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# Molecular investigation of two contrasting genotypes of *Medicago truncatula* to salt stress using two expressed sequence tag-simple sequence repeat (EST-SSRs) markers

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**Two expressed sequence EST-SSRs primers were used to show genetic variation and determine a potential link of these markers to salt stress tolerance on two contrasting *Medicago truncatula* genotypes (Tru 131 tolerant genotype, and *Jemalong,* sensitive one). The amplification of the DNA were isolated from 10 individual seedlings for each genotype (tolerant and sensitive) with two Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) primers (MTIC 044) and (MTIC 124) produced a total of 20 amplified products, of which MTIC 124 was polymorphic. The sizes of the alleles detected ranged from 100 to 280 bp. The EST-SSRs markers were polymorphic with an average of 1.33 alleles per primers and gave moderate values of polymorphic information content (PIC) that ranged from 0 to 0.267. The analysis of polymorphism loci for each genotype showed that the tolerant genotype (Tru 131) population had two alleles; genetic diversity index of 0.32 and PIC value of 0.267. The results obtained from unigene database of highly similarity proteins sequences with these loci showed that these two EST- SSRs loci MTIC 044 and MTIC 124 encode GATA transcription factor and cysteine proteinase inhibitor, respectively and were expressed principally in root in *M. truncatula*. This data suggest that these two loci are involved in salt stress tolerance and the two EST-SSR markers used are appropriate for the studying of salt stress tolerance in *M. truncatula*.**

**Key words:** *Medicago truncatula*, salt stress, *in silico* analysis, expressed sequence tag-simple sequence repeat (EST-SSR), UniGene / UniProt databases.

## INTRODUCTION

*Medicago truncatula* is widely used as a model legume plant for understanding tolerance to abiotic stress (Young and Udvardi, 2009). This legume is of great interest for sustainable agriculture and ecology. Salinity stress is an important abiotic stress which significantly affects legume growth and reduces crop production worldwide. Expressed sequence tags simple sequence repeats EST-SSRs are important sources for investigation of genetic diversity and

molecular marker development and they are useful markers for many applications in genetics and plant breeding because they show variation in the expressed part of the genome. EST-SSR primers have been reported to be less polymorphic compared with genomic SSRs in crop plants because of greater DNA sequence conservation in transcribed regions (Scott et al., 2000). The transcription factors are proteins that modulate gene expression by binding to specific cis-acting promoter elements, thus activating or repressing the transcription of target genes (Romano and Wray, 2003). Transcriptional regulation is also important for adaptation to abiotic stresses such as drought, cold, and high salinity, and for protection from biotic stresses (Shikata et al., 2004). Transcription factors are grouped into families based on the sequence of their DNA-binding domains (Luscombe and Thornton, 2002).

Our interest focuses on GATA transcription factors that are a group of DNA binding proteins broadly distributed in eukaryotes. In plants, GATA DNA motifs have been implicated in light-dependent and nitrate-dependent control of transcription (Reyes et al., 2004); they participate in nitrogen metabolism (Scazzocchio, 2000) but little information are available in relation to abiotic stress. Another interest focuses on enzymes like proteinases that are implicated in many cellular reactions involving protein degradation, such as degradation of storage proteins; their action can be inhibited by cysteine proteinase inhibitors, or cystatins superfamily. Expression of the proteinase inhibitor genes is usually limited to specific organs or to particular phases during plant growth: germination (Botella et al., 1996), early leaf senescence (Huang et al., 2001), drought (Waldron et al., 1993) or cold and salt stresses (Pernas et al., 2000; Van der Vyver et al., 2003). Information is still limited about the regulation of these inhibitors in plants and especially in the leguminous *M. truncatula* and their possible interaction with proteinases under salt stress conditions. The aim of this study was to find out if the two EST-SSR markers used (MTIC 044 and MTIC 124) encoding GATA transcriptor factors and cysteine proteinase inhibitors, respectively, are linked or no to salt stress tolerance on two contrasting *M. truncatula* genotypes (Tru 131 the tolerant genotype and *Jemalong* the sensitive one).

