KASP markers (Table 4). Five KASP markers (SS1, SS3, SS4, SS5, and SS6) showed clear separation of the three clusters (two homozygous and one heterozygous) and were polymorphic between the Pinto 114 and Aurora parents. These KASP markers were used to refine the Ur-3 gene map. Linkage analysis in the F2 population genotyped with 13 SSRs and the five KASP markers showed that Ur-3 was flanked by KASP marker SS5 and SSR marker BARCPVSSR14001 (46,535,562 bp) and BARCPVSSR14082 (47,291,606 bp), a 756,044 bp genomic region (data not shown). In addition, four positively associated SNPs from the BSA and two SNPs [retrieved from Song et al. (2015)] near the SSRs flanking the Ur-3 locus were selected and converted into KASP markers (Table 4).

Analysis of recombination in F3 and Ur-3 haplotype identification

KASP markers SS4 and SS6 were mapped at 0.6 and 1.0 cM from the Ur-3 locus, respectively (Figure 1A), in a 470,487 bp (470 kb) genomic region of chromosome Pv11, from 46,613,419 to 47,083,906 bp (Figure 1B).

Data availability

All data described in this manuscript related to bean rust phenotypes, Pinto 114 × Aurora F2 genetic map, F3 fine-mapping population, and haplotype analysis are available in Table S1, Table S2, Table S3, Table S4, Table S5, Table S6, and Table S7.
These markers were chosen to genotype 12 selected F3 families from the cross Pinto 114 × Aurora. Among the 12 families, four were derived from recombinant F2 plants between KASP markers SS4 and SS6, six families were heterozygous between markers SS4 and SS6 flanking Ur-3, and two families were used as internal controls: one homozygous resistant and the other homozygous susceptible. In addition, these 12 families (281 F3 plants) were inoculated with race 31-1 of *U. appendiculatus*. Genotyping the 281 F3 plants resulted in 87 F3 plants with recombination events between the SS4 and SS6 KASP markers (Table S4). These 87 F3 plants were selected for subsequent fine-mapping analysis with additional KASP markers (Table 4). SS5 (ss715647451 at position 46,667,862) was the only KASP marker derived from the BeanChip that was located between SS4 and SS6; thus, SS5 was also used to genotype the recombinant 87 F3 plants.

We then mined the SNP sequence data from the 18 common bean varieties (Song et al. 2015) to search for additional SNPs between SS4 and SS6. Based on the whole genome sequence of the 18 common bean varieties, ~6000 SNPs and small indels were found between SS4 and SS6 (Table S5). These SNPs were grouped into 10 major haplotypes (Table 5). Each of these haplotypes were then tagged with one or two KASP markers and were examined for their polymorphism between Pinto 114 (ur-3), Aurora (Ur-3), Mexico 235 (Ur-3+), and PI 181996 (Ur-11). The KASP markers polymorphic between the Pinto 114 and Aurora parents were tested on the set of 87 F3 recombinant plants identified previously with KASP markers SS4 and SS6. Analysis of the 87 F3 recombinant plants positioned the Ur-3 gene between KASP markers SS17 and SS21, in the 83,198 bp genomic region (Figure 1B and Table S7). Concurrently, a specific haplotype for Ur-3 was identified based on the reaction of the 18 sequenced varieties to race 31-1 of *U. appendiculatus*. Only the varieties C 20, Matterhorn, Stampede, T-39, and Sierra had a resistant phenotype (HR) to races 31-1 and 22-52, indicating that these cultivars have the Ur-3 gene (Table S7). The final genotyping analysis on the 87 recombinant plants mapped Ur-3 between KASP markers SS36 and SS21, in a specific genomic region of 46,563 bp, ranging from 46,967,787 to 47,014,350 bp of Pn1 (Table 6). Two F3 plants, one resistant and the other susceptible, had the same recombination breakpoint, demonstrating that the Ur-3 gene was located in the region flanked by SS36 and SS21 (Figure 1C and Table 6).

