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# *In silico* identification of putative expressed sequence tag (EST)-simple sequence repeats (SSRs) markers of resistance to *Meloidogyne* spp. in common bean

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**Expressed sequences are important sources in the development of functional heterologous microsatellite markers in phylogenetic related groups, that is, soybean and commom bean. The objective of this work was to identify and characterize expressed sequence tag (EST)-simple sequence repeats (SSRs) in silico candidates of resistance to *Meloidogyne* spp. in common bean. Seven DNA sequences from soybean associated with genetic resistance have been identified and obtained in the NCBI database. Its homology in common bean genome was verified using the BLAST tool. The cellular processes involved were also checked using the Blast2GO program. The identification of microsatellite markers and design of the primer pairs was performed using the SSRLocator and Primer3 programs, respectively. The transferability rate of common beans the target sequences identified was 86%, demonstrating the power of success of this method. All the cellular processes involved in the original DNA sequences were verified from EST on beans, with E-value between 0 and $2.9\times10^{-168}$. Fifteen EST-SSRs candidates for common bean resistance were identified, which have proved to be suitable for their amplification by PCR. The transferability analysis of ESTs related to resistance to *Meloidogyne* spp., especially among soybeans and common beans is efficient. Based on this study, 15 EST-SSRs candidates are available for validation and later use.**

**Key words:** simple sequence repeats (SSRs) markers, assisted selection, ontology, pre-breeding.

## INTRODUCTION

The common bean (*Phaseolus vulgaris*) is a leguminous vegetable cultivated in 113 tropical and subtropical countries, which has a high economic value and market acceptance due to its balanced chemical composition, including various proteins, complex carbohydrates, minerals and vitamin B complex (Broughton et al., 2003). Brazil is the world leader in the production and consumption of beans, although it is not yet self-sufficient

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in the production of the same (Wander, 2005). The bean is cultivated in most Brazilian states, in a variety of soils, climates, growing season and farming systems (Silva and Wander, 2013). The adversities which the beans have been exposed contribute to high diversity found in the culture, enabling the selection, that is, genetic variation for breeding (Burle et al., 2010).

This availability of variability generates demand of detailed information about their genetic resources for use, which will be required to meet a process of expansion and growing interest in improved cultivars increasingly productive and adapted to adverse conditions of development. Another point required and of greatest interest to breeders is the introduction of desired genetic variation in the Active Germplasm Bank (AGB) on which lineages are selected, reducing the segregation of characters already improved. The assisted selection allied to the DNA markers is a useful method for this purpose.

Over the past decades, bean culture is going through several changes in the form of management, production systems and breeding. Coupled with the landscape changes for planting, the various plant health problems arise, making diseases, caused by plant pathogens, to cause a strong deterrent to obtain better yields in the country in diverse cultures, like the beans (Vieira et al., 1998). These changes contributed to the introduction of emerging pathogens, and old diseases that were not considered problems for the culture started to be highlighted. An important example is the root-knot nematodes, considered bean pests only in some producing regions of Brazil. Though the diseases caused by nematodes are not widely distributed in the Brazilian states, their damage lead to the Brazilian production not meeting the supply demand of the domestic market (Yokoyama, 2007).

An important form of management of nematodes is the use of resistant varieties, which, despite having been found for *Meloidogyne* spp. some find themselves still not very exploited in bean crop (Carneiro et al., 1992; Walber et al., 2003), a fact that underscores the importance in the development of tools that help produce detailed information for using these varieties, in addition define preventive recommendations. In this context, DNA markers are biotechnological tool of quick evaluation that can help design strategies of use of available genetic resources.

The DNA markers, such as Simple Sequence Repeats (SSR) and Single Nucleotide Polymorphism (SNP), have often been used in bean breeding programs (Müller et al., 2015; Müller et al., 2014). Its use has involved studies of quantification and determination of gene diversity (Cardoso et al., 2014); characterization of Value for Cultivation and Use (VCU) (Cardoso et al., 2013), assisted selection (Alzate-Marian et al., 2005), among others. The SSRs have been widely used in recent years and had selected population studies and use of genetic resources in plants for breeding purposes because of their hypervariability and ease polymorphism detection (Li et al., 2015a; Li et al., 2015b; Kaur et al., 2015; Zhai et al., 2015).