## MATERIALS AND METHODS

### Plant material

Recently harvested seeds of two contrasting genotypes of *M. truncatula* to salt stress, Tru 131 (Tolerant) provided by the institute IDGC BelAbes (Algeria) and *Jemalong* (sensitive) as reference genotype, were used in this work for molecular characterization using the two EST-SSR markers.

### DNA Extraction and PCR amplification

Total genomic DNA was extracted for each genotype, from young seedling after 7 days of germination (10 seeds by genotype). DNA was isolated using a cetyl trimetrhylammonium bromide (CTAB) method adapted from Udupa et al. (1999). The two loci (EST-SSRs) located on the chromosome 3 (LG3) (Table 1), were chosen from the set of microsatellites developed by Journet et al. (2001) in *M. truncatula* (2n=16) available in GenBank EST (http://www.ncbi.nlm.nih.gov/dbEST/). Amplification of genomic DNA was done according to Udupa et al. (1999) in a PCR reactions (10 µL) containing 50 ng of template DNA, 1 × PCR Buffer, 0.2 mM dNTPs, 10 pmole of each primer and 1 unit of Taq polymerase. The amplification profile consisted of an initial period of DNA denaturation and Taq polymerase activation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. A final extension was done at 72°C for 7 min before cooling to 4°C. PCR products were resolved on a 6% denaturing polyacrylamide gel. After electrophoresis, the DNA bands were stained with ethidium bromide and visualized by UV. For each of the defined loci, SSR allelic composition was determined for each genotype.

Polymorphism information content (PIC) values which indicate the ability to distinguish between genotypes for each primer combination for polymorphic bands was calculated with the following formula (Anderson et al., 1993): PIC = 1 -Σ $P^2ij$ [Pij is the frequency of the allele i revealed using the primer j]. The genetic diversity at each locus was calculated as follows: $H_i = 1 - \Sigma Pi^2$, with $H_i$ and Pi denoting the genetic variation index and the frequency of the number of alleles at the locus, respectively (Nei, 1973). In order to find highly similarity sequences with EST SSRs, we used UniGene database (http://www.ncbi.nlm.nih.gov/UniGene/) to determine the selected proteins similarities involved in variability of salt stress tolerance and UniProt database (http://www.uniprot.org/uniprot/) to determine their principal function.

## RESULTS AND DISCUSSION

Two EST- SSRs markers of *M. truncatula* (legume model) were used to test polymorphism between two contrasting genotypes to salt stress (Tru 131 the tolerant genotype and *Jemalong* the sensitive one). Results show that the MTIC 124 locus was more polymorphic (Table 1). The amplification of the DNA isolated from 10 individual seedlings for each genotype produced a total of 20 amplified products (Figures 1 and 2). The sizes of the alleles detected ranged from 100 to 280 bp. The highest number of polymorphic bands was observed with MTIC124 locus, located on chromosome 3(LG3) and at this locus, two different alleles were observed in the tolerant genotype

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**Abbreviations: EST-SSR,** Expressed Sequence Tag-Simple Sequence Repeat; **LG,** Linkage Group; **CTAB,** Cetyl Trimetrhylammonium Bromide; **PIC,** Polymorphism information content; **$H_i$,** Genetic diversity at each locus.

**Table 1.** EST-SSR markers used for variability analysis of two contrasting genotypes of *M. truncatula* (Tru 131 tolerant genotype and *Jemalong* the sensitive one) to salt stress

| EST SSR markers | LG | Forward (F) and reverse (R) primers (5' - 3') | Repeat motif | Annealing temperature for PCR (°C) | GenBank EST name | References |
|---|---|---|---|---|---|---|
| MTIC 044 | 3 | F : CGCGCCTTCTAGTTCTCTC<br>R : GGGGTCTCTCTTTCTTGGA | [ACC]7 | 55 | MtBC10F10F1 MtBC *Medicago truncatula* cDNA clone MtBC10F10 T3, mRNA sequence | Journet et al. (2001) *Medicago truncatula* ESTs from endomycorrhizal roots |
| MTIC 124 | 3 | F : TGTCACGAGTGTTGGAATTTT<br>R : TGGGTTGTCAATAATGCTCA | [TG]7 | 55 | MtBC32B02R1 MtBC *Medicago truncatula* cDNA clone MtBC32B02 T7, mRNA sequence | |

LG, Linkage group.

