IDENTIFICATION AND ACTIVITY OF THE RETROTRANSPOSON TOS17 IN INDONESIAN JAVANICA RICE CV.ROJOLELE AND JAPONICA RICE CV. GAJAHMUNGKUR

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ABSTRACT

Retrotransposons are mobile genetic elements that transpose via an RNA intermediate that is reverse transcribed before integration into a new location within the host genome. They are ubiquitous in eukaryotic organisms and constitute a major portion of the nuclear genome (often more than half of the total DNA) in plants. *Tos17* is a rice endogenous retrotransposon that has been studied thoroughly. *Tos17* has been shown to be an efficient insertional mutagen and saturation mutagenesis tool for gene tagging and functional genomics in Japonica rice cv Nipponbare. In Javanica rice however, the presence and activity of *Tos17* has not been described thus far, while in some Indica rice *Tos17* has been found to be inactive. Javanica rice, also known as tropical Japonica rice, has many cultivars which may serve as potential genetic resources of great interest for breeding programmes. Here, the presence and activities of retrotransposon *Tos17* in Javanica rice cv Rojolele was described and compared to those of Japonica rice and Japonica rice cv Gajahmungkur. We identified five and three copies of *Tos17* in Rojolele and Gajahmungkur, respectively, with different activities.

Keywords: *Oryza sativa*, rice, javanica, retrotransposon, *Tos17*

INTRODUCTION

Rice is the third largest cereal crop behind maize and wheat, but the most important crop for human nutrition, being the staple food for more than half of the world population. Besides its importance, the production of rice is facing many challenges. Population increase, urbanization resulting in the loss of many good crop lands, and the occurrence of pests and diseases as well as abiotic stress factors such as drought, flooding, aluminum and salt, are among the major challenges in attempting to keep production high and to increase yield or at least achieve yield stability. To solve these problems, the development of new rice cultivars with improved agronomical traits such as tolerance to the above mentioned abiotic stress factors and resistance to pathogens (fungus, bacterial, virus) and pests (insect herbivory) will be very desirable. It is widely expected that this will be possible by studying the molecular basis of traits in rice, isolate the genes responsible and apply the knowledge gained to create improved rice cultivars, either by transgenic approaches or by marker-assisted breeding

Tropical Japonica rice is also known as Javanica rice and includes many cultivars grown mainly in Indonesia. There are many different Javanica cultivars grown in Indonesia and several of them possess important traits including superiority towards biotic and abiotic stresses. Examples are cv. Situpatenggang and Tenggulang which are tolerant to drought stress. Genes or genetic elements responsible for the agronomically important traits that are being utilized for crop improvement by traditional breeding could be isolated for faster crop improvement by genetic engineering and marker-assisted breeding. In order to be able to utilize any superior traits to improve rice quality using molecular breeding initial studies of the molecular base of those important traits should be done.

To study gene functions, reverse genetics is a powerful approach. This approach is dependent on the use of collections of mutants

which can be made by different methods such as chemical mutagenesis or insertion mutagenesis using transposons (endogeneous or exogeneous) or T-DNA. An example of the use of an endogeneous mutagen is the rice retrotransooson *Tos17*. The maize *Ac/Ds* transposon is being used as extrageneous transposon tagging system for random insertion mutagenesis of plant genomes. Similar to T-DNA mutagenesis (Jeon *et al*., 2000; Jeong *et al*., 2002; An *et al*., 2003; Chen *et al*., 2003; Sallaud *et al*., 2003, 2004; Sha *et al*., 2004; Ryu *et al*., 2004; An *et al*., 2005b), the *Ac/Ds* system has been proven to be an efficient, highly active and versatile system (Chin *et al*., 1999; Greco *et al*., 2001a, b, c, 2003; Kim *et al*., 2004; van Enckevort *et al*., 2004; Upadhyaya *et al*., 2006) that can be equipped with gene or enhancer traps (Eamens *et al*., 2004; Kolesnik *et al*., 2004) or activation -tagging (An *et al*., 2005a; Weigl *et al*., 2000; Marsch-Martinez *et al*., 2002) for efficient gene tagging. Central in these approaches is the development of large populations of mutants and the cloning and identifications of flanking sequence tags (FSTs) that lead to the identification of the tagged gene. Essential is that the resulting FST and other data are recorded in appropriate databases which are accessible for the scientific community (Zhang *et al*., 2006; Droc *et al*., 2006).