Obtaining selective microsatellite markers developed from databases, get easier, faster and economic compared to the ones developed by conventional methods (Buso et al., 2006). Thus, the recent full genomic sequencing advances and the increase in banks expressed sequence tag (EST) of different species, has facilitated studies of genetic similarity between phylogenetically related species (Schmutz et al., 2014; Wang et al., 2014). With this study we propose to identify and characterize *in silico* microsatellite markers candidates in *P. vulgaris* associated with genes that confer resistance to root-knot nematode (*Meloidogyne* spp.). The product generated from this study could be used as a preventive polymorphism identification tool related to the phenotypic behavior of bean caused by parasitism.

## MATERIALS AND METHODS

Initially this study used secondary source materials to find articles covering genes or DNA sequences identified that confer a phenotypic behavior of resistance to nematodes belonging to *Meloidogyne* spp. in soybean (*Glycine max*), within the following databases: NCBI (National Center for Biotechnology Information), Scopus, Science Direct and CAPES Journals. The search was conducted from 09/2014 to 03/2015 and used the following indexing terms in various combinations: (1) Resistance; (2) *Glycine*; (3) *Meloidogyne*; (4) Gene; (5) QTL; (6) Marker; all descriptors were searched in Portuguese and English languages. Articles, published between 1990 and 2014, were found to be related to the subject. From the first articles read other potential were identified according to the methodology "snowball" (Bernard, 2011).

In the selected articles, the #id sequence or genes related to a phenotypic behavior of resistance to root-knot nematode were transcribed. Based on these #id or genes sought to access their DNA target sequences in their entirety using the NCBI database. Then the "FASTA" file format of each target sequence was used separately for the identification of homologous regions in the common bean genome by the "BLAST" tool (Basic Local Alignment Search Tool) available on the online program PHYTOZOME 10.3. Homologous sequences in common beans were chosen by the value E-value <1e-50 with sizes above 1000 bp. Then, the functional annotation of target sequences identified in beans was performed using the program BLAST2GO (Conesa et al., 2005).

To identify putative markers SSRs in the identified target sequences in common bean was used the program SSRLocator (Maia et al., 2008). Only with microsatellite repeats larger than three motifs were used for the design of the foward (F) and reverse (R) primers using the program Primer 3 (Rozen and Skaletsky, 2000). The designed primer pairs for the localized SSRs were used on an *in silico* PCR test model by primer- BLAST tool available in NCBI. This tool is used to check specificity of the pair primers against a database of interest, which at the time was the genome of *P. vulgaris*.

To verify the presence of dimers between the forward and reverse sequences of each primer was used AutoDimer, a program that makes use of a similar slide algorithm capable of comparing two overlapping strands of DNA (Vallone and Butler, 2004). Then the pairs designed EST-SSR primers were named "IFRTXX" being

IFRT relative to 'Federal Institute Goiano - Campus Urutaí' and XX the SSR primer number (Table 1).

## RESULTS AND DISCUSSION

Seven genes described in the literature which confer resistance to root-knot nematode (*Meloidogyne* spp.) in Soybean (*Glycine max*) were found namely: Rhg1, Rhg4 (Concibido et al., 2004), Rmi1 (Luzzi et al., 1994), Extensin 1, Extensin 2, Pectin Esterase 1 and EREBP (Pham et al., 2013) (Table 1). From the sequences of the selected genes were identified regions of about 70% homology in common beans, except for the Pectin Esterase 1 gene, generating a sequence transferability rate of 87%. This success of genomics synteny between the two species is already well reported in the literature, which is due to the fact that they are phylogenetically sister groups, favoring the high occurrence of orthologous genes between them (Schmutz et al., 2014). A result that confirms the story of divergence between species from a complete duplication of the genome event approximately 56.5 million years ago (Lavin et al., 2005).

Using homologous target sequences in common bean was possible to recover the functional processes checked in selected genes in soybeans, with the exception of Ext1 gene, averaging 82.5% similarity and E-value values ranging from 0 to 2.9 and e-168. In general, the functional processes involved five categories: Cell wall structure, organization of the cell wall, integral membrane component, protein kinase activity and transcriptional regulation (Table 2). This comparison confirms the functional completeness homology, obtained using the transferability of soybean ESTs in common beans.

The identification of heterologous EST through *in silico* approach on comparative studies of intraspecific genes evolutionarily has become an easy and low-cost strategy, not practiced before the Post-Genomic Era (Zane et al., 2002; Pandey and Sharma, 2012). This understanding has allowed advances in the development of efficient functional SSRs markers (Sterky et al., 1998; Kaliswamy et al., 2015), as this work and others (Gupta et al., 2010; Victoria et al., 2011; Wang et al., 2014). Demonstrating that ESTs are good sources of sequences for prospecting functional microsatellite markers (Andersen and Lubberstedt, 2003), in this study, and associated with resistance to root-knot nematodes in bean.

