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Expression of modified 7SL RNA gene in transgenic *Solanum tuberosum* plants

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Abstract

A modified plant 7SL RNA gene from *Arabidopsis thaliana* designated AHIIA63M was introduced into potato plants *via Agrobacterium*-mediated transformation. No transgenic plants could be obtained using pGPTV-based binary vectors where AHIIA63M gene driven by polIII promoter was located close to the polII promoter of the selection gene. Special binary vectors with matrix attachment region (MAR) elements had to be used for transformation to insulate polII and polIII promoters within T-DNA. The level of AHIIA63M RNA in transgenic plants was lower than the levels of transcripts of transgenes driven by RNA polymerase II. The level of AHIIA63M transcript in transgenic potato plants was tissue specific. The highest expression was detected in roots and gynoecium and the lowest in tubers. Moreover, non-specific promoter activity within the MAR element was revealed. This activity contributed to AHIIA63M transcription. This is the first report of expression of a modified 7SL RNA gene in transgenic plants and promoter activity within the MAR element.

Additional key words: RNA polymerase III, polIII expression cassette, MAR element.

Introduction

7SL RNA is an abundant molecule that forms an RNA component (Walter and Blobel 1982) of the signal recognition particle (SRP) (for review see Lütcke 1995). There are few molecular forms of 7SL RNA in animals. Four genes and about 500 pseudogenes have been detected in humans (Ullu and Weiner 1984). On the other hand, multiple molecular forms of 7SL RNA genes have been discovered in plants (Campos *et al.* 1989, Heard *et al.* 1995, Riedel *et al.* 1995, Matoušek *et al.* 1999).

Potato spindle tuber viroid (PSTVd) is a species of viroids from the family *Pospiviroidae*. Viroids are the smallest known plant parasites. A single circular RNA molecule 246 - 401 nt in length is the only component of each viroid (for recent review see Flores *et al.* 2004). Viroids do not code for any proteins and due to the intramolecular base pairing form a rod-like secondary structure (Gross *et al.* 1978). The replication of PSTVd in the nucleus of host plant cells is catalyzed by host DNA dependent RNA polymerase II (Schindler and Mühlbach 1992). Metastable secondary structures are formed during replication. One of these structures denoted hairpin II (HII) is supposed to serve as a promoter element for initiation of plus strand synthesis on the minus replication intermediate (Loss *et al.* 1991).

CaMV 35S promoter-driven antisense RNA systems targeted against PSTVd were used in our laboratory in the early nineties (Matoušek *et al.* 1994), but they caused transcriptional gene silencing of antisense transgenes.

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Abbreviations: A element - chicken lysozyme A element; GUS - β-glucuronidase; MAR - matrix attachment region; NPTII - neomycin phosphotransferase II; P-35S - cauliflower mosaic virus 35S promoter; P-nos - nopaline synthase promoter; polII - RNA polymerase II; polIII - RNA polymerase III; SRP - signal recognition particle.

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This phenomenon is supposed to be caused by RNA directed *de novo* methylation of DNA described by Wassenegger *et al.* (1994). Cassettes for expression of antisense RNA based on promoters recognized by RNA polymerase III were designed in our lab recently (Matoušek *et al.* 2000) as an alternative to RNA polymerase II-driven antisense RNA genes. These polIII transcribed cassettes should be less sensitive to transcriptional silencing due to DNA methylation. Moreover, polIII genes are present in the genome in multiple copies of homologous or analogous sequences (Matoušek *et al.* 1999) and their transcripts are usually highly abundant in the cell (Poritz *et al.* 1988). This fact could decrease the probability of co-suppression caused by the presence of multiple gene copies or by the overproduction of homologous RNA gene products (Meyer 1995).

