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ORIGINAL PAPER

Increasing the density of markers around a major QTL controlling resistance to angular leaf spot in common bean

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Abstract Angular leaf spot (ALS) causes major yield losses in the common bean (*Phaseolus vulgaris* L.), an important protein source in the human diet. This study describes the saturation around a major quantitative trait locus (QTL) region, ALS10.1, controlling resistance to ALS located on linkage group Pv10 and explores the genomic context of this region using available data from the *P. vulgaris* genome sequence. DArT-derived markers (STS-DArT) selected by bulk segregant analysis and SCAR and SSR markers were used to increase the resolution of the QTL, reducing the confidence interval of ALS10.1 from 13.4 to 3.0 cM. The position of the SSR ATA220 coincided with the maximum LOD score of the QTL. Moreover, a new QTL (ALS10.2^{UC}) was identified at the end of the same linkage group. Sequence analysis using the *P. vulgaris* genome located ten SSRs and seven STS-DArT on chromosome 10 (Pv10). Coincident linkage and genome positions of five markers enabled the definition of a core region for ALS10.1 spanning 5.3 Mb. These markers are linked to putative genes related to disease resistance such as glycosyl transferase, ankyrin repeat-containing, phospholipase, and squamosa-promoter binding protein. Synteny analysis between ALS10.1 markers and the genome of soybean suggested a dynamic evolution of this locus in the common bean. The present study resulted in the identification of new candidate genes and markers closely linked to a major ALS disease resistance QTL, which can be used in marker-assisted selection, fine mapping and positional QTL cloning.

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Introduction

The common bean (*Phaseolus vulgaris* L.) represents an important source of protein in the human diet, especially in developing countries (Broughton et al. 2003; Gepts et al. 2008). Angular leaf spot (ALS), which has been reported in more than 60 countries worldwide, is one of the most devastating diseases of this crop, leading to yield losses up to 80 % (Stenglein et al. 2003; Singh and Schwartz 2010). This disease is caused by the hemibiotrophic fungus *Pseudocercospora griseola* (Sacc.) Crous & Braun (syn. *Phaeoisariopsis griseola* (Sacc.) Ferraris) (Crous et al. 2006), which causes angular necrotic spots on the plant leaves and pods (Allorent and Savary 2005). Infection occurs within 3 days of inoculation, with conidia penetrating either through the leaf epidermis or stomata, followed by intercellular colonization of the mesophyll, leading to plasmolysis of the host cells (Monda et al. 2001).

The most effective approach to controlling this disease is through the use of resistant cultivars. Therefore, it is important to develop molecular markers for plant breeding and to understand the mechanisms controlling host–pathogen interactions (Dodds and Rathjen 2010). In the case of ALS, two dominant resistance genes, known as *Phg-1* and *Phg-2*, have been described (Carvalho et al. 1998; Sietsche et al. 2000; Gonçalves-Vidigal et al. 2011; Mahuku et al. 2011). In addition, dominant monogenic inheritance for resistance to ALS has been reported (Corrêa et al. 2001; Mahuku et al. 2004; Namayanja et al. 2006), but the relationship between the genes underlying this resistance and *Phg-1* and *Phg-2* remains unknown. In fact, resistance to ALS can be controlled by both qualitative resistance genes and quantitative trait loci (QTL) (López et al. 2003; Mahuku et al. 2009, 2011; Oblessuc et al. 2012a), which explains the complex inheritance mode of ALS resistance observed by Caixeta et al. (2005). These authors reported the existence of three additional genes (*Phg-3, Phg-4* and *Phg-5*) with two alleles each controlling resistance to ALS in four bean varieties that were previously characterized as possessing monogenic resistance.

Recently, Oblessuc et al. (2012a) reported the existence of an ALS resistance QTL on linkage group Pv10 (ALS10.1^{DG,UC}) that controls 22 and 16 % of the resistance under both field and greenhouse infection conditions, respectively. In addition, the study reported six other ALS resistance QTLs in the IAC-UNA × CAL 143 recombinant inbred line population (UC RIL) developed by Campos et al. (2011). Although these results were important to the understanding of ALS resistance, none of the markers located near ALS10.1 coincided with the maximum LOD score of the QTL; the closest marker was the microsatellite (SSR) GATS11b located 6.0 cM away from the QTL (Oblessuc et al. 2012a).

The increase of markers density in QTLs region such as the ALS10.1 is very important to further study the QTL genomic context and to facilitate marker-assisted selection. Therefore, the development of new molecular markers is needed. Recently, the diversity array technology (DArT) was developed for beans (Briñez et al. 2012). This technique is based on restriction enzyme digestion and adapter ligation of a representative DNA pool of a species, followed by amplification and cloning of this DNA; the DNA is then used as a target for array hybridization of the query sample (Wenzl et al. 2004). Among other applications, the DArT has been used in QTL mapping of resistance loci (Hickey et al. 2011; Vazquez et al. 2012) and bulked segregant analysis (BSA) in various plant species (Wenzl et al. 2007; Lillemo et al. 2008; Golegaonkar et al. 2009; Tah et al. 2010; Satish et al. 2012).

The present study aimed to increase the marker saturation on the genetic region of the major QTL involved in ALS resistance, the ALS10.1 (Oblessuc et al. 2012a, 2012b). The goal was to develop PCR-based markers linked to the ALS10.1 and therefore, to obtain markers that could be efficiently used by breeders in marker-assisted selection toward ALS resistance. In addition, the genomic region of this QTL was investigated using the common bean genomic data from Phytozome. The results of this study will also facilitate further map-based approaches to cloning resistance genes.

Materials and methods

Plant material and marker-based composition of resistant and susceptible bulks

The recombinant inbred lines (RILs) from the IAC-UNA × CAL 143 cross were developed by advancing the F_2 generation through F_8, adopting the principles of single-pod descent for mapping (Funada et al. 2012). From F_8 till F_{10}, single seed descent method was used, resulting in 380 F_{10} plants that generated the UC map (Campos et al. 2011). In this study, 346 UC RILs in the F_{12} generation were used. For the BSA approach, resistant and susceptible RILs were selected from this population to compose the bulks based on previously available data on their respective responses to ALS (Oblessuc et al. 2012a). Briefly, the RILs were screened for ALS resistance under two different experimental conditions consisting of either exposure to natural infection in the field or inoculation with race 0–39 of *P. griseola* in the greenhouse. Disease severity was evaluated based on disease scores that ranged from 1 (no symptoms) to 9 (30 % or more of the leaf area with symptoms). After identifying the most resistant and most susceptible RILs in both the field and greenhouse, 50 lines from

each class were selected based on their genotypes for the SSR markers GATS11b and IAC137, which flank the confidence interval of ALS10.1, as reported by Oblessuc et al. (2012a). Therefore, the resistant bulk was composed of RILs that combined resistant marker alleles and phenotypic resistance to *P. griseola*; similarly, the susceptible bulk was composed of RILs that combined susceptible genotype and susceptible phenotype.