Subsequent genotyping of the 129 F2 plants from the Pinto 114 × Aurora cross using KASP SS36 and KASP marker SS68, which was targeting the Ur-3 haplotype and only ~200 bp downstream from SS36, showed that these markers were linked to the Ur-3 rust resistance gene, with no recombination observed between bean rust phenotype and genotype (Table S2). SNP for KASP marker SS68 (46,967,980 bp in Pn1) is a transversion nucleotide change from A to T, where A is homozygous-resistant, homozygous-susceptible, and heterozygous plants (Figure 2). Conversely, the KASP marker SS36 did not always differentiate homozygous-resistant from heterozygous plants (data not shown). KASP marker SS68 is located proximal (~500 bp) to the leucine-rich repeat–containing gene, Phvul.011G193100.

**Validation of KASP marker SS68 linked to the Ur-3 gene**

We used the SS68 KASP marker to genotype a panel of 130 common bean cultivars that included dry and snap beans. Some of these common beans possessed the Ur-3 gene alone, while others had Ur-3 in combination with other rust resistance genes. In addition, other cultivars had single or combinations of the other 10 rust resistance genes in common bean. The results of this validation showed that SS68 was highly accurate for the identification of the Ur-3 locus (Table 7). No false positives or false negatives were observed when comparing the genotypic
The Ur-3 locus contains six candidate genes
The genomic region delimited by markers SS36 and SS21, defined as the Ur-3 locus, contained six candidate genes according to the Phytozome net database for P. vulgaris assembly V1.0. The names of these genes are: Phvul.011G193100, Phvul.011G193200, Phvul.011G193300, Phvul.011G193400, Phvul.011G193500, and Phvul.011G193600. Three of these Ur-3 genes (Phvul.011G193100, Phvul.011G193500, and Phvul.011G193600) are classified as containing NB-ARC domains and leucine-rich repeat (LRR) regions. Genes Phvul.011G193200 and Phvul.011G193400 are annotated as serine/threonine kinases, and Phvul.011G193300 is a tyrosine kinase with salt/stress response-related and antifungal function. All these candidate genes, except Phvul.011G193600, were highly expressed in common bean leaves, according to the expression level experiments recorded in the JGI genome browser for P. vulgaris.

DISCUSSION
Development of accurate SNP markers linked to the Ur-3 locus
The historically important Ur-3 gene confers resistance to the pathogen that causes the rust disease of common bean. The effective incorporation of Ur-3 into dry and snap beans using molecular markers has been limited by the inaccuracy of the molecular markers linked to this gene (Haley et al. 1994; Nemchinova and Stavely 1998; Stavely 2000). The authors that reported the RAPD (OK14620) and SCAR (SK14) markers linked to Ur-3 indicated that these markers produced both false negatives and false positives results (Haley et al. 1994; Nemchinova and Stavely 1998).

More recently, we have used BSA, SNP assay, and whole genome sequencing to discover SSR markers closely linked to the Ur-3 and other disease resistance genes. However, even the use of closely linked BARCPVSSR14007, an SSR marker reported in this study positioned at 0.2 cM from the Ur-3 locus, resulted in >3% false positive results when this marker was used on the panel of 130 common bean lines (data not shown). Additionally, as indicated earlier, the inability to find specific molecular markers linked to Ur-3 may have been exacerbated by the presence of the Ur-11 rust resistance gene that is closely linked to Ur-3 on the terminal position of chromosome Pv11. Currently, the most reliable method to monitor for the presence of the Ur-3 gene in dry and snap bean cultivars continues to be race 31-1 (53) of U. appendiculatus. Race 31-1 is used as a phenotypic marker that effectively identifies common bean plants with Ur-3 alone or in combination (Pastor-Corrales 2002). However, phenotypic evaluations under greenhouse conditions are very laborious and time consuming (~21 d). Moreover, due mostly but not only to the biotrophic condition of the rust pathogen, most breeders of dry and snap beans do not have the option of using this methodology.

