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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 interaction of several factors that control DNA silencing of intergenic regions and siRNA production. DNA silencing is an important process during cell differentiation, nuclear structure and viral control. However, RNA pol IV and V are yet to be study in model monocot systems like *Oryza sativa* that can provide further information on genetic silence mechanism in plats. We show the expression pattern of these polymerases in tissues extracts of *Oryza sativa*. Detectable amounts of these polymerases are found in specific adult plant tissues and particularly expressed during somatic embryogenesis but not during early stages of normal embryo development. The use of synthetic auxin leads to an induction of both RNA pol IV and V in scutellum tissue where nuclear localization may be required for genome reorganization and gene silencing.

Keywords: *Oryza sativa*, RNA pol IV, RNA pol V, somatic embryogenesis, zygotic embryogenesis

INTRODUCTION

Gene silencing has become an important field of research in recent years. During the last decade, the emerging view of the RNA-directed DNA methylation (RdDM) pathway was defined (Herr et al. 2005; Onodera et al. 2005; Matzke et al. 2009; Law et al. 2010). The study of RNA polymerase IV and V have become highlighted within the field as being mayor players for 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 processed by RNA-dependent RNA polymerase 2 and dicer-like 3 to produce a 24 nt siRNAs. Methylation on their 3' ends is carried out by HUA ENHANCER 1 (Li et al. 2005), followed by binding to Argonaute 4 which is an effector protein for silencing (Matzke et al. 2009; Wierzbicki et al. 2009; Durán-Figueroa and Vielle-Calzada, 2010; Law et al. 2010; Olmedo-Monfil et al. 2010). In addition to the generation of 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 how they work during cell differentiation (Havecker et al. 2010; Olmedo-Monfil et al. 2010). Currently must of the research done to understand the mechanism underling these polymerases have been carried out in

Arabidopsis thaliana. However, the study in other plant models like *Oryza sativa* remains largely uncharacterized. Monocots and dicots diverged long ago and the only studies of pol IV and V in monocots have been done in *Zea mays,* where it already showed clues about how paramutation takes place (Pikaard and Tucker, 2009). Our results show high amounts pol IV expressed in stem and pol V predominantly on stem and inflorescence. Surprisingly very low level of pol IV or V are detected 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 maintained 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 were sterilized with 70% ethanol for 5 min, followed by 50% chlorine treatment for 1 hr and 3 washes with water. The seeds were incubated according to the set time in the legend before extraction was carried out. As previously publish by Mariani et al. 1998, the scutellum was obtained after 3 days, by dissecting the embryos and transferring them 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}) incubations were carried out in the dark room for the number of days as stated in the legends.

Antibodies

The peptide sequence selected for antibody production was obtained by comparing possible structure similarities between RNA pol II and IV, and looking for exposed domains. Surface sequence highlighted the peptide sequence INPLCCDPFKGDFDGDCLHG (432-451) for RNA pol V subunit I (NCBI Reference Sequence: NP_914279.1). For pol IV subunit I, the unique sequence chosen was MAVRPNDSRPSDVRPPFSQI (1-20) NCBI reference sequence: XP_473570.1. 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-González et al. 2007). Protein content was measured by Bradford assay and extracts were normalized by protein content.

Western blot analysis

Equivalent amount of total proteins from different tissues were separated on an 8% SDS-PAGE and transferred to nitrocellulose membrane (Pall Corporation, USA). After blocking 1 hr with 5% non-fat milk in TBST (TBS, 0.1% Tween-20), the membrane was incubated with either anti-RNA pol IV or V 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 Labelling Reagents (Amersham).

RT-PCR

The PCR reactions were with PIVF; GGT ATA TGG GAG AAC ATC ATT and 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 for actin AcF; atg gct gac ggc gag gac at and ActR atc ttc tcc atg tca tcc ca. Which amplify a 200 bp sequence of actin (GenBank: AY212324.1).