DArT-BSA analysis and development of STS-DArT markers

DNA from the selected RILs was pooled in equimolar amounts to compose the bulks for DArT analysis. Both the resistant (CAL 143) and the susceptible (IAC-UNA) parents were also included in the array hybridization. The hybridization was performed at the Diversity Arrays Technology Pty Ltd (DArT P/L, Canberra, Australia; http://www.diversityarrays.com/) with all 15,360 clones available for *P. vulgaris*. The bulks and the parents were scored for the presence (1) or absence (0) of hybridization based on fluorescence intensities, and a polymorphism information content of 0.5 was used to assess the distribution of the marker scores between the two bulks according to the method described by Anderson et al. (1993). Only clones with quality scores of 100 % (average reproducibility and call rate) were selected.

The inserts of selected polymorphic DArT clones were sequenced at the DArT P/L facility. The NEBcutter V2.0 software (http://tools.neb.com/NEBcutter2/, Vincze et al. 2003) was used to identify possible chimeric sequences based on the presence of *Pst*I, *Bst*NI or *Taq*I restriction sites. In addition, DArT sequences were compared pairwise using the BLASTn software from NCBI (http://www.ncbi.nlm.nih.gov/). Clones with sequence identities ≥95 % and E values $\leq 1 \times 10^{-5}$ were considered redundant, and the clone with the longest sequence and an adequate PCR band pattern was used for the linkage analyses. Putative functions of the DArTs were inferred by performing tBLASTx searches (E values $\leq 1 \times 10^{-5}$) in the non-redundant sequences and the EST databases of NCBI (Altschul et al. 1990).

Insert sequences of selected DArTs were used to design primers and convert them into co-dominant sequence-tagged site (STS-DArT) markers. Primers were designed using the Primer3 v0.4.0 software (http://frodo.wi.mit.edu/primer3/input.htm, Rozen and Skaletsky 2000) with the following criteria: (1) primer size between 18 and 22 bases, (2) maximum annealing temperature difference of 1 °C between forward and reverse primers, and (3) CG content of 40–60 %. Primers were tested using the DNA of the bulks and of the parents in a PCR experiment containing 30 ng of DNA, 1 U of *Taq* DNA polymerase (Promega®), 1.5 mM of $MgCl_2$, 0.25 mM of each dNTP, and 0.8 pmol/µl of each primer for a final concentration of 1× reaction buffer in a final volume of 15 µl. The amplification followed a touchdown protocol with a hot start of 94 °C for 4 min, followed by 30 cycles of 30 s at 94 °C, 45 s at 60 °C (decreasing 0.5 °C each cycle), and 45 s at 72 °C, and 30 cycles with the same denaturation and extension conditions but annealing temperature set at 45 °C. A final elongation step was performed at 72 °C for 7 min. STS-DArTs were resolved in 3 % agarose gels, and those sequences that were polymorphic between bulks and parents were used to genotype the 346 UC RILs in 6 % acrylamide gel electrophoresis followed by silver nitrate staining (Creste et al. 2001).

Genotyping of RILs with additional SSR and SCAR markers

In addition to STS-DArT markers, 6 sequence-characterized amplified markers (SCARs) known to be linked to ALS resistance loci (SN02—Sietsche et al. 2000; SH13, SAA19, SBA16, and SM02—Queiroz et al. 2004; $PF5_{330}$-Mahuku et al. 2004) and SSR markers described in the literature (Gaitán-Solís et al. 2002; Caixeta et al. 2005; Benchimol et al. 2007; Grisi et al. 2007; Hanai et al. 2007, 2010; Blair et al. 2008; Cardoso et al. 2008; Campos et al. 2011) were tested for polymorphisms between the IAC-UNA and CAL 143 parents. The polymorphic markers were genotyped in the 346 RILs of the UC population (Campos et al. 2011). PCR amplification and electrophoresis conditions were the same as described in the respective articles.

Linkage and QTL mapping analyses

Linkage analysis of new STS-DArTs, SCARs and SSRs was performed using the Pv10 framework from Campos et al. (2011). New markers were added using the *try* command of MAPMAKER 3.0b (Lander et al. 1987) with a LOD score of 3.0 and a maximum genetic distance of 37.5 cM calculated by the Kosambi (1944) mapping function as thresholds. The final order of the markers was tested with the *ripple* command with a window of six markers. Finally, multipoint distance estimates were obtained using the *map* command.

Composite interval mapping (CIM) analysis was used to redefine the position of the ALS 10.1 resistance QTL in group Pv10 using the available ALS severity scores of the RILs (Oblessuc et al. 2012a). Analyses were performed with QTL Cartographer v1.17 (CIM; model 6—Zmapqtl; Wang et al. 2005); evidence of the QTL was checked at 1.0 cM intervals with a 10.0-cM window using the likelihood ratio test (LRT) and converted to LOD values by the equation LOD = 0.2172*LRT. Stepwise multiple regressions ($p \leq 0.05$) were also used to obtain the cofactors for

the CIM analysis. The LOD threshold was determined by computing 1,000 permutations ($p \leq 0.05$) for each experimental condition (field or greenhouse evaluations). The 95 % confidence intervals were determined by the two-LOD support interval method (Van Ooijen 1992; Lynch and Walsh 1998).

Sequence analyses of markers linked to ALS10.1

To verify the genome location of all markers of linkage group Pv10, marker sequences were aligned to the draft sequences of the bean chromosomes of the Phytozome v1.0 assembly (http://www.phytozome.net/). The criteria used to assign putative regions to the markers included *E* values $\leq 1 \times 10^{-10}$ and a minimum identity of 50 % between query and database sequences. Transcripts predicted by Phytozome located within 10.0 kb of each marker linked to ALS10.1 were annotated for their putative function, with the goal of analyzing the genomic context of the QTL region. Markers were also aligned to the soybean (*Glycine max*) genome using the PhaseolusGenes website (http://phaseolusgenes.bioinformatics.ucdavis.edu; Gepts and Lin 2011) to assess the synteny between these species. Hits with *E* values $\leq 1 \times 10^{-10}$ were considered significant.