Figure 2 KASP marker SS68 analyzed on 129 F2 plants from the cross Pinto 114 (susceptible) x Aurora (resistant with the U-3 locus) cross inoculated with races of the bean rust pathogen (Uromyces appendiculatus). AA, ur-3 alleles; AB, heterozygous alleles; BB, Ur-3 alleles; NTC, nontarget control.
### Table 7 Validation of the KASP marker SS68 tightly linked with the Ur-3 rust resistance locus on 130 common bean cultivars

| Genotype       | Ur Gene<sup>a</sup> | Dry/Snap Bean | SS68<sup>b</sup> |
|----------------|---------------------|---------------|-----------------|
| Pinto 114      | ur-3                | Dry bean      | AA              |
| Aurora         | Ur-3                | Dry bean      | BB              |
| Mexico 235     | Ur-3                | Dry bean      | BB              |
| Ecuador 299    | Ur-3                | Dry bean      | BB              |
| NEP 2          | Ur-3                | Dry bean      | BB              |
| 51051          | Ur-3                | Dry bean      | BB              |
| Early Gallatin | Ur-4                | Snap bean     | AA              |
| Mexico 309     | Ur-5                | Dry bean      | AA              |
| Golden Gate Wax| Ur-6                | Snap bean     | AA              |
| GN 1140        | Ur-7                | Dry bean      | AA              |
| PI 181996      | Ur-11               | Dry bean      | AA              |
| PC 50          | Ur-9, Ur-12         | Dry bean      | AA              |
| Redlands Pioneer| Ur-13               | Dry bean      | AA              |
| Ouro Negro     | Ur-14               | Dry bean      | AA              |
| Condor         | Susc; reported with Ur-3 | Dry bean    | AA              |
| Vista          | Susc; reported with Ur-3 | Dry bean    | AA              |
| Raven          | Susc; reported with Ur-3 | Dry bean    | AA              |
| Jaguar         | Susc; reported with Ur-3 | Dry bean    | AA              |
| Santa Fe       | Ur-3                | Dry bean      | BB              |
| Merlot         | Ur-3                | Dry bean      | BB              |
| Stampede       | Ur-3                | Dry bean      | BB              |
| Alpine         | Ur-3                | Dry bean      | BB              |
| Starlight      | Ur-3                | Dry bean      | BB              |
| CO-54150       | Ur-3                | Dry bean      | BB              |
| C 20           | Ur-3                | Dry bean      | BB              |
| Matterhorn     | Ur-3                | Dry bean      | BB              |
| Chase          | Ur-3                | Dry bean      | BB              |
| Apache         | Ur-3                | Dry bean      | BB              |
| Burke          | Ur-3                | Dry bean      | BB              |
| La Paz         | Ur-3                | Dry bean      | BB              |
| Aztec          | Ur-3                | Dry bean      | BB              |
| T-39           | Ur-3, Ur-7          | Dry bean      | BB              |
| BelJersay-RR-1 | Ur-3, Ur-4          | Snap bean     | BB              |
| BelJersay-RR-4 | Ur-3, Ur-4          | Snap bean     | BB              |
| BelJersay-RR-5 | Ur-3, Ur-4          | Snap bean     | BB              |
| BelJersay-RR-6 | Ur-3, Ur-4          | Snap bean     | BB              |
| BelDade-RR-1   | Ur-3, Ur-4          | Snap bean     | BB              |
| BelDade-RR-2   | Ur-3, Ur-4          | Snap bean     | BB              |
| BelDade-RR-3   | Ur-3, Ur-4          | Snap bean     | BB              |
| BelDade-RGMR-4 | Ur-3, Ur-4          | Snap bean     | BB              |
| BelDade-RGMR-5 | Ur-3, Ur-4          | Snap bean     | BB              |
| Centennial     | Ur-3, Ur-6          | Dry bean      | BB              |
| Croissant      | Ur-3, Ur-6          | Dry bean      | BB              |
| CO-33875       | Ur-3, Ur-6          | Dry bean      | BB              |
| CO-34142       | Ur-3, Ur-6          | Dry bean      | BB              |
| CO-55119       | Ur-3, Ur-6          | Dry bean      | BB              |
| Kodiak         | Ur-3, Ur-6          | Dry bean      | BB              |
| Coyne          | Ur-3, Ur-6          | Dry bean      | BB              |
| ABC Weihing    | Ur-3, Ur-6          | Dry bean      | BB              |
| ABCP 8         | Ur-3, Ur-6          | Dry bean      | BB              |
| Stampede-R     | Ur-3, Ur-11         | Dry bean      | BB              |
| BelDak-RR-1    | Ur-3, Ur-6, CNC     | Dry bean      | BB              |
| BelDak-RR-2    | Ur-3, Ur-6, CNC     | Dry bean      | BB              |
| BelMiNeb-RMR-7 | Ur-3, Ur-4, Ur-11   | Dry bean      | BB              |
| BelDakMi-RMR-14| Ur-3, Ur-6, Ur-11   | Dry bean      | BB              |
| BelDakMi-RMR-16| Ur-3, Ur-6, Ur-11   | Dry bean      | BB              |
| BelDakMi-RMR-17| Ur-3, Ur-6, Ur-11   | Dry bean      | BB              |