Total RNA was extracted using the RNeasy plant mini kit (Qiagen). Reverse transcription reactions were performed using 2 µg of RNA, 200 U of M-MLV, 0.4 µM oligo dT primer and 2.5 µM dNTPs for 1 hr at 37ºC. The PCR reactions were with PIVF; GGT ATA TGG GAG AAC ATC ATT and 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 for actin AcF atg gct gac ggc gag gac at and ActR atc ttc tcc atg tca tcc ca. Which amplify a 200 bp sequence of actin (GenBank: AY212324.1). All primers were used at 0.4 µM each, Taq platinum DNA polymerase 2.5 U (Invitrogen), $MgCl_2$ 0.5 mM, 200 nM each dNTP. Cycling conditions were 94ºC 2 min 1 cycle; 94ºC 30 sec, 45ºC 30 sec and 72ºC 1 min for the indicated number of cycles. The PCR products were separated on 1% agarose gel.

Histology

Samples of representative somatic and zygotic embryos from all the stages were removed from culture or seeds respectively and fixed in a FAA: solution [formaldehyde (10%) ethanol (50%) and acetic acid glacial (5%)] for 48 hrs at room temperature, progressively dehydrated in an alcohol series, and infiltrated with paraffin (Kendall). Sections (5 µm) were cut using a Leica microtome and stained with a 0.5% (w/v) aqueous solution of Toluidine Blue O (TBO) (Sigma). Sections were examined with an Axioplan microscope coupled to a camera.

Immunofluorescence

The somatic embryos after 15 days of induction were fixed in tubes containing FAA with aspiration for 24 hrs. They were dehydrated through an ethyl alcohol series and embedded in paraffin (melting point 54-56ºC) with a graded series of tertiary butyl alcohol. The paraffin blocks were sectioned serially at 5 µm thickness using a microtome. Removal of paraffin was carried out as follows 4x washed with histology grade xylene for 2 min, removal of xylene with absolute ethanol followed by 70% ethanol and then water for 1 min each. Somatic embryos and surrounding tissue were permeabilized with 0.1% Triton X-100 in PBS for 15 min, respectively. Block with 1% antigoat serum in PBST and wash with PBST. Overnight incubation with either anti-RNA pol IV or ant-RNA pol V was followed by PBST washes and 2 hrs incubation with secondary antibodies donkey anti-rabbit IgG conjugated with Alexa 488 (Invitrogen) for studies with pol IV, goat anti-rabbit IgG conjugated with Alexa 647 (Invitrogen) for pol V work. The excess of antibodies were washed for 30 min with PBST two times. The cells were mounted with moviol (DAPI-DABCO). Images were taken in confocal microscope (Leica TCS SP5 AOBS TANDEM).

RESULTS

Expression of RNA polymerase IV and V in adult tissues

The first subunit of pol IV from *Oryza sativa* (NCBI Reference Sequence: XP_473570.1.) has a distinct sequence at the amino terminus which is unique in the data base and was selected for antibody production. For the largest subunit of pol V (NCBI Reference Sequence: NP_914279.1). We compared the surface region with the largest subunit of pol II which bears sequence similarity with this subunit of pol V. Alignments of both polymerases were made and conserved regions with similar secondary structure predictions were used to define possible exposed regions for the synthesis of the immunogenic peptide used. Western blots of both RNA polymerases subunits 1 were carried out and clean signals were obtained at appropriate molecular weights corresponding to 186 kDa for pol IV and 167 kDa for pol V. The highest detection of pol IV was in stem, while pol V was immunodetected in stem and inflorescence extracts. Protein expression correlated well with semi-quantitative RT-PCR (Figure 1c and 1d), the integrity of cDNA was check by RT-PCR for actin expression for every tissue extraction. The expression of actin is higher therefore lower number of PCR cycles were used. Anti-pol V antibody immunodetected an additional band in inflorescence extract as compared to the single polypeptide stained in stem extract. The second band was slightly larger in mass appearing just below the 180 kDa.