Results

BSA-DArT, STS-DArT, SCAR and SSR marker analyses

The BSA-DArT approach was used in an attempt to saturate the ALS10.1 region. Twenty-one DArT clones were found to be polymorphic between the resistant and the susceptible bulks (Supplemental Table S1). After trimming the vector sequence, the average clone insert was 490 bp long (Table 1); only one chimeric insert, inferred from the presence of a *Taq*I restriction enzyme site, was separated into two independent sequences (864174a and 864174b). Five inserts (23.8 %) had the *Taq*I restriction site at the end of the insert; of these, only one clone (clone 866317) also had the *Bst*NI site. Pairwise comparisons indicated no significant differences between DArT sequences in 59.1 % of the cases (Table 1). Therefore, 13 unique sequences were selected to verify their putative function. Of these, eight had no significant matches in the non-redundant and EST databases, and the remaining showed similarity with either *G. max* or *P. vulgaris* sequences deposited in the GenBank. Primers were developed for these 13 DArT clones in an attempt to convert them into STS-DArTs markers (Table 2); eight of these clones, displaying well-defined fragments in

Table 1 Redundancy analysis of DArT clones based on pairwise alignments with BLASTn and putative functions inferred by tBLASTx searches

Redundancy (BLASTn)					Putative function (tBLASTx)		
Dart clones	Size (bp)	*E* value	Identities[a]	Annotation	Organism[a]	GI number[a]	*E* value
827737/864415	507/421	1×10^{-120}	99 %	No hit	–	–	$>1 \times 10^{-5}$
827737/880625	507/413	1×10^{-120}	99 %	No hit	–	–	$>1 \times 10^{-5}$
827737/880625	507/413	1×10^{-120}	99 %	No hit	–	–	$>1 \times 10^{-5}$
827737/883634	507/704	0.0	91 %	No hit	–	–	$>1 \times 10^{-5}$
827887/882462	562/554	0.0	100 %	No hit	–	–	$>1 \times 10^{-5}$
882462/866012	554/600	0.0	99 %	No hit	–	–	$>1 \times 10^{-5}$
882462/883131	554/556	0.0	99 %	No hit	–	–	$>1 \times 10^{-5}$
864174a/866317	436/287	1×10^{-130}	95 %	Transcription factor bHLH-like	*G. max*	gi|356496190|	5×10^{-43}
864174b	496	No hit	–	PVUSE1NG-RP-025_M22 mRNA	*P. vulgaris*	gi|312036377|	2×10^{-20}
864614	881	No hit	–	No hit	–	–	$>1 \times 10^{-5}$
865197/879964	419/447	0.0	99 %	No hit	–	–	$>1 \times 10^{-5}$
873626/883124	491/491	0.0	100 %	CCA tRNA nucleotidyl transferase	*G. max*	gi|356576136|	1×10^{-13}
880279	389	No hit	–	No hit	–	–	$>1 \times 10^{-5}$
880323	531	No hit	–	Sprite BAC 78L17	*P. vulgaris*	gi|38194906|	2×10^{-64}
880463	481	No hit	–	No hit	–	–	$>1 \times 10^{-5}$
881808	527	No hit	–	No hit	–	–	$>1 \times 10^{-5}$
882636	712	No hit	–	PVUSE4NG-RP-010_A08 mRNA	*P. vulgaris*	gi|312044997|	2×10^{-6}
883703	360	No hit	–	No hit	–	–	$>1 \times 10^{-5}$

[a] "–" indicates absence of information, since the *E* value was not significant (*E* value $>10^{-5}$)

Table 2 Primer sequences for PCR amplification of non-redundant DArT clones polymorphic between the resistant and susceptible bulks

STS-Dart marker	Primer sequences (5′–3′)	Annealing temperature (°C)	Predict PCR product (bp)	Polymorphism[a]
D864174a	F-AGCACCTTTTCCACCCTGTA R-TGACCATCAAGTCTGAAGACAG	60	436	C+/U−
D864174b	F-CGAGAGTTTACTCTGACTCGTG R-CCAGAAATTTCGCCCATAG	58	496	No amplicon
D864415	F-GGATCCAGTGCAGTAGCACA R-GAACGGTTCTGTTCACAAGCTA	60	421	C+/U−
D864614	F-GCAGACTTATGTCCAATTGTGG R-CAGCATGAAAGTACCAAAACCA	60	881	No amplicon
D865197	F-GATTGCATTCAACACAATAGGC R-AGGGGCTGGAGTAGAAAAGG	60	419	C+/U−
D873626	F-ATCCAGTGCAGTTCCAGATG R-TGCAGCTGTTTGAACTAGTGA	58	491	C−/U+
D880279	F-TCCAGTGCAGAGTCAGAAAA R-TTGCGTATAATTGCGTACAA	56	389	C−/U+
D880323	F-CCAACCATTGGTATCAGAGC R-TGGATCCAGTGCAGTTCAAA	60	531	No amplicon
D880463	F-GCTACGAGCTCGGATCACTA R-AGTGCAGTAGCACAATGCAGA	58	481	No amplicon
D881808	F-GGATCCAGTGCAGAAGGATA R-TGAAAGCTGCCTTCTTAAGTGT	58	527	No amplicon
D882462	F-CAGTGCAGCTTCAAGCAAAA R-AGTGCAGTAGCACAATGCAGA	60	554	C+/U−
D882636	F-CAGTGCAGCTTCAAGCAAAA R-TGCAGTGTTTTGTGTGCCTA	60	712	C+/U−
D883703	F-AGTGCAGTTGTATCCGTTGCT R-CCGCCAGTGTGATGGATATT	60	360	C−/U+

[a] The plus and minus signals indicate the presence (+) or absence (−) of PCR product in CAL 143 (C) or IAC-UNA (U) parental lines

agarose gels and found to be polymorphic between IAC-UNA, CAL 143 and the bulks, were used in the linkage analysis. The primers designed for the remaining five DArTs clones did not generate an amplification product.

SCAR and SSR markers were used to increase the number of markers in linkage group Pv10. Of the six SCAR markers analyzed, three were polymorphic between the UC parents (SH13, SBA16, and $PF5_{330}$) and were used to genotype the RILs. The population was also genotyped with 94 polymorphic SSRs, 49 of which were previously screened but not mapped by Campos et al. (2011) and 45 of which were screened in this study (Supplemental Table S2).

Addition of new markers to linkage group Pv10 increased the resolution of ALS10.1

Linkage analyses incorporated three of the eight STS-DArTs, one SCAR ($PF5_{330}$), and six SSRs into linkage group Pv10, increasing the total number of markers reported in the previous study (Campos et al. 2011) from 9 to 19 (Fig. 1). For all three STS-DArTs (D864415, D865197 and D882462), both the resistant parent CAL 143 and the resistant bulk displayed the presence of amplicons (scored as '1' in Supplemental Table S1). The number of markers in the ALS10.1 region increased from four to ten markers: STS-DArT marker D865197 and five new SSRs were incorporated to the region, thus reducing the average distance between markers from 6.0 cM (reported in the previous analysis) to 3.1 cM (Fig. 1). The position of a newly added SSR ATA220 coincided with the peak LOD score of the QTL (Fig. 2), whereas in the previous study, the closest marker (GATS11b) was positioned 6.0 cM away. The addition of new markers reduced the confidence interval of ALS10.1 from 13.4 cM to 3.0 cM and increased the maximum LOD score of the field screening data from 17.7 to 20.0, but decreased the maximum LOD score of the greenhouse data from 10.1 to 9.3. Similarly, the R^2 values of the QTL increased from 21.2 to 22.8 in the field analysis and decreased from 15.9 to 10.2 in the greenhouse analysis.

