GENE SILENCING OF SOME NUCLEAR rDNA UNITS IN V. UNGUICULATA SUBSPECIES TENUIS

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Abstract

Transcriptional status of two intra-genomic ITS variants (Type-I and Type-II) identified in Vigna unguiculata ssp. tenuis (NI 1637) was analysed by RT-PCR. Total RNA was isolated from leaves, stem and roots of 15-day old seedlings and was reverse transcribed using specific primers for amplifying ITS-1 and ITS-2. Gel electrophoresis on a high-resolution agarose revealed that only Type-II variant was transcriptionally active in all the tissues analysed, while Type-I variant has been silenced. Sequence analysis revealed that Type-I variant in NI 1637 was of ssp. pubescens origin, whereas Type-II variant was of ssp. tenuis origin. These results indicate that NI 1637 is a hybrid between ssp. tenuis and ssp. pubescens; and further showed that either the hybridization is of recent origin or subspecies tenuis has a slow rate of homogenization. Our results suggest that although the rRNA genes of ssp. pubescens origin are present in NI 1637 genome, they have been rendered transcriptionally silent probably by epigenetic mechanisms.

Keywords: Epigenetic mechanisms; Gene silencing; Homogenization; Hybridization; Internal Transcribed Spacer (ITS); Reverse Transcriptase (RT) PCR; Vigna unguiculata.

Introduction

Vigna unguiculata (2n=22) belonging to genus Vigna (family Leguminosae) is a globally and economically important grain legume. It is also referred as black-eyed peas, field peas, or chawli (in India). The cultivated forms of V. unguiculata are further divided into five groups: Unguiculata, Biflora, Sesquipedalis, Textilis and Melanophthalmus. Cowpea is drought tolerant and grows well under conditions where most other food legumes do not (Singh et. al. 1997). Vigna unguiculata has 11 subspecies that includes ssp. unguiculata having the cultivated forms (var. unguiculata) and wild forms (var. spontanea) and 10 wild perennial subspecies (Pasquet 1999). The perennial subspecies have been grouped into five that are allogamous viz. ssp. baoulensis, ssp. burundiensis, ssp. letouzeyi, ssp. aduensis and ssp. pawekiae; and five autogamous viz. ssp. dekindtiana (var. spontanea), ssp. stenophylla, ssp. tenuis, ssp. alba and ssp. pubescens. The cultivated forms of V. unguiculata are further divided into five groups: Unguiculata, Biflora, Sesquipedalis, Textilis and Melanophthalmus (Pasquet 2000). Diversity among V. unguiculata and its subspecies has been analysed by morphological (Padulosi 1993), biochemical such as seed storage proteins (Fotso et al 1994), isoymes (Panella and Gepts 1992; Vaillancourt et al. 1993 and Pasquet 1999, 2000) and DNA markers such as chloroplast DNA (Vaillancourt and Weeden 1992), AFLP (Coulibaly et al. 2002) and RAPD (Ba et al. 2004).

Ribosomal RNA genes present as tandem repeats (Fig. 1) at one or more chromosomal locations in most eukaryotes (Long and Dawid 1980) are established as important tools for inferring molecular phylogeny at different divergence levels. While the coding genes (18S, 5.8S, 26S) are conserved and useful for analysis at higher taxonomic levels, the spacer regions (ITS and IGS) are variable and
hence useful at lower taxonomic levels. Nuclear ribosomal (nrDNA) internal transcribed spacer (ITS), present between the 18S and 26S genes is an established region for phylogenetic analysis at genus and species levels (Alvarez and Wendel 2003).

Internal transcribed spacer has been used for understanding relationships among some species in the Phaseolus-Vigna complex (Delgado-Salinas et al. 2006, Goel et al. 2002). However, in these studies mostly one representative accession (or two in a few case) per species has been analysed. Intra-species relationship within V. unguiculata has not yet been studied using nrDNA ITS region. Recently, analysis of species belonging to subgenus Ceratotropis (Asian Vigna) had shown presence of multiple intra-individual ITS variants specifically in V. radiata and several of them were also transcriptionally active (Saini et al. 2008).

In view of the above findings, the present study was carried out with an objective,

a) To analyse whether V. unguiculata subspecies show presence of multiple rDNA sequences;
b) To investigate their transcriptional status.