A strategy to do reverse genetics in rice without having to create transgenic plants is possible by utilizing endogenous transposable elements. In rice there are many endogenous transposable elements that have been identified such as the *mping*, *ping* and *pong* elements (Kikuchi *et al*., 2003; Jiang *et al*., 2003), and the thoroughly described *Tos17* (Hirochika *et al*., 1997). *Tos17* is a retrotransposon of which the activity was described firstly in the Japonica cultivars Nipponbare and Koshihikari, in which it is present in two and one copies, respectively (Hirochika, 1997). Activation of this transposable element was found to be induced by tissue culture (Hirochika *et al*., 1996). In cv. Nipponbare, *Tos17* activity was found only in one copy while the other was found to be inactive after tissue-culture. The ability of *Tos17* to transpose in rice is being used as a powerful tool to study gene functions by its ability to function as mutagen for reverse genetics purposes (Agrawal *et al*., 2001; Miyao *et al*., 2003). The convenience of *Tos17* as a tool for reverse genetics is facilitated by its ability to transpose following tissue cultures eliminating the requirements for generating transgenic plants for obtaining mutants. Therefore, the identification of the presence and activation of *Tos17* in Indica and Javanica rice will be important in order to study gene functions genome wide for the studies of genes involved in many different important traits. The application of non-transgenic approach for reverse genetics can be used to complement transgenomic approaches using T-DNA and/or *Ac/Ds* mutagenesis.

Here, we report the presence and the activity of *Tos17*, followed by the mapping of some of the locations of *Tos17* in Javanica rice cv. Rojolele and compare the results to that obtained with Japonica rice cv. Gajahmungkur. *Tos17* activity in Japonica rice cv. Gajahmungkur has also not been reported before. The purpose of this experiment is to analyze the possibility of using *Tos17* to study, isolate and analyze gene in Javanica rice cv. Rojolele and Japonica rice cv. Gajahmungkur in order to establish functional genomics in Javanica.

MATERIALS AND METHODS

Plant material

Rice seeds representing independent an M_1 generation of Javanica rice cv. Rojolele and Japonica rice cv. Gajahmungkur (originally named as accession cv. IRAT 112 from Ivory Coast) derived from tissue cultures following the modified method described by Hiei *et al*. (1997) were sown on soil media containing Osmocote as fertilizer. Plants were grown in phytotrons with continuous water supply at 28°C and a 12-hour light/12-hour dark cycle.

DNA isolation

High-throughput DNA extraction was based on Pereira and Aarts (1998). Ten centimeter leaves of three week-old plants were harvested and stored in 96 deep-well blocks (Qiagen) containing a stainless steel ball-bearing. The samples were immediately frozen in liquid nitrogen and shaken for 1 minute using a Retsch Miller MM301. To the resulting leave powders, 400 µl 1X isolation buffer (2X isolation buffer: 0.6 M NaCl, 100 mM Tris pH 7.5, 40 mM EDTA, 4% N-Lauryl sarcosine, 1% sodium dodecyl

sulfate. 1X isolation buffer: 1 volume of 2X isolation buffer, 1 volume of 10 M urea, 5% phenol pH 8.0 (0.1% 8-hydroxy-chinolin), 1% iso-amylalcohol) was added followed by thorough mixing. Four-hundred micro liter of phenol: chloroform: iso-amylalcohol (25:24:1) were added into the mixtures and mixed well. The samples were centrifuges at 6,000 rpm for 20 minutes. The supernatants were recovered into new deep-well blocks and 250 µl of isopropanol was added. The DNA was precipitated by mixing the mixtures gently for five minutes followed by centrifugation at 6,000 rpm for 20 minutes. Next, the pelleted DNA was washed with 70% ethanol and allowed to dry to the air overnight at room temperature. Finally, 150 µl TE was added to dissolve the pellets. RNA contamination was removed by RNAse treatment following the standard protocol (Sambrook et al., 1989).