The graphical genotypes of the ten most resistant and ten most susceptible RILs (Table 3) indicated the absence of recombination among six markers located in the core of ALS10.1 because the genotypes had the same marker alleles as the resistant or the susceptible parental line, respectively (bold markers in Table 3). In addition to increasing the resolution of ALS10.1, CIM analysis

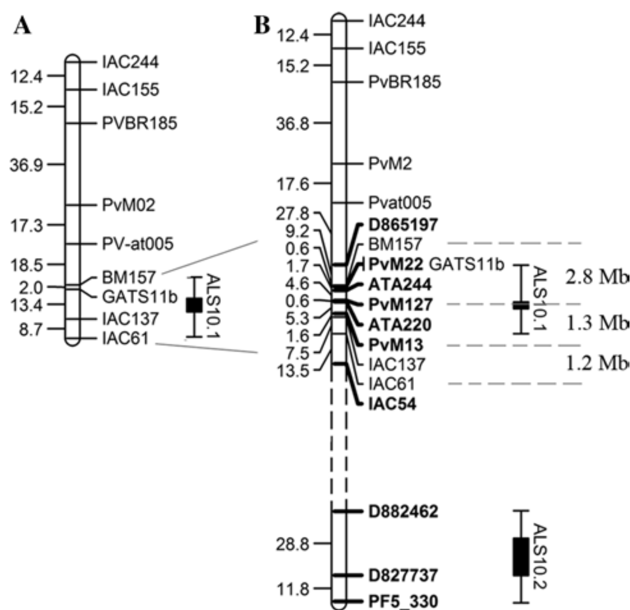

Fig. 1 Previous (**a**) and actual (**b**) versions of linkage group Pv10 after the addition of six SSRs, three STS-DArTs and one SCAR marker. The confidence intervals of QTLs ALS10.1 and ALS10.2 are indicated in *black boxes* and the region with LOD values above the threshold (3.0) are indicated by the *whiskers*. Physical distances between markers linked to ALS10.1 in the new map are represented by *gray dashed lines* and were estimated based on alignments with the draft genome sequence of Phytozome v1.0. The *black dashed lines* on the new Pv10 indicate a distance larger than 37.5 cM

identified a new QTL in this group, hereafter referred to as ALS10.2[uc] (Figs. 1, 2), which explained 5 % and 15 % of the phenotypic variance in the field and greenhouse experiments, respectively.

Alignment of ALS10.1 markers with the draft genome sequence of *P. vulgaris* assigned its core to the end portion of Pv10

The genome locations of markers from linkage group Pv10 and from the DArT-BSA that were not mapped were analyzed through the alignment of all available sequences with the draft sequence of the common bean chromosomes from Phytozome v1.0. In total, 17 markers were positioned on chromosome 10 (Pv10), being distributed throughout the chromosome and covering approximately 40 Mb of Pv10, with an average distance of 2.5 Mb (Fig. 2). The sequence alignment of all eight STS-DArTs indicated that five STS-DArTs are located on Pv10, in addition with other two DArTs which were not successfully converted in STS-DArT markers. Interestingly, linkage analysis indicated that only one of these STS-DArTs was linked to QTL ALS10.1 (D865197, Fig. 2). Of the three STS-DArTs that did not align with Pv10, the D882636 marker was assigned to Pv01 (E value = 2×10^{-39}), D882462 aligned with Pv08 (E value = 1×10^{-19}), and no similar sequence from this version of the genome assembly was found for D864415 (E value > 1×10^{-10}), even though D882462 and D864415 mapped to linkage group Pv10 close to the ALS10.2 QTL (Table 4). Moreover, of the DArTs that generated no amplicons in UC parents, two (D864614 and D881808) were located in the 40 Mb stretch of Pv10, with the D881808 marker only 0.2 Mb apart from the D865197 ALS10.1 marker (Fig. 2). Finally, the D864174b marker aligned with Pv05 (E value = 5×10^{-57}), and no similar sequences in the genome were found for either D880323 or D880463 (E value > 1×10^{-10}).

Of the 15 SSR markers mapped on linkage group Pv10, 13 were aligned with the genome; the ATA220 and ATA244 markers had no sequences available. Ten of the SSRs were located on Pv10 (Table 4; Fig. 2). The linkage and chromosomal orders of four markers linked to ALS10.1 were in agreement (from IAC161 to BM157), while the GATS11b marker was positioned outside of the QTL region in the genome sequence (Fig. 2). In addition, the order of PvM02 and PvBR185, which mapped outside of the confidence interval of ALS10.1, was inverted. Interestingly, more than one copy of PvM13 and IAC137 were identified on Pv10, resulting in two clusters 2.7 kb apart from each other, in which the markers were located in tandem. The two identical copies of PvM13 spanned approximately 14.3 kb, and the three highly similar copies of SSR IAC137 spanned approximately 22.2 kb (Supplemental Fig. S1 and Table S3). Markers IAC155 and IAC244 aligned with Pv09 (E value = 0.0), and PvM22 was the only marker linked to ALS101.1 that was located on a different chromosome (Pv03; E value = 0.0) (Table 4).

Taking into consideration the genome and map locations of the markers linked to ALS10.1, the genomic core of this QTL was defined as the region between IAC61 and BM157 spanning 5.3 Mb at one end of the Pv10 assembly (Fig. 2). Although the ATA220 was the marker which coincided with the LOD peak of the ALS10.1, the marker with available sequence closest to the QTL LOD peak was the PvM127 (0.6 cM; Fig. 1b); and it was located 6.1 Mb from the end of the chromosome (Fig. 2). Because the core of ALS10.1 spans 21.9 cM, a ratio of 242 kb/cM can be estimated for this region.