Twenty-six different SSRs were identified for the ESTs found in common beans from genes of genetic resistance to root-knot nematodes in soybean. Of those twenty-six only fifteen were considered because present a number of repetitions larger than three. For each of the six genes have been identified SSRs with at least two different repeat motifs, with the exception of Rhg1, Rhg4 and Rmi1 genes identified for which only one SSR (Table 1). The length of the pairs of designed primers was suitable, ranging from 18 to 26 base pairs (bp), whereas the approximate length of 20 bp defined as optimal (Wang et al., 2014). The expected size of amplification product by PCR ranged between 182 and 357 pb, with a range of melting temperature (Tm) from 59 to 61ºC, which allows to estimate an average value approximately to 60°C among the sequences (forward and reverse) and primer pairs.

The small average difference in Tm between the sequences facilitates adjustment of the annealing temperature (Ta) of the F and R primers defined by "Ta = Tm ± 5", which should be similar. Defining Ta as the temperature at which pairing occurs between the primer and the flanking region of the target in the DNA of interest. The wide temperature range and length of the sequences of SSR primers >18 bp gives to the designed markers a high stringency (Bornet and Branchard, 2001). This increased reliability and reproducibility provides the certainty to be evaluating specific loci, and to facilitate the comparison of data by different researchers.

We expect that the fifteen EST-SSRs show a high amplification rate, since there was no competition between F and R sequences of each primer, either the formation of self-dimers. It was still possible to recover ESTs from 87% of the designed primers using the PrimerBLAST tool, allowing us to perform better thorough pre-selection, seeking to increase this amplification rate (Table 1). The success of this rate transferability studies using ESTs has been high, reported in literature values from 60 to 90% in different species (Wang et al., 2014; Luro et al., 2008; Saha et al., 2004).

Considering the SSRs classification based on the motifs identified, was verified the occurrence almost exclusively of trinucleotide markers (n = 13) and only two (02) dinucleotide. As the number of tandem repeats found a range from four to seventeen repetitions. Of the 15 SSRs, eight were classified as perfect, five compounds and one discontinued. The perfect SSRs are those with uninterrupted sequence motif; the interrupted show a sequence of discontinuous motif for a small and different sequence; and the compounds are those formed with at least two distinct motifs (Oliveira et al., 2006).

The high abundance of di- and trinucleotide and small amplitude in the length of motifs in the SSR target structure are related to the sequence of EST and its mutational dynamic (Schlötterer, 2000). In expressed plant sequences, the prevalence of these repetitions has been widely reported (Gupta et al., 2010; Wang et al., 2014; Hong et al., 2015; Boccacci et al., 2015). The regions of ESTs are transcriptional targets rich in dimers in the untranslated regions (UTR) and in the predominance of trimers in the open reading frame (ORF) (Ellegren, 2004). These regions exhibit less mutational rate compared to the others repeating regions of the genome not associated with genes (Li et al., 2002), thus supporting a lower amplitude of motifs, since abrupt changes in ORFs were being selected negatively (Metzgar et al., 2002). This indicates that the reduced size is due to the action selection designed to preserve

**Table 1.** Description of 26 EST-SSRs candidate to nematode resistance in beans with their Forward (F) and Reverse (R) sequences,