Southern hybridization

Approximately 8 µg of Rojolele, Gajahmungkur and Nipponbare (control) total genomic DNA samples were overnight digested with *XbaI*. The samples were run on a 0.7% agarose gel in 1X TAE. After digestion, DNA's were transferred to GeneScreen by alkaline transfer (0.5 M NaOH) and fixed by baking at 80°C for two hours. The DNA probe was prepared by PCR amplification of a 1 kb *Tos17* fragment from wild-type Nipponbare rice total genomic DNA using the primers *Tos17*F (GCG TGCTCGTGATGCTCTTCGT) and *Tos17*R (TAGCCAGTGACAGAG CGACGAT). The fragment obtained was gel-purified and used as template for probe preparation. DNA hybridization was performed at 65°C for overnight. After hybridization, the membrane was washed in 50 ml of low stringency washing solution (2xSSC, 1%SDS), followed by a wash in 50 ml of medium stringency solution (1xSSC, 0.5% SDS), and then a wash in 50 ml of high stringency solution (0.5X SSC, 0.25%SDS). Each wash was performed for 20 minutes at 65°C in a rotating tube in a hybridization oven. Signals were detected by exposing the membrane to an X-ray film.

PCR

All PCR amplifications were performed with the RedTaq (Sigma) following the manufacturer recommendations. The primers to amplify *Tos17* are; TAGCCAGTG ACAGAGCGACGAT (formard) and GCGTGCTCGTGATGCTCTTCGT (reverse).

TAIL-PCR

Three different *Tos17* TAIL-PCR specific primers were used (Yamazaki et al., 2001). The primers were T17TAIL2 (AGTCGC TGAT TTCTTCACCAAGG), T17TAIL3 (GAGAGCATCATCGGTTAC ATCTTCTC) and T17TAIL4 (ATC CACCTT GAGTTTGA AGGG).Degenerate primers used were AD1 (NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT), AD2 (NGTCGA (G/C)(A/T)GANA(A/T)GAA) and AD3 ((A/T)GTGNAG(A/T)ANCA NAGA). The PCR reactions were performed following the method described by Liu et al. (1995) using RedTaq DNA polymerase (Sigma).

DNA sequencing

DNA fragments obtained from the TAIL-PCR were separated on agarose gel, purified using the Qiaquick Gel Extraction Kit (Qiagen) following the manufacturer's protocol and sent for sequencing (Baseclear, Leiden, The Netherlands).

Cloning

DNA PCR fragments were cloned by using the Topo Cloning Kit (Invitrogen). The fragments of interest were isolated and purified using the Qiaquick columns and fragments were cloned using a Topo cloning vector (Invitrogen). Minipreps of colonies obtained were extracted using the Birnboim-Doly alkaline-SDS method. To confirm presence of the right insert, digestions with *EcoRI* (flanking sites to the cloning site) were performed.

Results and Discussion

Rice cultivars Gajahmungkur and Rojolele contain three and five copies of Tos17, respectively

The retrotransposon *Tos17* is a 4.2 kb DNA fragment carrying the reverse transciptase domain flanked by two identical 138 bp long terminal repeats (3' LTR and 5' LTR) (Fig. 1;

Hirochika *et al*., 1996). To identify the presence and activity of *Tos17* in Javanica rice cv. Rojolele, Southern hybridizations were performed. The number of bands obtained from the wild-type non-tissue cultured generated plants will determine the copy number of *Tos17* prior to its activation and the number and patterns of bands obtained from the DNA sample from tissue-culture generated plants will demonstrate activation of *Tos17*.