RNA pol IV and V induced after auxin addition in scutellum

Since inflorescence tissue contains a large amount of tissue in different stages of differentiation we decided to test for the expression of these polymerases during the earlier stages of somatic embryo induction. Somatic embryogenesis requires a high level of nuclear reorganization therefore we tested if pol IV or pol V increased after adding 2-4D as source of auxin induction. We evaluated up to 30 days

after induction when large sections of the scutellum showed somatic embryos and calli as seen in Figure 2a. We check the formation of somatic embryos by histological sectioning the tissues, followed by staining with Toluidine Blue O a metachromatic dye used for examining general cell reorganization. After 15 days several structures are generated from the scutellum and after 30 days many of them can be seen independent of the mother tissue as normally seen in somatic embryos of other species (Figure 2b).

Protein extracts were carried out at different times and large amounts of both polymerases were immunodetected as would be expected from chromosomal rearrangement, particular after 15 days (Figure 2d). Extracts from different days were normalized by total protein content before loading.

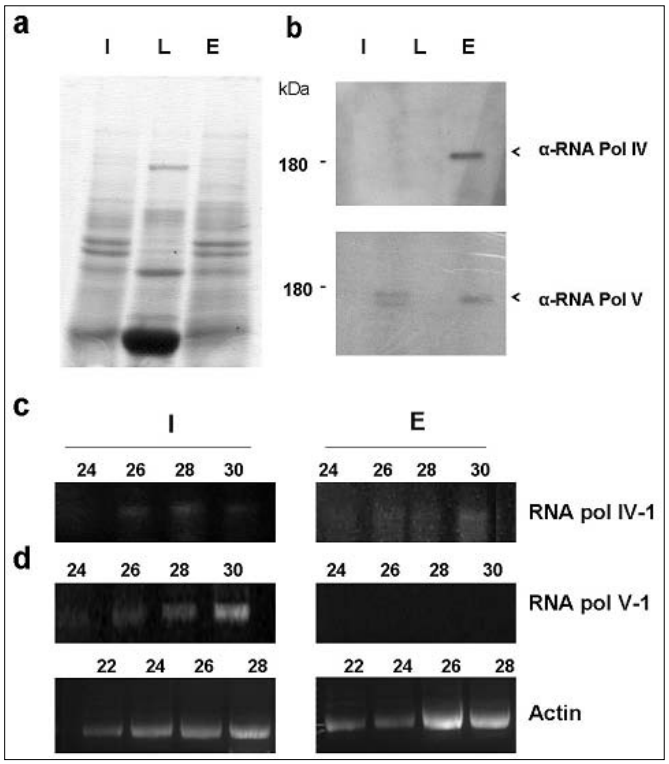

Fig. 1 Tissue expression of pol IV and V in *Oryza sativa*. (a) Coomassie stain of protein extracts from adult plant tissues. Inflorescence (I), leaf (L) and stem (E). (b) Western blot from the same selected tissues show pol IV at the expected 186 kDa and pol V at 167 kDa. (c) RT-PCR from tissues expressing (inflorescence and stem) pol IV subunit 1 and (d) for RT-PCR for pol V subunit 1. RT-PCR of actin from each cDNA extraction was used for control of cDNA production.

RNA pol IV and V are not detected during germination of zygotic embryos

Taking into account the somatic embryo results we check if this was also true during the first stages of zygotic embryo germination. Extracted embryos from 0, 12, 24 and 36 hrs after hydration as seen in Figure 3a and 3b triplicate in size after 36 hrs after hydration. Dissected embryos were used for immunolocalization. Figure 3c shows the dissected embryo cells, nucleus and two different sections of the same block were used for immunostained with either anti pol IV or anti pol V. The experiment was repeated after 36 hrs where the embryo has clearly generated new cell types and defined the radicle and plumule as seen in Figure 3d from which a faint signal was obtained for pol V. 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 3e).