| No. | Primer ID | Gene | Cr. | TM (°C) | ES | Primer Sequence (5' - 3') | Motifs | *NCBI* access number |
|---|---|---|---|---|---|---|---|---|
| 1 | IFRT02 | EREBP | 9 | 59<br>59 | 206 | F: GGGTGGTAACCTCACCTTCA<br>R: GGCGAGGAAAACAGACACTC | $(ATT)_3(ATT)_3$ | XM_007133308.1 |
| 2 | IFRT03 | EREBP | 9 | 59<br>60 | 197 | F: TCGTTCATGGAGTTCATAGCA<br>R: AATCCACAGAGCCATCCTTG | $(CTT)_4$ | No match |
| 3 | IFRT06 | Ext1 | 3 | 60<br>59 | 200 | F: CACCTCCTTCCACCACAAAC<br>R: TCTGGGGTAGTTTCCATTGC | $(CGG)_3(CTA)_3(CCG)_4(CTA)_3$ | XM_007154277.1 |
| 4 | IFRT08 | Ext1 | 3 | 59<br>59 | 242 | F: CCTACTACTCACCACCTCCACC<br>R: AAGCAAGAACACCAGAAAGGAG | $(CCG)_4$ | XM_007154277.1 |
| 5 | IFRT09 | Ext1 | 3 | 59<br>60 | 291 | F: CAAGAAACGAGAAAAGAAGGGA<br>R: GGTGCTGACCAAATAAGACTCAC | $(AT)_5$ | XM_007154277.1 |
| 6 | IFRT10 | Ext2 | 8 | 59<br>59 | 198 | F: AGTCTCCTCCTCCACCATCA<br>R: GAGACTTGTAGTAGTAGGGAGGTGGT | $(CTA)_3(CAC)_3$ | XM_007139060.1 |
| 7 | IFRT11 | Ext2 | 8 | 59<br>60 | 197 | F: CATCACCACCACCACCATAC<br>R: TGATGGATCAGGTGGAGGA | $(TAC)_3(CCA)_3(TAC)_6(CTA)_3$ | XM_007139060.1 |
| 8 | IFRT12 | Ext2 | 8 | 59<br>60 | 211 | F: CACCACCACCCCCATACTAC<br>R: TGGTGAGGGTGAGGATAAGC | $(CTA)_3(CCA)_3(CAC)_4(CTA)_3(CTC)_3$ | XM_007139060.1 |
| 9 | IFRT13 | Ext2 | 8 | 61<br>59 | 182 | F: TCCTCCTCCACCTTCTCCAT<br>R: AGTAGGGTGGTGGTGGTGAT | $(CTA)_3(CCA)_3(CAC)_3$ | XM_007139060.1 |
| 10 | IFRT14 | Ext2 | 8 | 59<br>60 | 394 | F: ATCACCACCACCACCCTACTAC<br>R: ACCGACAACCTTAACGATCAAT | $(CTC)_4$ | XM_007139060.1 |
| 11 | IFRT15 | Ext2 | 8 | 60<br>60 | 190 | F: GTCGCTTATCCTCACCCTCA<br>R: GTCGAAGCATCAGCATCAGA | $(ATT)_5$ | XM_007135485.1 |
| 12 | IFRT16 | Ext2 | 8 | 61<br>60 | 208 | F: ACCTCCTCCACCTGATCCAT<br>R: AGATGGTGGTGGTGGTGACT | $(CAC)_4$ | XM_007139060.1 |
| 13 | IFRT20 | Rhg1 | 1 | 59<br>59 | 302 | F: ATGCTCCTCAATGGTTTCAACT<br>R:AGGTTGGTTTTCTCCACTACCA | $(TC)_8(TC)_5$ | XM_007163524.1 |
| 14 | IFRT25 | Rhg4 | 1 | 59<br>59 | 212 | F: AACCCTACCAAAGGCCAGAT<br>R: TGCAGGAATGCTTGATTGAG | $(GAA)_4$ | XM_007163741.1 |
| 15 | IFRT26 | Rmi1 | 2 | 60<br>61 | 352 | F: AAAATTCCCATTGTCCTCTCCT<br>R: GAAACAACTTTTGGCTTTGGTG | $(CGA)_4$ | XM_007159942.1 |

the structure of the transcript because abrupt changes easily affect the corresponding gene product.

## Conclusion

With this early work, fifteen (15) EST-SSRs candidate markers in six different genes that give soybean root-knot nematode resistance are available for validation in common bean.

**Table 2.** Cellular processes linked to homologous sequences.

| Gene/sequence | Protein | Cellular processes |
|---|---|---|
| EREBPm | Ethylene-responsive transcription factor rap2-12 | transcription regulation |
| Ext1 | Leucine-rich repeat extensin-like protein 6 | - |
| Ext2 | Pollen protein ole e i-like protein | Structure and organization of the cell wall |
| Rhg1 | Receptor-like kinase | Activity of kinase protein |
| Rhg4 | Receptor-like kinase tmk4 | Integral component of membrane |
| Rmi1 | Recq-mediated genome instability protein 1 | Formation of floral organs |

Moreover, it was possible to demonstrate that transferability between different species, but close evolutionarily following the example soybean and common bean, can increase the efficiency in the transfer of genomic information by homology ESTs gene of interest. Finally, it is noteworthy the promising results of this herein study, once validated the genetic link by phenotypic evaluation essays of hospitability to root-knot nematode in beans and association of polymorphism amplified by suggested markers studies, they can help programs improvement, outlining their crosses directed to the phenotype of interest, in this case the resistance to root-knot nematode. Yet it is noteworthy that these markers will facilitate the development of superior genotypes with multiple sources of resistance.

## Conflict of Interests

The authors have not declared any conflict of interests.

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