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# SEQUENCE POLYMORPHISM AND GENETIC MAPPING OF CANDIDATE GENES FOR AERIAL MORPHOGENESIS IN *MEDICAGO TRUNCATULA*

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## Abstract

**Description of the subject:** Despite progress in genome sequencing of the legume model species *Medicago truncatula*, some genes described in other species are not mapped.
**Objective :** Four genes described in the literature to be involved in the genetic variation for aerial morphogenesis in *Arabidopsis thaliana* and pea (*Pisum sativum*) were thus identified. Three of them are known to be involved in growth process, particularly in stem branching (*Ramosus or Rms1*) and stem elongation (*Spindly* or *Spy* and *Gibberellin Insensitive Acid* or *GAI*). The fourth gene *Lumini dependens* (*LD*) is involved in flowering pathway.
**Methods :** Specific primers were designed to amplify by PCR mutation-rich intronic regions of these genes. The amplified sequences in *M. truncatula* were similar to the gene sequences of *A. thaliana* and *P. sativum*.
**Results :** SNP polymorphisms were detected on four parental lines of mapping populations for *Rms1*, *GAI* and *LD*, but no polymorphism was found for *Spy*. PCR markers were developed to genotype the three polymorphic genes in mapping populations: either specific primers used in stringent conditions to get a presence/absence of amplification for *GAI* and *LD* or CAPS marker for *Rms1*.
**Conclusion :** *Rms1* was mapped on chromosome 3, *GAI* on chromosome 4 and *LD* on chromosome 7.
**Keywords:** SNP; molecular marker; *Medicago truncatula;* genetic map; *Spindly*; *Ramosus*; *Lumini dependens*; *Gibberellin Acid Insensitive*.

# POLYMORPHISME DE SÉQUENCE ET CARTOGRAPHIE GÉNÉTIQUE DE GÈNES CANDIDATS INTERVENANT DANS LA MORPHOGENÈSE AERIENNE CHEZ *MEDICAGO TRUNCATULA*

## Résumé

**Description du sujet :** Les ressources actuellement disponibles offrent l'opportunité d'étudier l'espèce modèle (*M. truncatula*) avant un transfert des connaissances vers les espèces cultivées, possédant généralement des structures génétiques complexes (polyploidie, allogamie).
**Objectifs :** Quatre populations de RILS sont disponibles ainsi que des QTLs de morphogenèse. En utilisant une stratégie « gènes candidats », quatre gènes intervenant dans les variations génétiques pour la morphogenèse chez *A. thaliana* et *P. sativum* ont été selectionnées. Trois d'entre eux sont connus pour être impliqués dans la croissance : *gène Ramosus (Rms1)* (ramification des tiges) ; *gène Spindly (Spy)* et *Gibberellin Acid Insensitive (GAI)* (élongation des tiges). Le quatrième gène *Luminis dependens (Ld)* est impliqué dans la floraison.
**Méthodes :** Des couples d'amorces spécifiques sont testés sur quatre génotypes parentaux afin d'amplifier, par, des régions introniques des gènes, riches en variabilité. Les séquences amplifiées pour l'ensemble des gènes s'alignent parfaitement avec les séquences d'origine d'*A. thaliana* et de *P. sativum*. Le polymorphisme détecté est de type SNP. Ce dernier est révélé par deux techniques différentes : CAPS et l'utilisation des amorces hyper-spécifiques.
**Résultats :** *Rms1* est cartographié sur le groupe de liaison 3. Le gène *Ld* (sur le groupe de liaison 7) et *GAI* (sur le groupe de liaison 4).
**Conclusion :** Les résultats obtenus sont transposés, par synthénie, sur la luzerne cultivée (*Medicago sativa*).
**Mots clés:** SNP; marqueurs moléculaires; *Medicago truncatula;* Cartographie; *Spindly*; *Ramosus*; *Lumini dependens*; *Gibberellin Acid Insensitive*