Nuclear localization of RNA pol IV and V in cells of somatic embryos

Immunolocalization of both polymerases was carried 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 were it appears in the cytoplasm in a non diffuse manner. Figure 4f and 4I shows pol V speckle pattern in the nucleus of all cell types observed. Figure 4 (a,c,e,g) show only the DAPI channel to visualize DNA clearly, DAPI also weakly interacts with the cell walls of the cells giving a slide edge of the cells. These results were obtained by confocal microscopy to ensure the signal observed was in the same plane between DNA and the polymerases. Pol IV appears to be intermingling with large portions of the DNA while pol V shows a nuclear speckle pattern suggesting specific locations of action as is the case of pol IIo.

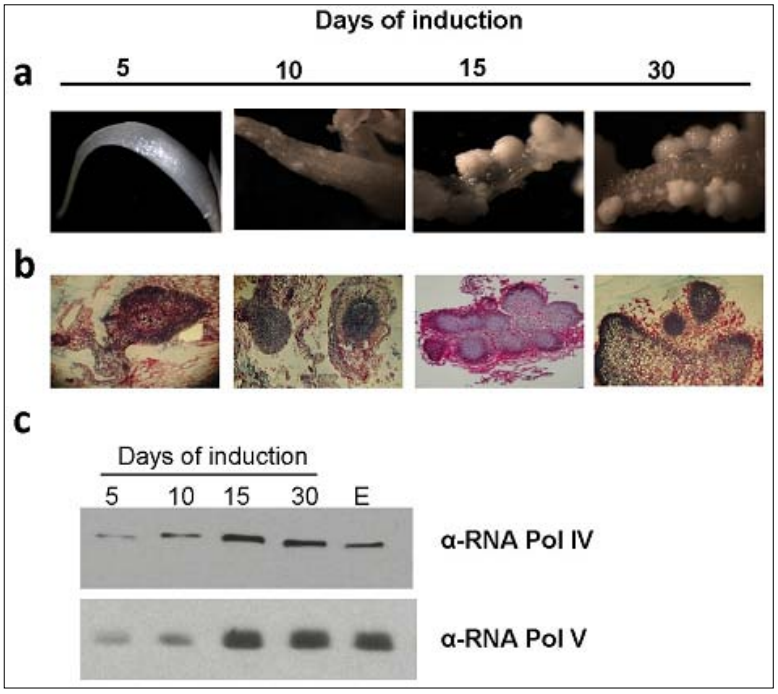

Fig. 2 Somatic embryo induction in *Oryza sativa*. (a) Stereoscopy visualization of the somatic embryos from 5, 10, 15 and 30 days respectively. (b) Histological sections from somatic embryos from 5, 10, 15 and 30 days respectively. (c) Western blot of the extracts from somatic embryos with anti pol IV or anti pol V as stated in the figure. Stem extract was used as control in lane 5.

DISCUSSION

Pol IV and V are known to be key players in gene silencing either in siRNA production or IGS methylation. The observation that mutant pol IV *A. thaliana* cells had an altered nuclear structure led to their involvement in DNA methylation (Onodera et al. 2005). Their study in *A. thaliana* has shown particular expression in root and inflorescence tissue while our result are similar for the inflorescence, suggesting the need of gene silencing for proper nuclear restructure at the early stages of gametogenesis, meristem formation and cell differentiation. The inflorescence extracts from rice also showed an additional band of higher molecular mass than expected, and was also seen in a time dependent manner after auxin induction. Three possible scenarios are likely to be the case: (1) the expression of an alternate transcription start site or splicing that produces a larger form of pol V. (2) a posttranslational modification occurs in this tissue like pol IIa phosphorylation conversion to pol IIo that shows a large change in mobility upon phosphorylation (Castaño et al. 2000); (3) the expression of another protein expressed in inflorescence tissue with an epitope that is recognize by the antibody. It will be interesting in the future to elucidate its identity and define its biological role in rice plants.