The strategy that was used to identify the presence and the activity of *Tos17* in the Javanica rice was essentially the same as that of Hirochika *et al*. (1996). Genomic DNA samples of wild-type and tissue culture generated plants of cv. Rojolele and cv. Gajahmungkur were digested overnight with *Xba*I. The digestion should yield two fragment of *Tos17* since in *Tos17* there is one internal *Xba*I site. Southern hybridization using the probe corresponding to a 1 kb fragment covering the reverse transcriptase domain of *Tos17* (Hirochika *et al*., 1996) should result in a single band in rice plant with one copy of *Tos17* (Koshihikari). In Nipponbare rice, which was used as control, two bands will be obtained since there are two copies of *Tos17* which are located in different chromosomes (7 and 10). Southern hybridization of genomic DNA isolated from the wild-type non-tissue culture derived plants indicated that there are three and five copies of *Tos17* in cv. Gajahmungkur and cv. Rojolele, respectively (Fig. 2). As previously described (Hirochika *et al*., 1996), analysis of DNA isolated from cv. Nipponbare showed two copies of *Tos17*.

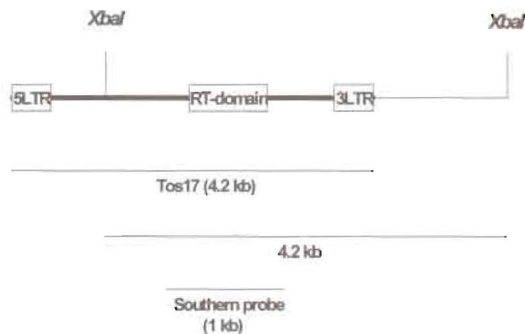

Figure 1. Schematic description of *Tos17* located in rice chromosome 10 showing the reverse transcriptase domain (RT-domain), 5'- and 3'-LTR (long terminal direct repeat). The *XbaI* sites are indicated to describe the fragment sized obtained by Southern hybridization using the probe as indicated.

Tos17 is active in Gajahmungkur *and Rojolele after tissue culture*

Southern hybridization indicated that after tissue culture and regeneration of plants, *Tos17* hybridization patterns changed both in cv. Rojolele and cv. Gajahmungkur (Fig. 2). However, it may seem that the activities are different among the cultivar tested. As shown by the number of *Tos17* copies obtained from the Southern hybridization, *Tos17* seems to be more active in Nipponbare than in the Javanica rice cv. Rojolele and Japonica cv. Gajahmungkur. Using the same method of tissue culture and plant regeneration, there were more *Tos17* copies observed in cv. Nipponbare observed than in cv. Rojolele and Gajahmungkur (Fig. 2). This might indicate that cv. Nipponbare is more sensitive to mutagenesis through tissue culture by the activity of *Tos17*. *Tos17* may seem to be more active in cv. Gajahmungkur than in cv. Rojolele, following the same tissue culture protocol (Fig. 2).

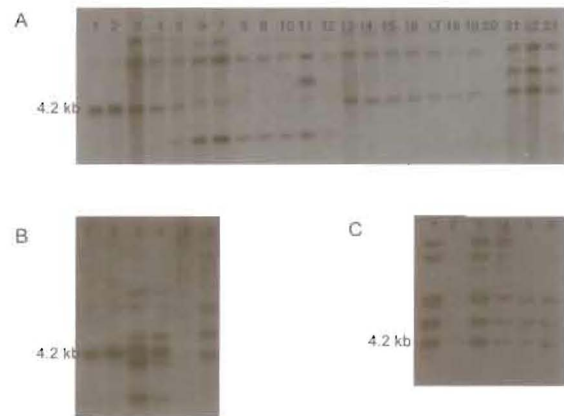

Figure 2. The copy numbers and activity of *Tos17*. A. In cv. Gajahmungkur there are three copies of *Tos17* identified in wild-type non tissue-culture derived plant (lane 3 and 4). Cv. Nipponbare *Tos17* was used as control (lane 1 and 2). Lane 5-23 showed that after tissue culture *Tos17* was activated indicated by the presence of new or missing DNA bands. B. *Tos17* is more active in cv. Nipponbare after tissue culture than in Javanica rice cv Rojolele or Indica rice cv. Gajahmungkur. Lane 1 and 2 non-tissue-cultured Nipponbare plant. Lane 3 and 4, tissue culture-generated Nipponbare plant and lane 6 tissue culture-generated Rojolele plant. C. Rojolele has five copies of *Tos17* (lane 1). The activity of *Tos17* after tissue culture was also observed in Rojolele (lane 3-6). Lane 2 represents Nipponbare.