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## INTRODUCTION

One of the main objectives of molecular genetics is to identify and isolate genes governing genetic variation for important traits. The strategy of positional cloning in which there is no hypothesis on gene function, is often conducted. Another strategy is to have an *a priori* hypothesis of the involvement of some genes in trait variation. This candidate gene strategy supposes that structural or regulatory genes already described in other species to govern a trait pathway could be involved in the target species [1]. In the candidate gene approach, the working hypothesis assumes that a sequence polymorphism within the gene may be related to phenotypic variation. This approach has been successfully used in human and animal genetics [2] and since 1990, in plant genetics [3]. The legume species *Medicago truncatula* is a model plant world-widely used for molecular genetic studies. Large-scale projects in *M. truncatula* genomics have been initiated within the international community [4, 5] and essential tools have been developed for structural genomics (genetic mapping, BAC librairies, genome sequencing) [6, 7] and functional genomics (ESTs, microarrays, mutant collection) [8] along with the development of bioinformatics resources. Comparative genomic studies suggest a high level of synteny between genomes of model and crop legumes, as proven between *M. truncatula* and tetraploid or diploid alfalfa (*M. sativa*) [6, 9], between *M. truncatula* and *P. sativum* [10] and among six legume species [11]. *M. truncatula* is close from a phylogenetic point of view to legumes grown in temperate areas: it is part of the Galegoid family that contains the Trifolieae (*Medicago sativa*, *Trifolium repens*), the Vicieae (*Pisum stivum*, *Vicia faba*, *Lens esculata*, *V. sativa*) and Cicereae (*Cicer arietinum*) tribes. It is hoped that the active development of research on this model legume provides important tools in genetics and improvement of legume crops. In grain and forage legumes, the characteristics of aerial morphogenesis are fundamental elements of population structure (number of stems, number of leaves, stem height) and its agricultural value (resistance of stems to lodging, biomass yield). In model species, mainly *A. thaliana* [12] but also in pea [13], genes are described to govern basic processes involved in the plant aerial morphogenesis.

Using the candidate gene approach, four genes described in the literature to be involved in genetic variation for aerial morphogenesis in *A. thaliana* and *P. sativum* were selected. Three of them are known to be involved in the growth process. *Ramosus* 1 (*Rms*1) is involved in stem branching [14, 15, 16]; *Gibberellin Acid Insensitive* (*GAI*) [17, 18, 19, 20] and *Spindly* (*Spy*) [21, 22, 23, 24] are involved in stem elongation. The fourth gene, *Luminidependens* (*LD*), is involved in the flowering pathway [25]. The purpose of this study was to map these four candidate genes involved in aerial morphogenesis in *M. truncatula*, by the analysis of sequence polymorphism among four parental lines of recombinant inbred lines (RIL) populations, the development of markers and their genotyping in mapping populations.

## MATERIAL ET METHODS

### *1. Plant material*

Four *M. truncatula* lines, parents of mapping populations and originating from different origins were used (Pierre 2008). DZA315.16 [7] and DZA45.5 were collected in Algeria, F83005.5 [26] was collected in South of France and Jemalong6 originates from an Australian cultivar. These four lines were provided by Biological Ressources Center of INRA Montpellier in France (www.montpellier.inra.fr/BRC-MTR/). Seeds were sown in greenhouse. DNA was extracted using DNAeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions, from 200 mg of young leaves previously grind with liquid nitrogen. Two RIL populations of *M. truncatula* were used: LR3 (F83005.5 X DZA 45.5; 179 lines) and mini-LR4 (Jemalong6 X DZA315.16; a subsample of 99 lines over 199) and their DNA was extracted using a 96 well-plates rapid extraction protocol [27] from 50 mg of leaves previously grind with liquid nitrogen.

### *2. Methods*

#### *2.1. Design of primer sequences*

A bioinformatics analysis was carried out to align the sequences of *A. thaliana* or *P. sativum* genes with *M. truncatula* sequenced clones and ESTs. Rms1 gene available in *P. sativum* (AY557342) was aligned with *M. truncatula* clone mth2-52p12 (CR956392) and with *M. truncatula* TC 81879. *A. thaliana* LD gene (At402560) was aligned with *M. truncatula* clone mth2-34C9 (AC144504) and with TC91556.

*A. thaliana* Spindly (*Spy*) gene (At3g11540) was aligned with *M. truncatula* clone mth2-12e5 (AC146559) and TC84147. Making the assumption that introns are richer in sequence polymorphism than exons, Primers were defined for each gene on both sides of an intron, in exonic sequences. They were synthesized by MWG Biotech company (Table 1).