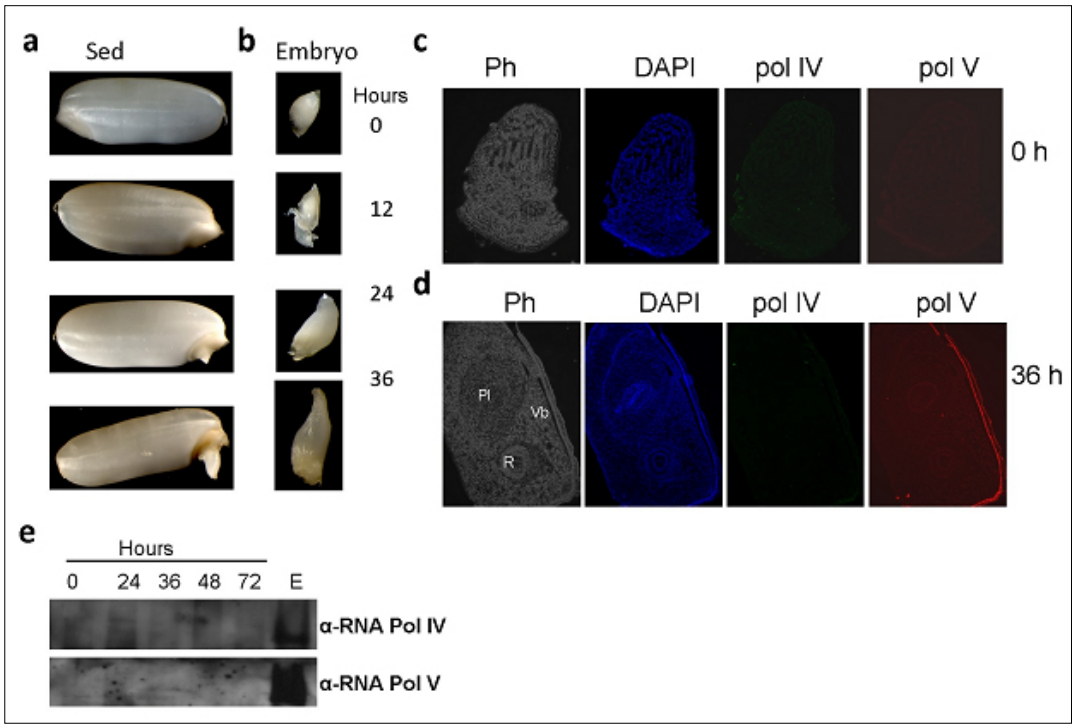

Fig. 3 Zygotic *Oryza sativa* embryos. (a) Whole seeds after 0, 12, 24 and 36 hrs of hydration. (b) Stereoscopy visualization of dissected embryos used for extract analysis after 0, 12, 24 and 36 hrs. (c) Histological section of zygotic embryo before hydration showing either phase contrast, DAPI stain or immunostained against pol IV and V. (d) Histological section of zygotic embryo after 36 of hydration showing either phase contrast, DAPI stain or immunostained against pol IV and V. Plumule (Pl), radicle (R) and Vascular bundle are shown. (e) Western blot of the zygotic embryo extract from the written time period with either anti pol IV or anti pol V. Stem extract was used as control in lane 6.