Putative genes linked to markers of the ALS10.1 core indicated enrichment of genes involved in disease resistance

The genomic context of ALS10.1 was analyzed by investigating the function of putative genes located within 10.0 kb of each marker (Table 4). At the core of the QTL, 13 transcripts were observed (Table 4); interestingly, the majority

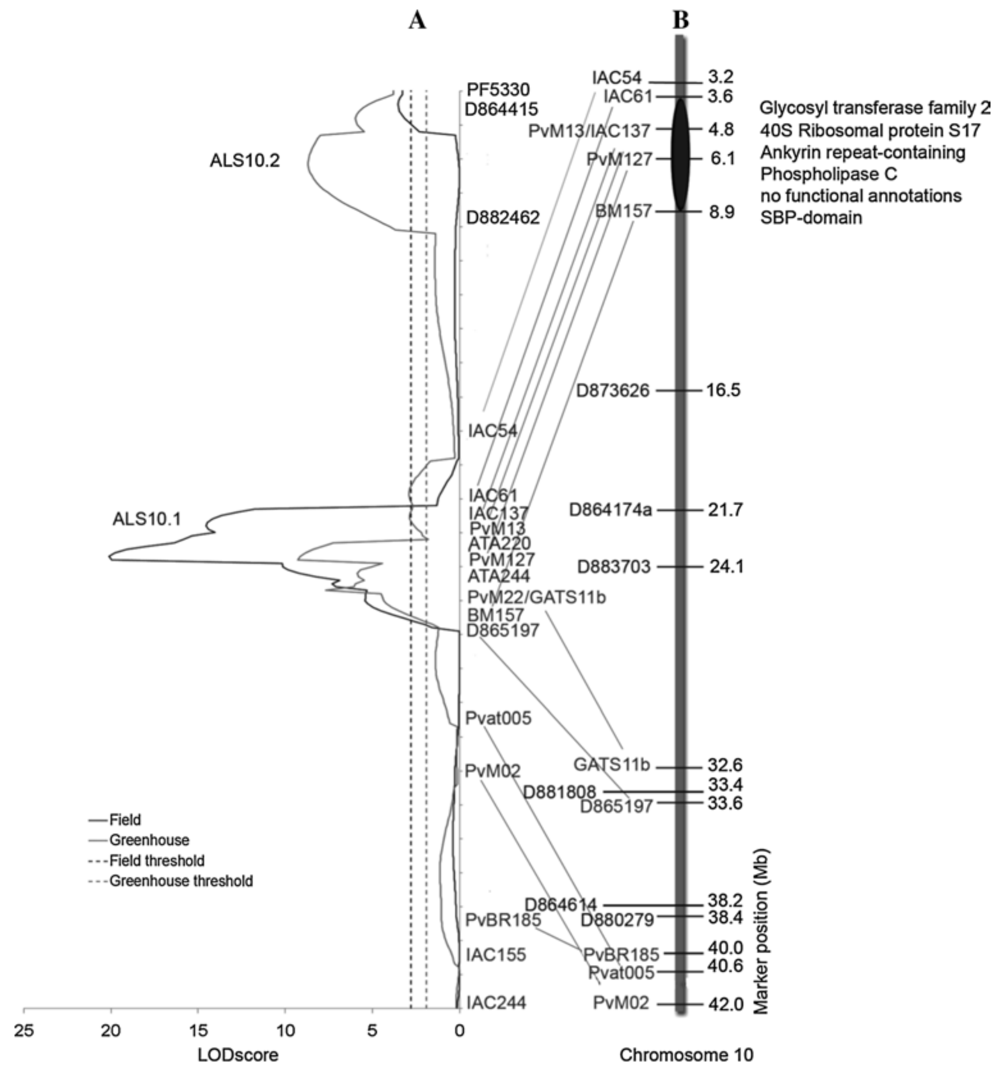

Fig. 2 Inferred positions of markers mapped on linkage group Pv10 based on linkage analysis and alignments with the draft sequence of chromosome 10 according to Phytozome v1.0. **a** Composite interval mapping LOD scores of the angular leaf spot resistance QTLs ALS10.1 and ALS10.2 on linkage group Pv10 based on disease screenings of the UC RIL population under natural conditions of infection in the field (*black line*) and under controlled conditions in the greenhouse (*gray line*). *Traced horizontal lines* indicate LOD thresholds. **b** Physical positions on the chromosome are indicated in *front of each marker*. The *region highlighted in black* represents the core of the QTL ALS10.1, with the linked genes listed alongside the markers' positions

of the transcripts (60 %) were genes known to be related to the immune response in plants, including genes coding for glycosyl transferase, ankyrin repeat-containing protein, phospholipase, and SBP-binding protein. Three genes coding for ribosomal proteins and two with no functional annotation were also detected in this region. In addition, the transcripts linked to other markers of Pv10 were analyzed, and a total of 26 transcripts were identified (Table 4). Disease-related genes, including kinase and nucleotide-binding adaptor (NB-ARC), were observed throughout the entire chromosome.

Synteny analysis between ALS10.1 markers and the soybean genome suggested the presence of duplicated regions in the QTL

The synteny between the common bean and soybean genomes in the ALS10.1 QTL region was investigated. Markers of the ALS10.1 core were distributed on six different *G. max* chromosomes (Gm) (Table 5). One cluster was composed of markers BM157, PvM22 and GATS11b, located at one end of ALS10.1 and spanning 0.6 cM (Fig. 1). It was identified on chromosomes Gm03 and Gm07. In addition, PvM127 aligned to Gm05 and Gm17, while IAC137 and PvM13 aligned to Gm16 and Gm20, respectively.

Discussion

In previous work, the same UC RILs used in this study were scored for resistance to ALS under field and greenhouse conditions; the study identified a major QTL on linkage group Pv10 (ALS10.1) that controlled ALS resistance and spanned a large confidence interval with four markers (Oblessuc et al. 2012a). In the present study, STS-DArT, SSR and SCAR markers were added to this linkage group, thus reducing the confidence interval of the QTL (Fig. 1).

Table 3 Graphical genotypes of markers from linkage group Pv10 and ALS severity scores of the field and greenhouse trials for the ten most resistant and most susceptible UC RILs

		Resistant RILs[a]										Susceptible RILs[a]									
Markers position (cM)	**Markers names**	208	162	100	73	215	64	148	354	353	322	371	375	379	270	26	139	177	27	55	262
0.0	IAC244																				
12.4	IAC155																				
27.6	PvBR185																				
64.4	PvM2																				
82.0	Pvat005																				
109.8	D865197																				
119.0	BM157																				
119.6	PvM22																				
119.6	**GATS11**																				
121.3	**ATA244**																				
125.9	**PVM127**																				
126.5	**ATA220**																				
131.8	**PVM13**																				
133.4	**IAC137**																				
140.9	IAC61																				
154.4	IAC54																				
220.8	D882462																				
249.6	D864415																				
261.4	$PF5_{330}$																				
disease severity field		1.06	1.11	1.06	1.00	1.25	1.33	1.79	1.71	1.08	1.50	7.04	6.79	6.75	5.63	5.58	5.50	5.04	5.02	5.00	4.83
disease severity greenhouse		1.00	1.33	1.42	1.53	1.56	1.68	1.22	1.32	1.96	1.67	7.83	6.38	5.58	5.12	5.64	5.16	5.99	5.06	5.89	5.53

