**Expression of RNA polymerase IV and V in *Oryza sativa***

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**Abstract**

RNA polymerase IV and V are principal players in the RdDM pathway, where their current study has shown the interaction of several factors that control DNA silencing of intergenic regions. DNA silencing is an important process during cell differentiation, nuclear structure and viral control. RNA pol IV and V are yet to be study in dodel monocot systems like *Oryza sativa* and can provide further data to define if the genetic silencing mechanism has diverged over time as compared to Dicots. Here we showed the expression pattern of these polymerases in *Oryza sativa*. Detectable amounts of these polymerases are found in specific adult plant tissues and particularly during somatic embryogenesis but not during the early stages of normal embryo development. Synthetic auxin leads to induction of both RNA pol IV and V. Their nuclear localization which may be required for genome reorganization and gene silencing.

Keywords: RNA POL IV, RNA POL V, *Oriza sativa*, Somatic embryogenesis, zygotic embryogenesis

**Introduction**

Gene silencing has become important in the field of research in recent years. During the last decade with the emerging view of the RNA-directed DNA methylation (RdDM) pathway (Herr et al. 2005; Law et al. 2010; Matzke et al. 2009). The study of RNA polymerase IV and V have become highlighted within the field of gene silencing. The complete mechanism remains unclear but it is known that generation and targeting of siRNAs requires RNA Polymerase IV (pol IV), which is proposed to generate single stranded RNA transcripts (Pikaard et al. 2008; Lahmy et al. 2010). These transcripts are then processed by RNA-DEPENDENT RNA POLYMERASE 2 and DICER-LIKE 3 to produce a 24 nt siRNAs which are methylated on their 3′ ends by HUA ENHANCER 1 (Li et al. 2005), followed by the union to ARGONAUTE 4 which is an effector protein for silencing (Law et al. 2010; Matzke et al. 2009; Duran-Figueroa et al. 2010; Olmedo-Monfil et al. 2010; Wierzbicki et al. 2009). In addition to siRNAs, RdDM is also associated with the presence of intergenic noncoding (IGN) RNA transcripts. The accumulation of IGN transcripts has been shown to depend on RNA polymerase V (Pol V) in *Arabidopsis thaliana* (Wierzbicki et al. 2008). These transcripts may act as scaffolds to recruit RdDM effector proteins, which in turn aid in the recruitment of DOMAINS REARRANGED METHYLTRANSFERASE 2 to loci that produce both siRNAs and IGN transcripts. Regardless of these advances in understanding the RdDM pathways there are many questions unsolved about the two plant specific RNA polymerases (Pol IV and Pol V) in particular during cell differentiation (Havecker et al. 2010; Olmedo-Monfil et al. 2010). Currently must of the research trying to understand the mechanism underling these polymerases has been carried out in *Arabidopsis thaliana*. However, the study in other plant models like *Oryza* *sativa* remains largely uncharacterized. Monocots have long diverged and their main study has been in *Zea mays* where it already showed clues about how paramutation takes place (Pikaard et al. 2009). Our results show that high amounts pol IV are expressed in esteem and pol V is expressed predominantly on inflorescence but surprisingly very low level of pol IV or V are express during zygotic embryogenesis, unlike that of somatic embryogenesis were large amounts are produced. Early somatic embryos show nuclear localization of both polymerases while older more develop tissue show that pol IV is found in the cytoplasm while RNA pol V maintains its nuclear pattern.

**Materials and methods**

**Plant material.**

Seeds from *Oryza sativa var. Filipina* were obtained from the commercial producer Arroz Covadonga, Champoton Campeche. Seeds sterilized with 70% ethanol for 5 min, followed by chlorine at 50% for 1 hour and 3 water washes. The seeds are incubated for the number of days for extraction. As previously publish by Mariani *et al.,* 1998 the scutellum was obtain after the 3 day by dissecting the embryos and transferring it to an induction media EIM (sucrose 4%; MS Media 4.3 g/L; 2,4-dichlorophenoxy acetic acid (2,4-D) 2 mg/L-1) following incubations were carried out in the dark room for the number of days stated in the legends.

**Antibodies**

Peptide sequence was selected for antibody production by comparing possible structure similarities between RNA pol II and V. Surface sequence highlighted the peptide sequence INPLCCDPFKGDFDGDCLHG (432-451) for RNA pol IV subunit I. For Pol V subunit I the unique sequence chosen was MAVRPNDS RPSDVRPPFSQI (1-20). Polyclonal antibodies were raised in rabbit as published by Vaitukaitis et al 1971.