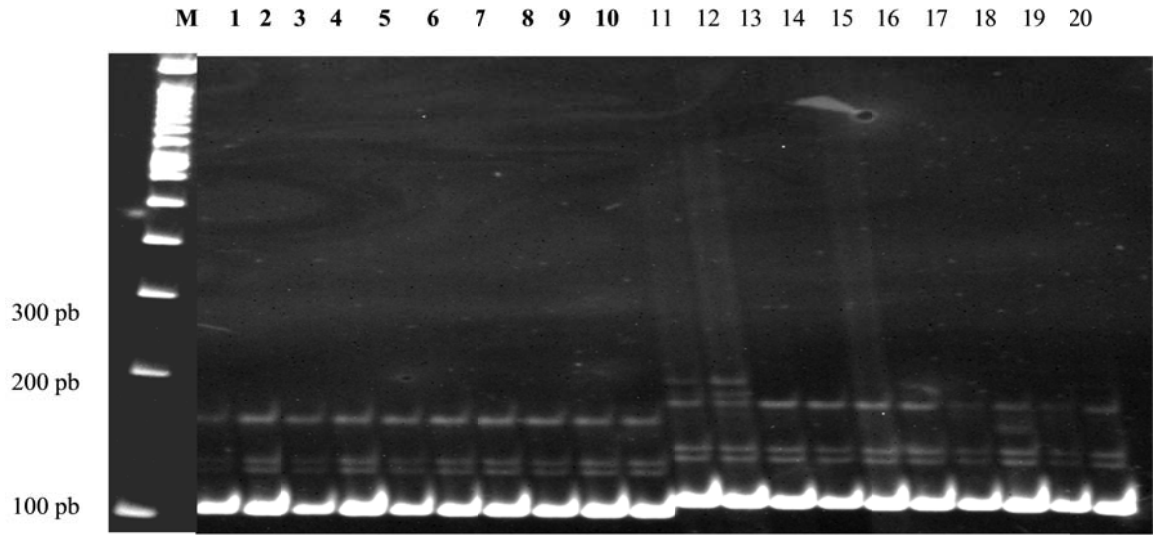


**Figure 1**. EST-SSR markers profile of the two contrasting genotypes of *M.truncatula* to salt stress [*Jemalong* 'sensitive': 1 to 10] and [Tru 131 ' tolerant' : 11 to 20] generated by the primer MTIC 044. M, Molecular weight marker.

(Tru 131) with genetic diversity index of 0.32 and PIC value of 0.267 (Table 2). The locus MTIC 044 located on the same chromosome 3 yielded one allele. The two EST-SSRs markers used were polymorphic with an average of 1.33 alleles per primers and gave moderate values of polymorphic information content (PIC) that ranged from 0 to 0.267. The results of EST profiles (Mtr.1896 - MTR_3g109760: GATA transcription factor and Mtr.5874 - MTR_3g043750: cysteine proteinase inhibitor) obtained from UniGene database which are of highly similarity proteins sequences to these loci showed that these two EST- SSRs loci(MTIC 044 and MTIC 124) encode GATA transcription factor and cysteine proteinase inhibitor, respectively, and were expressed principally in root in *M.truncatula* (Table 2). Their principal function was obtained from UniProt database. The EST-SSR locus