[a] Filled squares represent the allele from the susceptible parent IAC-UNA and the empty the allele from the resistant CAL 143 parent. Markers spanning the core of the ALS10.1 are marked in bold

This analysis identified an SSR marker (ATA220) with a position that coincided with the peak LOD score of the QTL (Fig. 2); therefore, this co-dominant marker can be used in marker-assisted breeding for resistance to ALS and can aid in positional gene cloning. The study of the genomic context of ALS10.1 identified many genes related to plant immunity in that region, which highlights the importance of this QTL. In addition, a new resistance QTL was identified in this group; this finding could explain the fact that the R^2 value of ALS10.1 in the greenhouse data analysis decreased by 6 % compared to the previously reported value because the effects of both QTLs were confounded due to the lack of markers in the previous study.

The STS-DArT markers were developed through the DArT-BSA approach (i.e., the DArT technique combined with marker-oriented BSA) to maximize the chances of finding DArT markers linked to ALS10.1. This is the first report on the use of this new class of marker in linkage analysis in the common bean. Sequence alignment analysis of DArT clones indicated a high redundancy (59.1 %) in the bean DArT libraries (Table 1). A similar percentage of redundancy (50 %) based on sequence analysis was observed in oats (*Avena sativa* L.) (Tinker et al. 2009). In beans, the large number of repetitive elements inferred from the sequence analysis of BAC inserts (Schlueter et al. 2008; Ribeiro et al. 2011) could explain the similarity between sequences observed in the DArT-BSA results. Nevertheless, it should be considered that the redundancy reported in this study might be overestimated due to the stringent conditions used in the analysis.

Combining DNA pool analysis with selective genotyping is the simplest and cheapest approach to identifying markers for major genes, although some problems have been associated with this method due to the insufficient marker density (i.e., 15–25 cM) and the high level of false positives observed (Xu and Crouch 2008). Indeed, of the eight non-redundant STS-DArTs developed based on the BSA results, three mapped to linkage group Pv10 and only one to the ALS10.1 region, 16.7 cM away from the QTL LOD peak. However, the combination of the BSA and mapping analyses used in this study reinforces the identification of D865197 as a marker flanking the ALS10.1 QTL region; this result was also confirmed by the sequence analysis which aligned this STS-DArT to Pv10. In addition, the small increase in the number of markers on the ALS10.1 region identified with the BSA approach may result from the absence of recombinant RILs for that region, which could render the linkage mapping of the new markers unfeasible. This lack of recombinant RILs also could explain why five STS-DArTs did not map to linkage group Pv10, but four of these aligned to Pv10, indicating that the BSA method actually identified DArT clones around the ALS10.1 region. The identification of markers by DArT-BSA near ALS10.1 but not within the QTL

Table 4 Chromosomal locations of markers from linkage group Pv10 and DArT-BSA clones, with functional annotation of putative genes linked to them based on the Phytozome database

Marker name[a]	Chr[b]	*E* value[c]	Chromosome position	Predicted gene	Distance (kb)[d]	Functional annotation
IAC54	10	1×10^{-176}	3,276,703..3,277,072	Phvul.010G022100	0	ER lumen retaining receptor
				Phvul.010G022200	6	ER lumen retaining receptor
IAC61	10	1×10^{-153}	3,614,814..3,615,256	Phvul.010G024700.1	0	Glycosyl transferase family 2
			4,872,149..4,872,394	Phvul.010G033000.1	0	Ankyrin repeat-containing
IAC137	10	4×10^{-135}	4,888,088..4,888,545	Phvul.010G033100.1	0	Ankyrin repeat-containing
			4,893,842..4,894,343	Phvul.010G033200.1	3	40S Ribosomal protein S17
PvM13 (CB543664)	10	0.0	4,897,032..4,897,540	Phvul.010G033200.1	0	40S Ribosomal protein S17
				Phvul.010G033100.1	8	Ankyrin repeat-containing
			4,910,779..4,911,290	Phvul.010G033300.1	0	40S Ribosomal protein S17
				Phvul.010G033400.1	3	Phospholipase C
PvM127 (CB450175)	10	0.0	6,174,932..6,175,465	Phvul.010G041200.1	0	No functional annotations
				Phvul.010G041300.1	7	No functional annotations
BM157 (AF483873)	10	4×10^{-18}	8,999,124..8,999,246	Phvul.010G056000.1	0.5	Squamosa-promoter binding protein (SBP) domain
D873626	10	0.0	16,505,342..16,505,821	–	–	–
D864174a	10	0.0	21,757,602..21,758,036	Phvul.010G073200.1	0	Aprataxin-related
D883703	10	3×10^{-64}	24,137,115..24,137,310	Phvul.010G076700.1	0	Cytochrome P450
GATS11b (AF483840)	10	5×10^{-111}	32,692,053..32,692,354	Phvul.010G087600.1	0	Glycosyl transferase family 8
				Phvul.010G087700.1	5	Sin3 associated polypeptide p18 (SAP18)
D881808	10	0.0	33,405,401..33,405,916	Phvul.010G089800.1	5	No functional annotations
D865197	10	0.0	33,667,342..33,667,758	Phvul.010G091200.1	1	Nucleotide-binding adaptor (NB-ARC) domain
D864614	10	0.0	38,257,070..38,257,940	Phvul.010G115900.1	1	Yippee putative zinc-binding protein
				Phvul.010G115800.1	3	Protein tyrosine kinase
D880279	10	0.0	38,464,656..38,467,110	Phvul.010G117200.1	8	Homeobox-associated leucine zipper
PvBR185	10	1×10^{-55}	40,020,362..40,020,544	Phvul.010G130100.1	6	No functional annotations
				Phvul.010G130200.1	7	AP2-like factor
Pvat005	10	0.0	40,678,008..40,685,937	Phvul.010G134800.1	0	Lipoxygenase
PvM02	10	3.3×10^{-128}	42,004,352..42,005,489	Phvul.010G149900.1	0	No functional annotation
D882636	01	2×10^{-39}	37,702,027..37,702,175	Phvul.001G133400.1	8	Leucine-rich repeat-containing protein 2
				Phvul.001G133500.1	9	Hypothetical protein
PvM22 (CB539406)	03	0.0	42,106,427..42,107,050	Phvul.003G206600.1	6	Transmembrane amino acid transporter protein
				Phvul.003G206700.1	0.5	RNA binding protein
				Phvul.003G206800.1	5	Protein-L-isoaspartate-methyltransferase (PCMT)
D864174b	05	5×10^{-57}	9,387,722..9,387,863	Phvul.005G063900.1	4	Cytochrome P450
D882462	08	1×10^{-19}	29,659,103..29,659,219	Phvul.008G147500.1	1	Cytochrome P450
IAC155	09	0.0	12,778,016..12,778,577	Phvul.009G078600.1	1	Q calmodulin-binding motif
				Phvul.009G078500.1	5	Mitochondrial carrier protein
IAC244	09	0.0	14,313,568..14,314,142	Phvul.009G092600.1	0	Clathrin assembly protein