**Protein extraction**

1 gr of selected tissues from Oryza sativa were grinded in liquid nitrogen, followed maceration as previously publish (Valadez-Gonsalez et al. 2007). Protein content was measured by Bradford and extracts were normalized by protein content.

**Western blot analysis**

Equivalent amount of total proteins from the different tissues were separated on an 8% SDS-PAGE and transferred to nitrocellulose membrane (Pall Corporation, USA). After 1 h of blocking with 5% non-fat milk in TBST (TBS, 0.1%Tween-20), the membrane was incubated with either anti-RNA pol IV or V as mention in the legends in TBST with 5% milk over night at 4oC then washed with TBST. Immunoreactive bands were detected with anti- rabbit antibodies conjugated with HRP followed by AlkPhos direct labeling reagents (Amersham).

# **RT-PCR**

Total RNA was extracted using the RNeasy plant mini kit (Qiagen). Reverse transcription reactions were performed using 2 g of RNA, 200 U of M-MLV, 0.4 M oligo dT primer and 2.5 M dNTPs for 1 hour at 37oC. The PCR reactions were performed with PIVF; GGT ATA TGG GAG AAC ATC ATT with PIVR; CCC ACT GCC TCC AAT ATG CAG for RNA pol IV subunit I and PVf; CTT GGA AGA GAA CTC CGT GCG with PVR; AGG AAC CTC CTC TGT AGG AAT for RNA pol V subunit 1 and primers 0.4 M each, Taq platinum DNA polymerase 2.5 U (Invitrogen), MgCl2 0.5 mM, 200 nM each dNTP. Cycling conditions were 94°C 2 min 1 cycle; 94°C 30 sec, 45°C 30 sec and 72°C 1 min for the indicated number of cycles. The PCR products were separated on 1% agarose gel

**Histology**

Samples representative somatic and zygotic embryos from all the stages were removed from culture and seeds respectively were fixed in a FAA: solution [formaldehyde (10%) ethanol (50%) and acetic acid glacial (5%)] for 48 h at room temperature, progressivelydehydrated in an alcohol series, and infiltrated with paraffin (Kendall). Sections (5 μm) werecut using a Leica microtome and stained with a 0.5% (w/v) aqueous solution of Toluidine blue O (TBO) (Sigma) for examining cell reorganization. Some sections were directly observed without any staining. Sections were examined with a Axioplan microscope coupled to camera.

**Immunofluorescence**

The Somatic embryos after 15 days of induction were fixed in tubes containing FAA with aspiration for 24 h. They were dehydrated through an ethyl alcohol series and embedded in paraffin (melting point 54–56 °C) with a graded series of tertiary butyl alcohol. The paraffin blocks were sectioned serially at 5 μm thickness using a microtome. Followed by deparaffinization with 4 washes with Histology grade Xylene for 2 min followed by removal of xylene with Absolute ethanol followed by 70% ethanol followed by water for 1 minute each. Somatic embryos and surrounding tissue were permeabilized with 0.1% Triton X-100 in PBS for 15 min, respectively. After washes with PBST they were either incubated with anti-RNA pol IV or ant-RNA pol V. As secondary antibodies donkey anti-rabbit IgG conjugated with Alexa 488 (Invitrogen), goat anti-rabbit IgG conjugated with Alexa 647 (Invitrogen). After being washed for 30 min with PBST cells were mounted with moviol (DAPI-DABCO). Images were taken in confocal microscope (Leica TCS SP5 AOBS TANDEM).

**Scanning electron microscopy**

Somatic and zygotic embryos were fixed in FAA solution and passed through increasing concentrations of ethanol (30%, 50%, 70%, 96%, 100% thrice [W/V[). The fixed embryos were dried in a Sandri-795 semi automatic critical point drying apparatus, mounted on stubs, coated with a 12 nm layer of gold in a Denton vacuum Desk II sputer coater, and visualized using a scanning electron microscope JEOL (6360 LV, Japan).