### *2.2. Detection of polymorphism in parental lines*

PCR was performed using a PTC-100 or PTC-200 thermocycler (MJ Research Peltier Thermal Cycler) for amplification with the program: 94°C for 5 min, followed by 40 cycles of 40 s at 94°C, 40 s at optimal temperature melting (table 1), 30 s at 72°C and a final extension of 10 min at 72°C. In a total volume of 25 µl, 50 ng of genomic DNA, 0.2 µM of each forward and reverse primer, 200 µM of dNTPs, 1X Buffer 10X, 3 mM of MgCl2 and 0.625 unit of Taq polymerase (Taq Platinum, Invitrogen) were used. PCR products were analyzed in 1.5% agarose gel in TBE 0.5X.

### *2.3. Sequencing and SNP detection*

Sequencing was performed by Millegen company (Labège, France) either directly from the PCR products if the profile presented a unique and intense band at the expected size or after cloning, if several bands were visible. In that case, the band of the expected size was cut from the agarose gel, purified using the Mini Elute Gel Extraction kit of Qiagen, and inserted into the pGEM-T Easy Vector using the pGEM-T II kit of Promega. Steps of cloning were conducted following the manufacturer's instructions. Sequences were processed using the Staden Package release 1.6.0 (http://sourceforge.net /projects/staden/). The sequences from the four parental lines were aligned using Multalin software (http://bioinfo.genotoul.fr/multalin/multalin.html). SNPs were detected by manual inspection.

### *2.4. SNP genotyping*

Two methods based on PCR were used for SNP genotyping. The first was based on specific PCR amplification, using one of the primers ending at the SNP position. This primer pairs perfectly with DNA of one parent, and does not pair, under stringent conditions, with the DNA of the other parent. The polymorphism obtained in the mapping population is thus presence/absence of the band. The second method was based on the development of CAPs markers, using restriction site polymorphism.

### *2.5. Mapping*

The markers were used on a mapping population made from polymorphic parents. The linkage analysis was done using JoinMap software [27].

Table 1: Sequences and Tm of primers used for testing amplification locus

| Gene | Forward and reverse primers (5'-3') | Tm (°C) | Theoretical size (pb) |
|---|---|---|---|
| *Rms1* | CTATGCTTGTGGAGCACAGCG<br>TGTTGGGTTCCAAAACAGTAA | 52 | 481 |
| *Spy* | GAAAGATATTTATGGGACTGA<br>GTGATAGTGTTAGACAGAGGT<br>AGCATTCCGTTTCCGATTCAC<br>TCGCCGCCGCTAACTCAGTGG | 49 | 761 |
| *GAI* | ATGGTGCTTCTCTTTGGTCCC<br>GTCACGCCAAATAAAGCACTG | 57 | 914 |
| *LD* | | 65 | 847 |

## RESULTS

### *1. PCR amplification and sequencing of candidate genes in the parental lines*

Primers described in table 1 gave amplification products for all four genes in the four parental lines (Jemalong6, DZA315.16, DZA45.5 and F83005.5). For *Rms1*, a single band of about 400 bp was observed, close to the theoretical size. For *GAI* gene, a band of about 900 bp, as expected, was observed for each line. *Spy* gene gave an amplification product of about 1400 bp, larger than the theoretical size of 761 bp. Finally, the *LD* gene produced by PCR an intense band of about 2400 bp, larger than the expected size of 847 bp. For *GAI*, *SPY* and *LD*, the most intense band was accompanied by pale bands.

### *2. Sequencing and sequences analysis*

For *Rms1* gene, a quantity of 1.58 µg DNA from PCR products of each line was directly sequenced. For *GAI*, *Spy* and *LD* genes, a step of cloning was applied, after extraction of the most intense band from agarose gels. Sequences (372 bp for *Rms1*, 918 bp for *GAI*, 2358 bp for *LD* and 1368 bp for *Spy*) were compared to *M. truncatula* clones, *A. thaliana* and *P. sativum* sequences by BlastN [29]. For *Rms1*, the sequences were aligned with the MTH2-52P12 sequence clone of *M. truncatula* (CR956392.5; e-value: 0.0) and with *P. sativum* cultivar Raman *Rms1* (AY557342.1; e-value: 6e-112). The *GAI* sequences were perfectly aligned with *P. sativum* Cry mRNA (DQ845340.1) with an e-value of 0.0 and also aligned with *A. thaliana GAI* (At1g14920; e-value: 1e-176). The *LD* sequence was aligned with *M. truncatula* clone mth2-34c9 (AC144504.14; e-value: 0.0). SNPs were identified among four lines in exonic sequences, for three genes: *Rms1* with 5 NPs (Fig. 1), *GAI* (5 SNPs) (Fig. 2) and *LD* (11 SNPs) (Fig. 3). *Spy* gene did not showed polymorphism in the sequenced portion of 1368 bp. SNP frequency varied from 0 for *Spy* to 1.34 for 100 bp in *Rms1*.