[a] Underlined markers were located on the ALS10.1 genomic core region

[b] Chromosome

[c] *E* values threshold $\leq 1 \times 10^{-10}$

[d] The approximated distance between the marker and its linked transcripts, which is around 10 kb from the marker

Table 5 Synteny analysis between markers in the ALS10.1 locus and the soybean genome

Glycine max chromosome[a]	*P. vulgaris* markers	*E* value[b]
Gm03	BM157	3×10^{-13}
	GATS11b	3×10^{-12}
	PvM22	2×10^{-42}
Gm05	PvM127	2×10^{-27}
Gm07	BM157	9×10^{-11}
	GATS11b	3×10^{-14}
	PvM22	9×10^{-36}
Gm16	IAC137	3×10^{-57}
Gm17	PvM127	8×10^{-18}
Gm20	PvM13	2×10^{-104}
–	D865197	$>1 \times 10^{-10}$

[a] Gm = Soybean chromosomes, based on PhaseolusGenes website (http://phaseolusgenes.bioinformatics.ucdavis.edu; Gepts and Lin 2011)

[b] *E* values threshold $\leq 1 \times 10^{-10}$

region may also be a result of the low resolution of the QTL obtained by Oblessuc et al. (2012a), with the bulks for DArT-BSA selected based on phenotype and marker genotypes of the only two SSRs available at the time (GATS11b and IAC137), which were 13.4 cM apart. Indeed, if the chromosomal location of these two SSRs were considered, five DArT sequences were assigned to a major QTL region, which would validate the DArT-BSA results.

SSRs represent an important source of markers in assisted breeding (Collard and Mackill 2008) and were therefore used in addition to the STS-DArT markers to increase the resolution of the ALS10.1. The graphical genotypes of the most resistant and most susceptible RILs illustrate the validity of these markers, with a clear distinction between resistant and susceptible lines (Table 3). In addition to being associated with ALS resistance, some of these markers were reported to be linked to other useful traits, including resistance to halo blight caused by *Pseudomonas syringae pv. phaseolicola* and seed weight. The halo blight resistance gene *Pse*-1 was mapped in the vicinity of BM157 and GATS11b (Miklas et al. 2009, 2011), whereas BM157 was reported to be linked to a QTL that accounts for approximately 19 % of the variation in seed weight (Davis et al. 2006). Therewith, the addition of these markers to ALS10.1 could be useful when breeding for ALS resistance and other relevant traits.

Similar to the SSRs, SCARs are useful markers for marker-assisted selection (Collard and Mackill 2008), even though only one ($PF5_{330}$) of the three polymorphic SCARs used was mapped on Pv10. It probably is result of the dominant character of the SCAR markers that are less informative than the SSRs, which are co-dominant markers. Nevertheless, the SCAR $PF5_{330}$ was previously linked to ALS resistance (Mahuku et al. 2004), and here it was linked in coupling phase to the newly identified QTL (Fig. 1b), demonstrating the utility of this marker in ALS QTL mapping. The ALS10.2 QTL was located at the end of the linkage group and spanned two STS-DArTs in addition to the SCAR. The SCAR $PF5_{330}$ was developed from an AFLP marker linked 5.0 cM apart from an ALS resistance locus located on the Pv08 linkage group of the DOR364 × G19833 map (Mahuku et al. 2004). Because $PF5_{330}$ was mapped on linkage group Pv10 in this study, we infer that these groups share a duplicated region and that these two QTLs could be paralogs. A duplication of these regions was also inferred by synteny analysis with soybean because similarities between markers present on the Pv08 and Pv10 groups of the common bean were identified on both Gm07 and Gm08 chromosomes of soybean (McClean et al. 2010). In contrast, the synteny analysis of the STS-DArT markers present on ALS10.2 and the soybean genome did not identify any similarities between these sequences. Therefore, it is important to note that even though the markers linked to ALS10.2 were linked to Pv10 based on the BSA evidence, they mapped 66.4 cM away from the other markers of this group, so it is possible that this QTL maps to group Pv08 and further studies should investigate this possibility.

As a preliminary version of the common bean genome was recently made available, the physical locations of the Pv10 markers and consequently the ALS10.1 genomic context were investigated along with the linkage analysis. Sequence analyses indicated that most STS-DArT and SSR markers of linkage group 10 were located on chromosome Pv10. Moreover, the order of the markers that comprise the core of ALS10.1 was in agreement with the common bean genome assembly with the exception of an inversion between the SSR markers BM157 and GATS11b. Similarly, the marker order for the entire chromosome was the same in the linkage and sequence analyses with the exception of an inversion on the end of Pv10 (Fig. 2). These differences might reflect genomic rearrangements in the divergent bean genotypes (Kwak and Gepts 2009). For instance, different positions of Pv10 markers analyzed using both draft genomes available (CYTED—http://www.cyted.org/ and Phytozome—http://www.phytozome.net/) resulted in different rearrangements of the markers (Supplemental Table 3), most likely due to the divergent *P. vulgaris* gene pools used [Mesoamerican (BAT93; CYTED) and Andean (G19833; Phytozome)]. Such rearrangements are suggested by inversions in marker order observed in different bean linkage maps, such as that of the six SSRs between BM157 and ATA220 (Fig. 1) on ALS10.1, which were mapped in a different order (but maintained as a linked cluster) in different RILs from a cross between DOR364 × G19833 (Blair et al. 2003). The GATS11b and PvM22 markers

were similarly mapped to linkage groups Pv08 and Pv09, respectively, instead of Pv10, in a RIL population from a BAT93 × Jalo EEP558 cross (Grisi et al. 2007; Hanai et al. 2010). Nevertheless, it is also important to consider the possibility of these rearrangements being in fact artifacts of the linkage mapping analysis or the genome assembly analysis, in which statistical probabilities are used.