**Results**

The first subunit of pol IV from *Oryza sativa* has a distinct sequence at the amino terminus which is unique in the data base and was selected for antibody production. Western blots of both RNA polymerases subunits 1 were carried out. The highest pol IV amount was in esteem, while pol V expression was higher in the inflorescence tissue showing an additional band in inflorescence and esteem tissue. Protein expression correlated well with semi-quantitative RT-PCR (figure 1c and 1d). Somatic embryogenesis requires a high level of nuclear reorganization therefore we tested if pol IV or pol V increased after 2-4D as source of auxin induction. We evaluated up to 30 days after induction of the scutellum. Protein extracts were carried out at different times and large amounts of both polymerases were found as would be expected from chromosomal rearrangement particular after 15 days (figure 2d). We therefore decided to check if this was also the case when compared to zygotic embryos. We extracted embryos 0- 36 hours after hydration as seen in figure 3 which triplicate in size after 36 hours after hydration. Repeated western analysis showed a lack of expression of both RNA polymerase IV and V in any of the early stages of zygotic development (figure 3d). Inmunolocalization of both polymerases wascarried out in somatic embryo slides as seen in figure 4. Pol IV is mainly located in the nucleus of somatic embryos but not in the nucleus of somatic nucleated cells. Figure 4f and 4I shows pol V speckle pattern in the nucleus of cell types.

**Discussion**

Pol IV and V are known to be key players in gene silencing either in RNAi production or IGS methylation. The observation that mutant pol IV cells had an alter nuclear estructure led to their involment in DNA methylation. Their study in A. thaliana has shown particular exprestion in the root and inflorescence while our result are similar for inflorescence that suggest the need of gene silencing and proper nuclear structure at the early stages of gametogenesis, meristem formation and cell differentiation. 2,4-D is widely used as a synthetic auxin that can induce organogeneesi or somatic embryos (Mariani et al. 1998). The analisis of early somatic embryo provided a good source of tissue with large amount of cells that were in the processes of differentiation. Western blot data from figure 2 correlated well with inmunolocalization seen in figure 4.

Our inmunolocalization correlate well with the known data from inmunolocalizatons in *Arabidopsis* as well as with the western blot profile seen in figure 1. The speckle pattern observed from large nucleated cells of *Oryza* somatic embryos from RNA Pol IV (figure 4) are similar to that observed in *Arabidopsis* (Pontes et al. 2005) in particular in somatic embryos with large nuclear compartments were most of the silencing and chromosome reorganization need to take place. However, periferial nucleated cells show a large nuclear exclusion of pol IV but not of pol V which always showed a nuclear pattern. Our tissue slides allowed cytoplasmic and tissues postion of the cells, unlike previous nuclei extraction for inmunolocalization (Pontes et al. 2005; He et al. 2009; Pontes et al. 2009) which may render higher resolution but lack the information from whole cells in the were is mainly in inflorescence and roots but not in esteem as in *Oryza sativa* tissue (Ondera et al. 2005; Havecker et al. 2010). Moreover, early stages of somatic embryos were used and tested for the expression of RNA pol IV and V. Expression increased during the formation of the somatic embryos as one would expect for nuclear reorganization in particular after the first stages. However to our surprise zygotic embryos did not showed any expression of either polymerase (figure 3). These are significant differences that may involve different cellular strategies to generate the new plants and how high levels of synthetic auxin hormones like 2-4D can affect the silencing machinery. It will be important in the future to define which regions of DNA required silencing during the reorganization during cell differentiation and how is the process control to generate of a new expression profiles.

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**Figure legends**

Figure 1. Tissue expression of RNA pol IV and V in Oriza sativa. a) Coomasie stain of protein extracts from adult plant tissues. Inflorescence (I), Leaf (L) and esteem (E) b) Western blot from the same selected tissues. c ) RT-PCR from tissues expressing (Inflorescence and esteem) RNA pol IV subunit 1 and d) for RT-PCR for RNA pol V subunit 1.

Figure 2. Somatic embryo induction in Oriza sativa. a) Electron microscopy from 5, 10, 15 and 30 days respectively. b) Histological sections from somatic embryos from 5, 10, 15 and 30 days respectively. c) Stereoscopy visualization of the somatic embryos from 5, 10, 15 and 30 days respectively. d) Western blot of the extracts from somatic embryos with anti RNA pol IV or anti RNA pol V for the written time period of the figure. Esteem extract was used as control in lane E.

Figure 3. Zygotic Oriza sativa embryos, a) whole seeds after 0, 12, 24 and 36 hours of hydration. b) stereoscopy visualization of dissected embryos used for extract analysis after 0,12, 24 and 36 hours. c) Western blot of the zygotic embryo extract from the written time period with either anti RNA pol IV or anti RNA pol V. Esteem extract was used as control in lane E.

Figure 4. Inmunolocalization of RNA pol IV and V. in somatic embryos a) Inmunolocalization of RNA pol IV in somatic embryos showing contrast to enhance visualization of the nucleus, b) Localization of RNA pol IV in somatic embryos is shown in green over DAPI stain cells. c) Somatic nucleated cells either with visual contrast e and g or with red label RNA pol V over DAPI stain cells f and h.