The reverse transcriptase domain of Rojolele Tos17 is identical to that of Nipponbare

To determine whether the *Tos17* in cv. Rojolele and cv. Nipponbare are conserved, the reverse transcriptase (RT) domains isolated from both cv. Rojolele and cv. Nipponbare were sequenced and compared. The RT domains were isolated by PCR amplification and cloned into Topo vectors (Invitrogen). The cloned fragments were then sequenced using M13 forward and reverse primers, and the *Tos17* forward and reverse primers. Comparison of the sequenced RT domains showed that both sequences were identical, indicating that they are conserved. The sequence of the RT domain of *Tos17* from cv. Rojolele, has been deposited in GenBank (GenBank Accession EU155081). However, since the complete sequence of the cv. Rojolele *Tos17* copy has not been obtained yet, we may not be able to conclude at this moment the homology of *Tos17* from cv. Rojolele to that of cv.Nipponbare.

Transposition sites were identified in cv. Gajahmungkur and cv. Rojolele

Positions of *Tos17* in cv. Rojolele and cv. Gajahmungkur genomes were identified by isolating 3' flanking regions of *Tos17* by TAIL-PCR. The forward nested primers (specific primers) used were the same as those described by Yamazaki *et al*. (2001). The reverse degenerate primers used were AD1, AD2 and AD3 as described by Liu *et al*. (1995). The TAIL-PCR reactions were as described previously (Liu *et al*., 1995). The results of the amplification were run on 1% agarose gels and the resulting bands were purified and sequenced. In our experiments, only combination of amplification using primer AD2 give good results (data not shown). Primers AD1 and AD3 failed to produce amplification products after round three of the TAIL protocol. The results of the amplifications after round three of TAIL-PCR using primers AD2 were purified and then sequenced to identify the flanking region of the fragments. Next, sequences were BLAST searched against the rice genome for validation. The results showed that in cv. Rojolele and Gajahmungkur new insertion sites were identified. One *Tos17* insertion was identified in chromosome 2 of cv. Gajahmungkur and one insertion was identified in chromosome 11 of cv. Rojolele. These two newly found insertions are different from those of the two copies of *Tos17* in cv. Nipponbarre which are located in chromosome 7 and 10.

Conclusions

We identified presence of *Tos17* in Javanica rice cv. Rojolele and upland Japonica cv. Gajahmungkur. We identify three and five copies of *Tos17* in cv. Gajahmungkur and cv. Rojolele, respectively, thus the copy numbers of *Tos17* in the Javanica rice are higher than that of Japonica rice cv. Nipponbare. The higher number of *Tos17* in Javanica rice may support the notions that Javanica rice is derived from Japonica rice and that *Tos17* in Javanica rice has undergone several transpositions and amplification or alternatively, Tos17 copies went lost during the breeding of cv. Nipponbare. Similar to the *Tos17* copies in Japonica rice, *Tos17* elements in cultivars Rojolele and Gajahmungkur were also active after tissue culture, although transposon activity seems not to be as high as in Nipponbare since we observed in one specific line six copies, thus four extra with respect to the two copies in wild type rice. These results however, suggest that there is a possibility of application of *Tos17* for functional genomics, without the requirements of creating transgenic Javanica rice plants.

However, to be able to use *Tos17* as a tool for functional genomics, further characterization of *Tos17* in Javanica rice needs to be conducted and a large population of mutants needs to be developed. However, our results were from a pilot experiment and indicate that *Tos17* is active and therefore it will be feasible to develop such a population for reverse genetics for discovering gene functions involved in important traits in Javanica rice.

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