### *3. SNP Genotyping*

For *GAI* and *LD*, the design of primers for specific amplification was chosen. For *GAI*, the primer pair 5' GTCACCGGAAACGGAATC 3' and 5' CTGTTATTCGTCAGATTCGGC 3' gave an amplification product of 109 bp using a melting temperature of 77°C and a MgCl2 concentration of 6mM in Jemalong6 and not in DZA315.16. For *LD*, the primer pair 5' ATGTCTGGATATAAGCCC 3' and 5' CAACGAAGACTTACTGAT 3' gave a PCR product of 266 bp in Jemalong6 but not in DZA516.16 with melting temperature of 63°C This technique was applied on LR4 mapping population for RIL genotyping. For *Rms1*, the sequences of the four parental lines were aligned and restriction site polymorphism was determined (http://www.restrictionmapper.org/). The HaeIII enzyme, a type 2 enzyme whose restriction site is GG/CC, theoretically generates two bands of 171 and 201 bp in DZA45.5 and has no effect on PCR product (372 bp) of F83005.5, Jemalong6 and DZA315.16. (Promega) according to the manufacturer's instructions. Genotyping of *Rms1* was carried out in LR3 mapping population.

### *4. Genetic mapping*

*GAI* gene was mapped at the bottom of chromosome 4 in LR4 population (Fig. 4A). *Rms1* gene was located on the chromosome 3 in LR3 population (Fig. 4B) and *LD* gene was mapped on chromosome 7 in LR4 population (Fig. 4C).

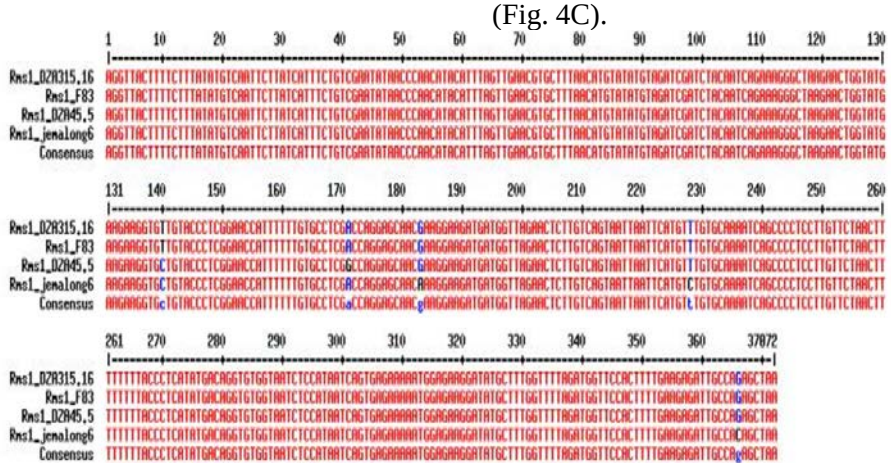


Figure 1 : Alignment of *Rms1* gene sequences between the four *M. truncatula* parental lines. Nucleotids in blue are SNPs


Figure 2 : Alignment of *GAI* gene sequences between the four *M. truncatula* parental lines. Nucleotids in blue are SNPs.