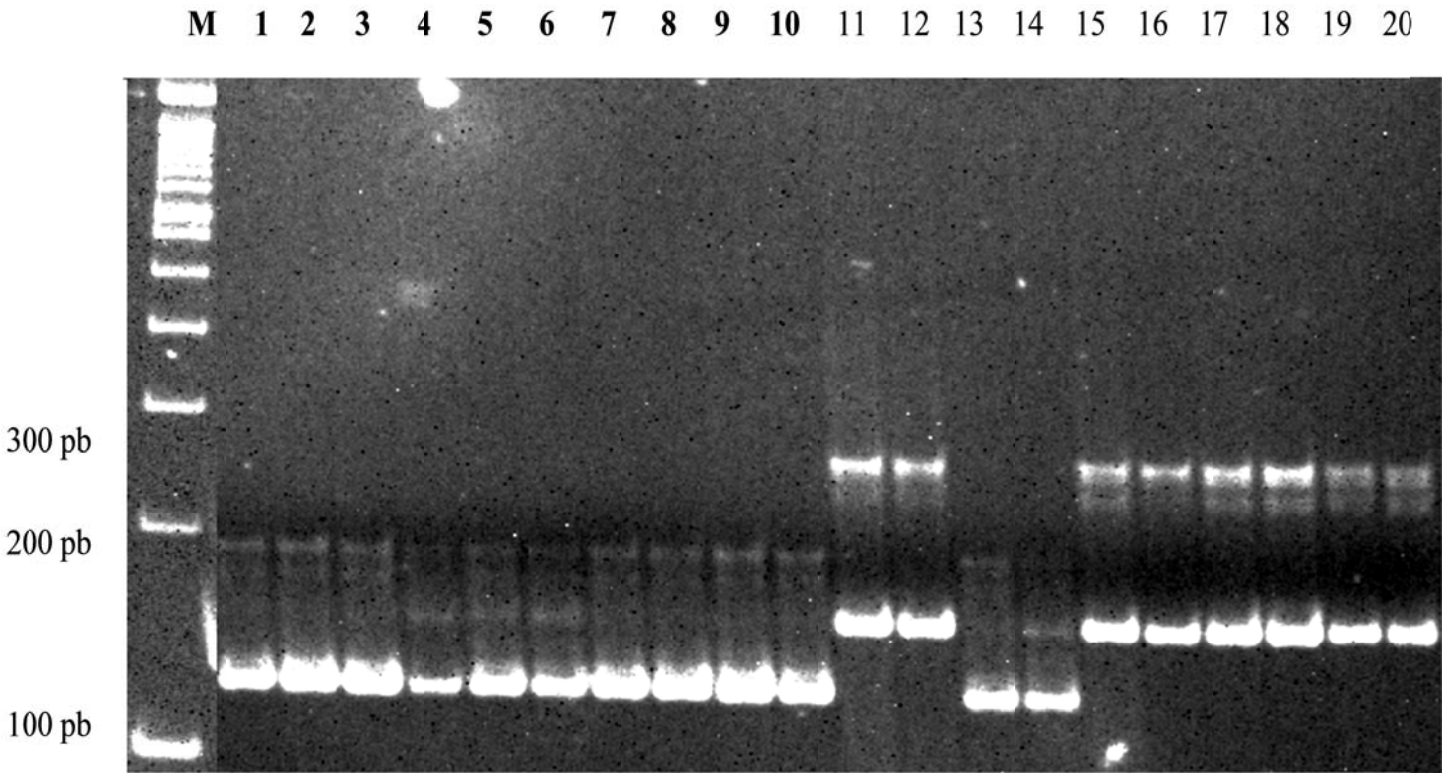


**Figure 2**. EST-SSR markers profile of the two contrasting genotypes of *M. truncatula* to salt stress [stress [*Jemalong* 'sensitive': 1 to 10] and [Tru 131 'tolerant' : 11 to 20] generated by the primer MTIC 124 (B). M, Molecular weight marker.