Materials and Methods

Plant Material

Three accessions of V. unguiculata ssp. tenuis (Table 1) obtained from National Botanic Garden, Belgium (Meise collection) were investigated in the present study.

DNA Isolation, PCR amplification and Agarose gel electrophoresis

Total DNA was isolated from the leaves of 15-day old seedlings according to Nalini et al. (2004). The DNA was treated with RNase A, further purified and quantitated according to Prasad et al. (1999). ITS region was PCR amplified using primers G1 (5’-GGAAGGAGAAGTCGTAACAAGG-3’) and C2 (5’-TCCTCCGCTTATTGATATGC-3’) as per Saini et al. 2008.
In 2008, separate PCR amplification was performed with primer VRD2 (5’-CGGGATTCTGCAATTCACACCAAG-3’) in combination with G1 and VRD1 (5’-CGTGAACCATCGAGTCTTTGAACGC-3’) in combination with C2 (Fig. 1) as described in Saini et al. 2008. PCR amplified products were separated on a 2.5% high-resolution agarose gel in 1X TBE at a constant voltage of 8 V/cm, stained with ethidium bromide according to Sambrook and Russell (2001) and photographed under UV light on a Gel-doc system from Syngene, Inc. (UK). Sizes of the PCR products were estimated by GeneTools software of the Gel-doc system by comparing with the DNA size standards. PCR products were purified using the PCR product purification kit (Qiagen, Germany) for subsequent use.

**Cloning and Sequencing**

The ITS products were directly sequenced on an ABI 377 automated DNA sequencer (Applied Biosystems, USA). Where mentioned, the PCR product was ligated to the EcoRV digested pBluescript vector (Stratagene) using the Rapid DNA Ligation Kit (Roche Diagnostics, Germany). Ligation mixture was used to transform DH5α and the recombinant colonies were identified by blue/white screening using isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Sambrook and Russell 2001). The insert from the recombinant colonies were amplified by colony PCR using primers and conditions specific for the amplification of ITS mentioned above.

**Secondary Structure Analysis**

Secondary structure analysis of ITS variants Type-I and Type-II of NI 1637 was carried out using Mfold program (Mathews et al. 1999; Zuker 2003).

**Transcript Analysis**

Total RNA was isolated from leaves, stem and roots of 15-day-old seedlings using Tri-Reagent (MRC, USA). RNA was treated with RNase-free DNAseI and subsequently the DNAseI was inactivated. Approximately, 100 ng of total RNA was reverse transcribed using First-Strand cDNA Synthesis Kit (Roche Diagnostics, Germany) and primer C2 as per the protocol provided by the manufacturer. First-strand cDNA product (1 μl) was used for PCR amplification of complete ITS as well as ITS1 and ITS2 separately. Positive (with genomic DNA) and negative (-RT) controls were also included for each set of reactions. The ITS products were analyzed on 2.5% high-resolution agarose gel and the transcribed product was sequenced.

**Results and Discussion**

**Characterisation of Internal Transcribed Spacer region**

The size of the PCR amplified ITS product/s from the three *V. unguiculata* ssp. *tenuis* accessions were found to be in the range of ~640 to ~700 bp (Fig. 2). Of the three accessions of ssp. *tenuis* two yielded single ITS fragment

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**Table 1: List of *Vigna unguiculata* ssp. *tenuis* accessions used in the study and their GenBank accession numbers**

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<tr>
<td>1.</td>
<td><em>V. ung.</em> ssp. <em>tenuis</em> NI 1712</td>
<td>EF530157</td>
<td>EF530370</td>
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<tr>
<td>2.</td>
<td><em>V. ung.</em> ssp. <em>tenuis</em> NI 1637</td>
<td># EF530155</td>
<td># EF530369</td>
</tr>
<tr>
<td>3.</td>
<td><em>V. ung.</em> ssp. <em>tenuis</em> NI 1636</td>
<td><em>EF530156</em></td>
<td><em>FJ390115</em></td>
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*V. ung.: Vigna unguiculata; ssp.: subspecies ‘#’-genbank accession number of type-I ITS variant and ‘*’-genbank accession number of type-II ITS variant in *V. ung.* ssp. *tenuis* NI 1637.
Sequence variation and Secondary structure analysis