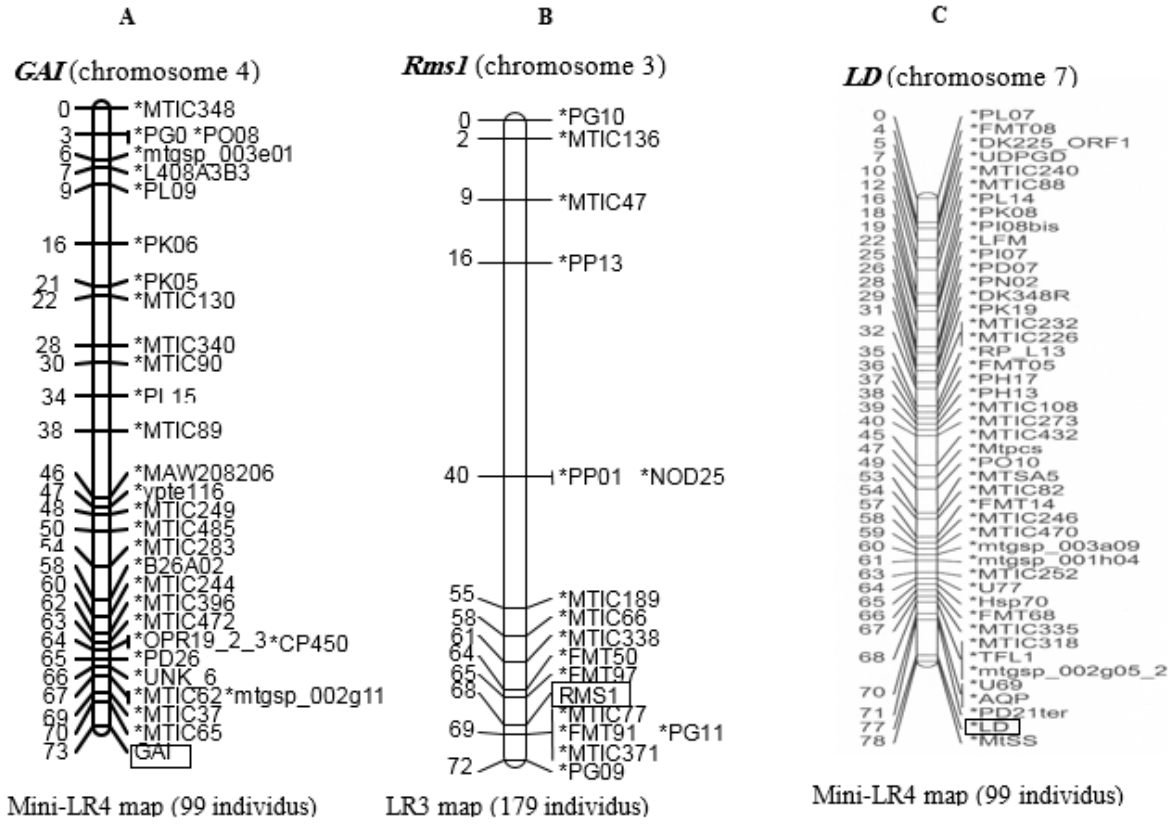


Figure 4 : Map of candidate gene of *M. truncatula* for aerial morphogenesis traits.
A : *GAI* gene (chromosome 4 -LR4). B : *Rms1* gene (chromosome 3-LR3). C : *LD* gene (chromosome 7 -LR4).