**Table 2.** Results of the EST-SSR markers revealed in the two contrasting genotypes of *M. truncatula* (Tru 131 tolerant and *Jemalong* sensitive one) to salt stress and data obtained from UniGene and UniProt databases of highly similarity proteins sequences with EST SSR markers used .

| Genotypes | EST SSR markers | S.Z | N.A | N.G | $H_i$ | PIC | Selected Protein Similarities | Identity % | G.A | R.E |
|---|---|---|---|---|---|---|---|---|---|---|
| Tru 131 (T) *Jemalong* (S) | MTIC 044 | 10 | 1 | 1 | 0 | 0 | GATA transcription factor (MTR_3g109760) mRNA, complete cds | 100 | XP_003603626.1 | Root |
| Tru 131 (T) *Jemalong* (S) | MTIC 124 | 10 | 2 | 2 | 0.32 | 0.26 | Cysteine proteinase inhibitor (MTR_3g043750) mRNA, complete cds | 100 | XP_003599710.1 | |

T, Tolerant; S, Sensitive. S.Z, Sample size; N.A, Number of alleles; N.G. number of genotypes. G.A, Gene bank accession; R.E, restricted expression; PIC, polymorphic information content; $H_i$, Genetic diversity; Highly informative: (PIC > 0.50); moderately informative: (0.25 < PIC <0.50) and slightly informative: (PIC < 0.25), non informative: (PIC = 0).

(MTIC 124) was more variable than the MTIC 044 locus and this variation was observed exclusively in the tolerant genotype (Tru 131); this information suggests the direct involvement of cysteine proteinase inhibitor in seedling development under salinity, especially in root.

Cysteine proteinases play an essential role in plant growth but also, in accumulation of seed storage proteins and in the response to biotic and abiotic stresses (Grudkowska and Zagdanska, 2004). Their action can be inhibited by proteinase inhibitors induced by abiotic stress. Amouri et al. (2014) showed that the tolerant genotype (Tru 131) had a higher storage protein content and increased root growth than the sensitive one (*jemalong*) suggesting the low synthesis of the cysteine proteinsae inhibitor (cystatins) in the tolerant genotype Tru 131 compared to *Jemalong*. Interestingly, this predicted data could be confirmed at transcriptomic level. Yamaguchi-Shinozaki et al. (1992) and Koizumi et al. (1993) noted that the clones rd19 and rd21 encoding different cysteine proteinases in *Arabidopsis thaliana* were induced by water deficit and were also responsive to salt stress. Several studies suggest that plant cystatins are responsive to abiotic stresses such as drought, salt, abscisic acid and cold treatment (Gaddour et al., 2001; Van der Vyver et al., 2003; Diop et al., 2004; Massonneau et al., 2005; Christova et al., 2006), although they have also been detected in vegetative tissues, including roots and leaves (Lim et al., 1996; Pernas et al., 2000).

In *A. thaliana*, two cysteine proteinase inhibitors (cystatins) designated AtCYSa and AtCYSb, were characterized. The northern blot analyses showed that the expressions of these two cystatins gene in cells and seedlings were strongly induced by multiple abiotic stresses from high salt, drought, oxidant, and cold (Zhang et al., 2008), suggesting the same mechanism in the legume model (*M. truncatula*). However, The GATA transcription factor encoded by the EST-SSR marker (MTIC 044), was not variable, wich explains the indirect involving activation of gene expression in relation to salt stress tolerance and may be implicated in common regulation network of gene expression related to plant growth and development. Members of GATA transcription factor family that have a role in development are found throughout eukaryotes, including plants, fungi, invertebrates and vertebrates. Little information was available in plant under abiotic stress (Haenlin and Waltzer, 2004). Sugimoto et al. (2003) illustrate that the family GATA transcription factor target genes respond to stress in tobacco.

## Conclusion

From all data analysis, we can propose that the two EST-SSR markers used in our study are suitable for the study of salt stress tolerance in the plant model (*M. truncatula*). The MTIC 124 locus that encode cysteine proteinase inhibitor (cystatins) is more polymorphic and implicated directly in salt tolerance than the MTIC 044 locus that encode GATA transcription factor. These two loci could be used for studying transcriptional regulation of gene expression involved in salt stress tolerance in *M. Truncatula*.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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