Although inversions in marker order were observed and despite the fact that multiple copies of certain markers of the ALS10.1 core occur both at the ALS10.1 core region and elsewhere in the genome (Supplemental Table S3), the physical location of ALS10.1 predicted in our analysis is further confirmed by fluorescence in situ hybridization (FISH) studies. The genetic and genomic position of SSR markers indicated that ALS10.1 is located between clusters of ribosomal DNA (rDNA) on the long arm of Pv10. FISH of BAC clones and plasmids containing 5S and 45S rDNA bean sequences showed that this chromosome contains a large region of 45S rDNA in the end of its long arm and a smaller 5S rDNA region close to the centromere (Fonsêca et al. 2010). Moreover, the BAC probe 173P6 was located on Pv10 between these rDNA clusters (Fonsêca et al. 2010). This clone contains the Bng200 SSR marker that was mapped to linkage group Pv10 near the ALS10.1 core markers PvM127 and PvM13 in the BAT93 × Jalo EEP558 population (Hanai et al. 2010). These two markers were located in the ALS10.1 core in both genomic and linkage analyses, which corroborates this putative physical position of the QTL. In addition, the PvM13 marker was developed from a putative 40S ribosomal EST (Hanai et al. 2010), and curiously, PvM13 and its closest marker, IAC137 (1.6 cM; Fig. 1), were positioned in a region within ankyrin repeat-containing paralogs (Table 4). These genes are composed of repetitive motifs like the rDNA, and play an important role in pathogen resistance and the promotion of reactive oxygen species (ROS) production (Yang et al. 2012). This points to the presence of pathogen resistance genes in the long arm of Pv10.

The genome location of ALS10.1 allowed us to investigate the genomic context of this region and identify disease resistance-related genes which may play a role in resistance to ALS. One of these genes codes for a phospholipase C was located 3.0 kb away from PvM13; phospholipids are structural components of cell membranes involved in plant signaling, particularly in immune responses (Canonne et al. 2011). When an invading microbe is detected, several phospholipases are activated and contribute to the establishment of an appropriate defense response consisting of the production of oxylipins, jasmonates and the potent second messenger phosphatidic acid, which has been shown to modulate the activity of a variety of proteins involved in defense signaling (Canonne et al. 2011). In *Oryza sativa*, the phospholipase C1 (*OsPLC1*) was shown to be activated by diverse chemical and biological inducers of plant defense pathways and by the incompatible interaction between rice and the pathogenic fungus *Magnaporthe oryzae* (Song and Goodman 2002). In addition, phospholipase C isoforms were required for the hypersensitive response and activation of disease resistance genes; in the tomato (*Solanum lycopersicum*), for example, the *SlPLC4* gene of the *PLC* gene family is needed to produce an immune response against the biotrophic fungus *Cladosporium fulvum* (Vossen et al. 2010).

Another resistance candidate gene in the QTL region was identified as a member of the glycosyl transferase family 2, located 6.0 kb away from IAC61. This gene family is involved in many aspects of plant primary and secondary metabolism, including protein glycosylation and cellulose biosynthesis (Coutinho et al. 2003). Reduction in the synthesis of cellulose leads to changes in the cell wall composition/structure and activates the synthesis of lignin (Caño-Delgado et al. 2003; Hamann et al. 2009) and of defense responses through the production of jasmonic acid (JA) and the activation of ethylene signaling pathways (Ellis et al. 2002; Hernández-Blanco et al. 2007). Studies have shown that glycosylation can also be important in pathogen recognition (Nekrasov et al. 2011). The Phvul.010G056000.1 transcript, located 0.5 kb away from BM157, is also potentially responsive to ethylene; this gene encodes for an SBP-binding domain protein, which is a transcription factor that acts upstream of the ethylene-mediated regulation of ripening in tomato (Manning et al. 2006). Plant defense against herbivores and necrotrophic fungi via the JA and ethylene pathways is an established response mechanism (Antico et al. 2012), and transcriptome analysis of the common bean showed a down-regulation of these hormone responses during infection with the hemibiotrophic fungus *Colletotrichum lindemuthianum* (Oblessuc et al. 2012b). Thus, because *P. griseola* is also a hemibiotroph, it is possible that this pathway is also responsive to the attack of this pathogen.

In addition to analyzing the genomic context of ALS10.1, we investigated transcripts linked to DArT sequences that aligned with Pv10 but were outside of the QTL core. The 40-Mb region covered by such markers encompasses almost the entire chromosome, and all identified transcripts with known functions are related to plant disease resistance, indicating that this class of genes is not restricted to the ALS10.1 region but is spread out over the entire chromosome. For instance, genes coding for Yippee-like proteins (He et al. 2005), NB-ARC domain proteins (van Ooijen et al. 2008), aprataxin-related proteins (Nanda et al. 2010; Oblessuc et al. 2012b), cytochrome P450 (Matthes et al. 2011) glycosyl transferase (Vorwerk et al. 2004),

protein kinases (Melotto et al. 2004; Schwessinger and Ronald 2012), and homeobox-associated leucine zippers (Vleeshouwers et al. 2011) were observed, which reinforce the importance of studying this chromosome for breeding disease resistance in the common bean.

Conserved sequences between common bean and soybean chromosomes involving Gm03, Gm07, Gm16 and the bean Pv10 were detected by McClean et al. (2010) and Miklas et al. (2011). Synteny analysis of the markers in ALS10.1 and the soybean genome was therefore performed. Complex evolutionary dynamics in ALS10.1, characterized by rearrangements and duplications not found in soybean was observed, suggesting a continuous adaptation of this locus. Previous studies have shown that the soybean genome experienced a whole genome duplication after diverging from the common bean (Lin et al. 2010) in such a way that almost all single-copy sequences of the common bean are present in two copies in the soybean genome (McClean et al. 2010). Indeed, the sequence of one single-copy marker of ALS10.1 (PvM127) aligned with two soybean chromosomes. However, this observation did not always hold true for other markers of this region, and in some cases the opposite situation was observed, i.e., multiple copy markers from the bean (PvM13 and IAC137) aligned as single markers in the soybean, illustrating that independent duplication events may have occurred within the *P. vulgaris* genome. In addition, the observation of multiple copies of sequences in common bean which are single copies in soybean could also be result of a significant diversion that took place on the soybean paralog sequences.

In conclusion, the incremental increase in resolution of the major QTL for ALS resistance (ALS10.1) from the addition of the newly developed STS-DArT and SSRs available in literature will be useful for molecular breeding, particularly at the ALS10.1 core region spanning the markers IAC61, PvM13, IAC137, PvM127 and BM157. The genomic context analysis of this QTL showed an enrichment of disease-related genes in this region; at the same time, the rearrangements and inversions observed either through comparison of genetic and genomic marker positions or through synteny analysis with the soybean hint at the dynamic evolution of the region. This report represents one of the first steps toward the cloning of ALS resistance genes.

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Conflict of interest The authors declare that they have no conflict of interests.

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