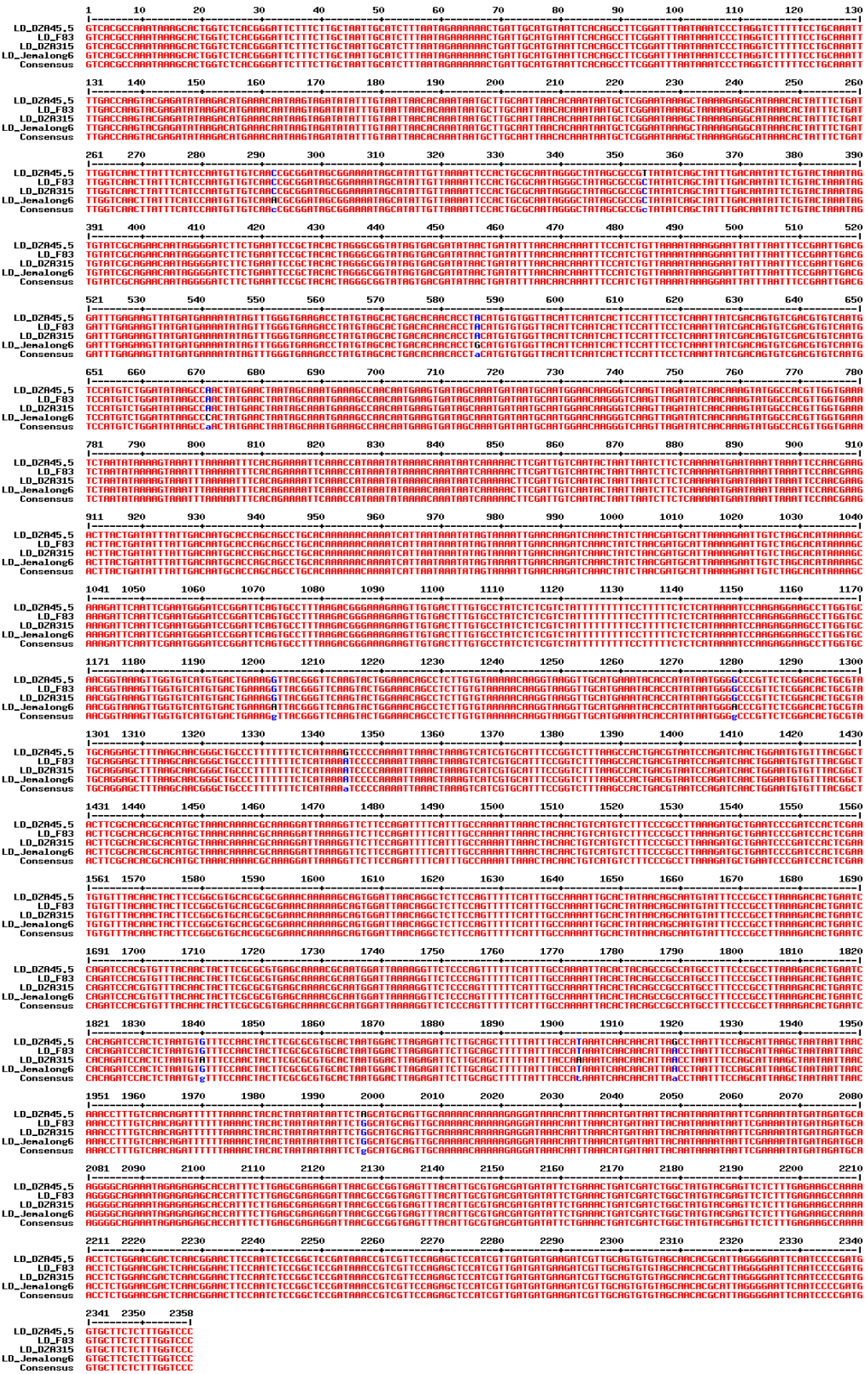

Figure 3 : Alignment of *LD* gene sequences between the four *M. truncatula* parental lines. Nucleotids in blue are SNPs.

## DISCUSSION

Mapping of candidate genes improves the information content of the *M. truncatula* genetic map. In this study, four genes described to be involved in aerial morphogenesis were sequenced in parental lines of RIL populations. The strategy was to sequence intronic regions, known to be more polymorphic than exons, to identify polymorphism and to use this polymorphism for RIL genotyping. These genes are presently not identified in genome sequence database of *M. truncatula*, but EST and clone sequences were available. Alignment of *A. thaliana* and *P. sativum* gene sequences to *M. truncatula* EST gave access to exons putatively separated by introns. In the exons, primers were successfully designed to amplify sequences in *M. truncatula*. The PCR products obtained was of the expected size, longer or shorter, depending on the gene. As the information of intron sequences was available from other plant species, these differences in intron length were not surprising. Among four parental lines, a sequence polymorphism was obtained for three genes but one gene was monomorphic at least in the sequenced gene portion among the four lines. Candidate genes often present low levels of polymorphism, because they often are relatively conserved. The polymorphisms detected were of SNP type, no length polymorphism was found. This is in accordance to the observations that the majority of sequence polymorphisms are SNPs. The SNPs were used to defined PCR markers for genotyping of a RIL population. PCR based methods were chosen, even if other methods, especially valuable when a large number of genotypes must be analysed, are described. The three polymorphic genes were mapped in two different mapping populations, the constraint being that the two parents of a mapping population are polymorphic. Gene position on *M. truncatula* was similar to that obtained in pea. In *M. truncatula*, several QTLs for morphogenetic traits were described [30]. On chromosome 7, a strong QTL for flowering date and stem elongation was identified, but *LD* gene was clearly outside the confidence interval of this QTL. *GAI* was mapped at the bottom of chromosome 4, close to QTL for morphological traits (branch length, flowering date and aerial dry weight). It could explain a part of variation for these traits as already found in other species, *GAI* homologues being genes of the green revolution in rice [31].

In the region of the chromosome 3 where *Rms1* was mapped, a QTL was found for the number of internodes.

## CONCLUSION

In conclusion, we found that the mapping of selected candidate genes in *M. truncatula* can be done quickly and efficiently with specific primers. This methodology in combination with a highly polymorphic reference population makes mapping of the vast majority of sequences of interest feasible.

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