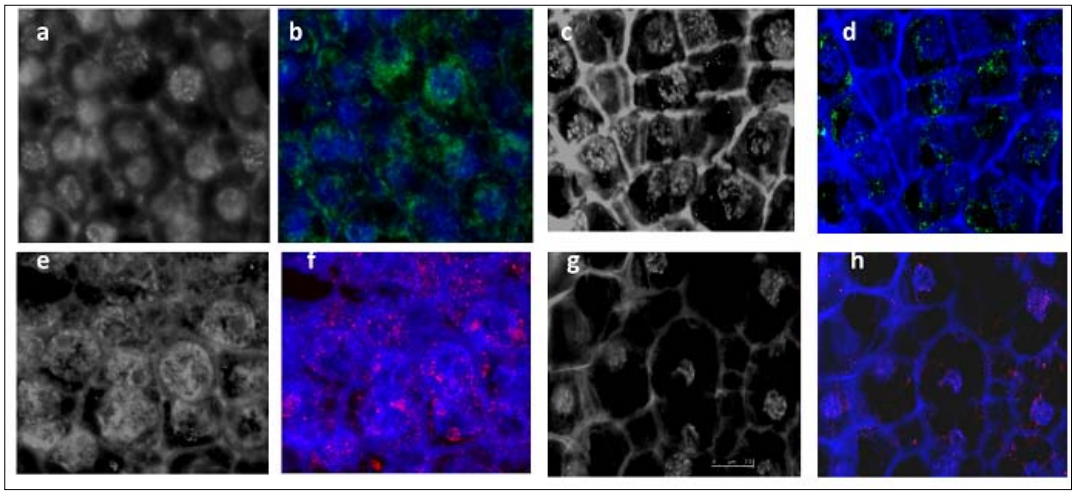

Fig. 4 Immunolocalization of RNA pol IV and V in somatic embryos. (a), (c), (e) and (g) show only the DAPI channel to observe the nucleus. Localization of pol IV is shown in green over DAPI stain in (b) somatic embryos cells and (d) somatic nucleated cells. Localization of pol V is shown in red over DAPI stain in (f) somatic embryos cells and (h) somatic nucleated cells.

The main difference from previous reports in *Arabidopsis* is the expression seen in stem tissue Figure 1 as compared to the lack of expression in *A. thaliana* (Onodera et al. 2005). One possibility is the difference in type of tissue. The stem in *Arabidopsis* is mainly a structural component, while in rice it contains adventitious root primordial at the inter nodes of the stem, usually covered by the epidermis (Steffens and Sauter, 2005). These internodes maintain large populations of meristematic root cells which may require the expression of these two polymerases during cell differentiation.

We check the expression of pol IV and V during dedifferentiation by the addition of 2, 4-D a widely used synthetic hormone that can induce organogenesis or somatic embryos in rice and other plants (Mariani et al. 1998). The analysis 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 seen in Figure 2 correlated well with immunolocalizations Figure 4, were large amount of pol IV and V are observed.

The pattern observed from large nucleated cells of *Oryza* somatic embryos from RNA pol IV (Figure 4) are similar to that observed in *Arabidopsis* (Onodera et al. 2005) in particular in somatic embryos with large nucleus were most of the silencing and chromosome reorganization need to take place. However, peripheral nucleated cells show a large portion of nuclear exclusion of pol IV but not of pol V which always showed a speckle nuclear pattern. Speckles are typical areas of large quantity of protein, either in a reserve zone or at functional sites. Our tissue slides allowed us to view whole cells and their location in the tissue, unlike previously publish nuclei extraction for immunolocalization (Onodera et al. 2005; He et al. 2009; Pontes et al. 2009) which may provide higher nuclear resolution but lack information from whole cells. The excess of pol IV in early somatic cells may indicate that it is targeted for degradation after differentiation while centre cells maintain a non differentiated state. Pol V may remain active in the nucleus during these stages to methylate intergenic sequences, transposons or viral sequences from expressing at any time. The early stages of somatic embryos tested for the expression of RNA pol IV and V show an increase during the first 15 days followed by a decline after 30 days induction. This may be due to nuclear reorganization and re-methylation of the DNA during the formation or stimulation of competent cells. However to our surprise zygotic embryos did not showed significant expression of either polymerase (Figure 3). These are significant differences which may be due to the actual number of cells requiring DNA silencing in early stages of germination from zygotic embryos. Compared to somatic embryogenesis where large number of embryos are generated from several competent cells, with little amount of somatic cells that may dilute the signal (Figure 2). Zygotic embryonic after 36 hrs show several new cells types and structures but only provide an immunolocalization signal with our antibodies for pol V in specific cells of the radicle and plumule. The bright stained line surrounding the embryo appears to be resin that was not removed more than a specific pol V signal. In the future it will be important to define which regions of DNA required silencing during the reorganization and cell differentiation, and how is the process control to generate of a new expression profiles during the early stages of embryo formation rather than of embryo development.

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