(continued)
**The Ur-3 locus maps to a 46 kb region possessing candidate genes with resistant gene motifs**

Through haplotypic analysis and KASP marker development, it was possible to determine a genomic region of 46,563 bp containing the Ur-3 locus and delimited by markers SS36 and SS21 on Pv11. Six candidate genes were identified within this 46.5 kb region in the *P. vulgaris* reference genome, obtained by sequencing the landrace G19833 of Andean origin. Among the six candidate genes, there were three genes with NB-ARC LRR domains. Proteins containing NB-ABC LRR domains are known to be involved in plant resistance and activation of innate immune responses to various types of pathogens (Hammond-Kosack and Jones 1997; Jones and Dangl 2006). Similarly, protein kinases (also found in the 46.5 kb region) are known to play a central role in signaling during pathogen recognition and the subsequent activation of plant defense mechanisms (Xue et al. 2015). The genomic region containing the Co-4 gene on chromosome Pv08, conferring resistance to *Colletotrichum lindemuthianum* in common bean, has been characterized and known to contain an open reading frame coding for a serine threonine kinase (Oblesescu et al. 2015), a type of protein which has also been identified in our studies. Additionally, serine threonine protein kinase constitutes candidate genes for controlling angular leaf spot resistance in the Andean landrace G5686 (Keller et al. 2015). Whether the phenotype of the Ur-3 locus is the result of the expression of one or more of the six candidate genes will be a matter of further investigation.

Sequence analysis of the Andean landrace G19833, used to sequence the reference genome of common bean, revealed that the 46.5 kb genomic region containing the Ur-3 locus is highly duplicated (Figure S1), and it includes repetitive elements in the intergenic spaces. Additionally, this genomic region is AT-rich (33% vs. 16% for GC), which suggests that it is highly unstable. Sequence analysis comparing the Middle American Aurora common bean and the Andean landrace G19833, will provide valuable insights into the structural differences and evolutionary history of the important Ur-3 rust resistance locus.

**Conclusions**

This study used a new approach to generate KASP SS68, the first highly accurate DNA marker linked to the Ur-3 rust resistance gene in common bean. We fine-mapped a 46.5 kb genomic region in chromosome Pv11, present in Middle American common bean cultivar Aurora. This was accomplished using the BARCBEAN6K_3 BeadChip, SSRs, KASP technology, and local association. The validation of this newly discovered KASP SS68 marker on a panel of 130 common bean lines revealed that SS68 was highly accurate in identifying Ur-3. This marker will be of value for combining Ur-3 with other Andean and Middle American genes with broad spectrum resistance to the highly variable bean rust pathogen. In addition, the utilization of the new marker SS68 will significantly reduce the time and labor associated with the transfer of the Ur-3 gene using inoculations of bean plants with specific races of the rust pathogen. The genomic region containing the Ur-3 locus included six genes annotated in the reference genome of *P. vulgaris*. These genes are likely candidates for the Ur-3 rust resistance gene. Gene expression analysis of these candidate genes and functional approaches will enhance our understanding of the mechanisms underlying the reaction of *P. vulgaris* to *U. appendiculatus*.

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