The Type-I and Type-II ITS variants of *V. unguiculata* ssp. *tenuis* NI 1637 showed high sequence similarity and differed with a six base indel, repeat motif – ATTTGG. This motif was absent in the Type-II variant in NI 1637. The sequence variations observed were compared with ITS sequences of other *V. unguiculata* subspecies from database. Sequence analysis showed that the ITS ‘Type-I’ variant of NI 1637 was similar to sequence of ssp. *pubescens* (‘pubescens’ type’ variant), and ‘Type-II’ sequence was similar to ssp. *tenuis* (‘tenuis’ type’ variant). Secondary structure analysis using Mfold program showed a free energy value of -182.03 and -179.47 for Type-I and Type-II variants respectively.

Transcriptional status of the rDNA ITS variants in NI 1637

Transcriptional status of the two intra-genomic ITS variants (Type-I and Type-II) identified in *V. unguiculata* ssp. *tenuis* (NI 1637) was analysed by RT-PCR as detailed in materials and methods. The profiles showed that only one of the two ITS variants was transcriptionally active in all the tissues viz. leaf, root and stem (Fig. 3).

Sequencing of the RT-PCR ITS product revealed it to be of ‘tenuis’ type (Type-II variant). Analysis of the *V. unguiculata* ITS sequences from database showed, of the two types of ribosomal RNA genes identified in NI 1637, the one harboring *tenuis* type of ITS variant are transcriptionally active. However, loss of expression of the rDNA units harbouring Type-I (pubescens type) ITS variant alone does not indicate that they are non-functional (pseudogenic). High sequence similarity in the coding (5.8S gene) and spacer (ITS1, ITS2) region between the type-I and type-II ITS variants indicate that both belong to functional rDNA repeat units. This is further supported from the results obtained from secondary structure analysis using Mfold (Mathews et al. 1999; Zuker 2003) that showed a small difference in the free energies (-182.03 and -179.47) between the two variants.

Ribosomal RNA genes of different parental origins have been shown to be differentially expressed in tissues like leaf, stem, root etc. as observed in *Gossypium* species (Adams et al. 2003). However, in NI 1637 only one of the variants i.e. ITS ‘type-II’ variant (*tenuis* type) was found to be transcriptionally active in all the tissues (lanes 2, 3 and 4 in Fig. 3). These results suggest that although the rRNA genes of ssp. *pubescens* origin (as evident from ITS similarity) are present in NI 1637 genome, they have been rendered transcriptionally silent probably by epigenetic mechanisms such as DNA methylation, histone acetylation etc. (Volkov et al. 2006 and Dadejová et al. 2007).
Different fates of the rDNA units from the parents have been documented in plants, subsequent to hybridization: 1) both the parental type sequences are maintained in the genome, 2) homogenization towards one of the parental types and 3) maintenance of sequence element of both the parental types in the resultant rDNA unit (Wendel 2000). In the present study, in V. unguiculata ssp. tenuis (NI 1637), the rDNA repeat units of two different parental origins are still maintained in the genome and have not been homogenized. However, only one of the two variants was found to be transcriptionally active and the second one has been rendered silent, probably by epigenetic gene silencing. This indicates that either NI 1637 is a product of a recent hybridization event between two subspecies (ssp. pubescens and ssp. tenuis) of V. unguiculata or the rate of homogenization of ribosomal DNA repeat units is relatively low in subspecies tenuis. However, analysis of more number of tenuis accessions would be needed to precisely conclude the role of the first or second or both the above-mentioned phenomena in subspecies tenuis.

We have proposed that epigenetic silencing of the rDNA unit of subspecies pubescens origin may be an important preceeding step in the process of genome homogenization in NI 1637. This variant may slowly get depleted from the genome in the course of evolution. The present study demonstrates gene silencing of some nuclear ribosomal DNA units in V. unguiculata accession.

Acknowledgements

We thank Dr. Thierry Vanderborght, Seed Bank Manager, National Botanic Garden, Belgium for providing plant material used in this study. We thank Dr. S. K. Apte, Head, Molecular Biology Division, BARC, for the encouragement and support during this study.

Note: This work with additional analysis on species and subspecies of Vigna has been published as a full-text article in Journal of Heredity 2010.

References