Matrix attachment region (MAR) elements are regions in chromosomal DNA that bind to the nuclear framework. They are believed to serve as border sequences of chromatin loops and thus insulate regulatory domains (Hall *et al.* 1991, Laemmli *et al.* 1992, Bode *et al.* 1996, Paul and Ferl 1998). Flanking of constructs for transformation by MAR elements has been shown to increase expression of transgenes in both animal (Stief *et al.* 1989, Poljak *et al.* 1994) and plant systems (Mlynárová *et al.* 1994, Allen *et al.* 1996, Liu and Tabe 1998, Ülker *et al.* 1999, Petersen *et al.* 2002, Brouwer *et al.* 2002, Fukuda and Nishikawa 2003). Some publications also report position independent expression of transgenes flanked by MAR elements in animals (Stief *et al.* 1989, Bonifer *et al.* 1990, Namciu *et al.* 1998) and plants (Mlynárová *et al.* 1994, 1995, 1996, Petersen *et al.* 2002). These reports indicate that certain MAR elements probably insulate transgenes from the effect of surrounding chromatin. One of the most commonly used MAR elements for these studies (Stief *et al.* 1989, Bonifer *et al.* 1990, Mlynárová *et al.* 1994, 1995, 1996) is the 5' MAR element of the chicken lysozyme gene locus (Phi-Van and Strätling 1988), called the A element. Similar to most MAR elements, the A element is AT-rich (Phi-Van and Strätling 1988). Thus, inserting the modified polIII genes into the A element could produce sequence proximity analogous to AT-rich genomic areas where these genes are often located (Matoušek *et al.* 1999).

The construction and *in vitro* transcription of the modified 7SL RNA gene in HeLa cell extract has been published previously (Matoušek *et al.* 2000). Here, we describe the expression of a modified 7SL RNA gene from *Arabidopsis thaliana* fused to an antisense RNA sequence targeted against hairpin II of PSTVd in transgenic potatoe plants.

Materials and methods

Preparation of vectors: Preparation of AHIIA63M (Fig. 1) has been previously described (Matoušek *et al.* 2000). *In vitro* transcription of AHIIA63M in plant extracts was performed as described by Yukawa *et al.* (2002).

The first group of plant transformation vectors was prepared by inserting AHIIA63M into a *Hind*III site of either modified pGPTV-KAN or pGPTV-HPT vectors (Becker *et al.* 1992) with P-35S inserted in front of the *uidA* coding sequence (Fig. 1). Vector pGPTVL was prepared from pGPTV by deleting the *NPTII* and *GUS* genes using *Bam*HI and *Eco*RI and inserting multiple cloning sites instead. AHIIA63M was cloned into a unique *Hind*III site within this polylinker (Fig. 1).

MAR vectors were based on the pLV-06 vector (Mlynárová *et al.* 2002) - a derivative of the binary vector pBinPLUS (Van Engelen *et al.* 1995). The *NPTII* coding sequence, under the control of the nopaline synthase promoter and terminator, was cloned into the *Nhe*I and *Xho*I sites of pLV-06 resulting in pLV-07. The *Eco*RI site of pLV-07 was disrupted by cutting, blunt-ending and religating; and the A element from pLM-04* was inserted into the *Kpn*I and *Xba*I sites resulting in pLV-10. pLM-04* contains a blunt-ended 2.95 kbp *Bam*HI - *Xba*I fragment of the chicken lysozyme A element (Phi-Van and Strätling 1988) inserted in the *Sma*I site of pUC19 (Mlynárová *et al.* 1995). The AHIIA63M cassette was blunt-end cloned into the *Eco*RI site of the A element within pLV-10 resulting in pLV-17 (Fig. 1). The pLV-16 vector was constructed similarly, utilizing a modified $tRNA^{Tyr}$ gene (Fig. 1), called pNtY1-aHPII as described earlier (Yukawa *et al.* 2002), instead of AHIIA63M.

Constructs pLV-33 and pLV-34 were also derived from pLV-07. The first the A element was inserted by blunt-end ligation into a *Sca*I site near the left border and the second one was inserted into the *Nae*I site near the right border resulting in the universal MAR vector pLV-25. GUS-AHII or -SHII fusion genes were cloned into the *Kpn*I and *Xba*I sites of pLV-25, resulting in pLV-33 (Fig. 1) or pLV-34, respectively. GUS-AHII and GUS-SHII fusion genes were prepared by inserting the synthetic AHII sequence
CCTACTCGCTCCCTTTGCGCTGTCGCT
in both orientations into an *Ecl*136II site located between the *uidA* coding sequence and the *nos* terminator of the P-35S-driven GUS marker gene in the pUC18 plasmid lacking the *Sac*I site.

Engineered binary vectors were introduced into the *Agrobacterium tumefaciens* strains EHA105 (pGPTV-based vectors) or LBA4404 (MAR vectors), respectively. The construct AHIIA63M in pGPTVL for co-transformation was introduced into the *A. tumefaciens* strain A4-24 (C58C1 bearing pRiA4).

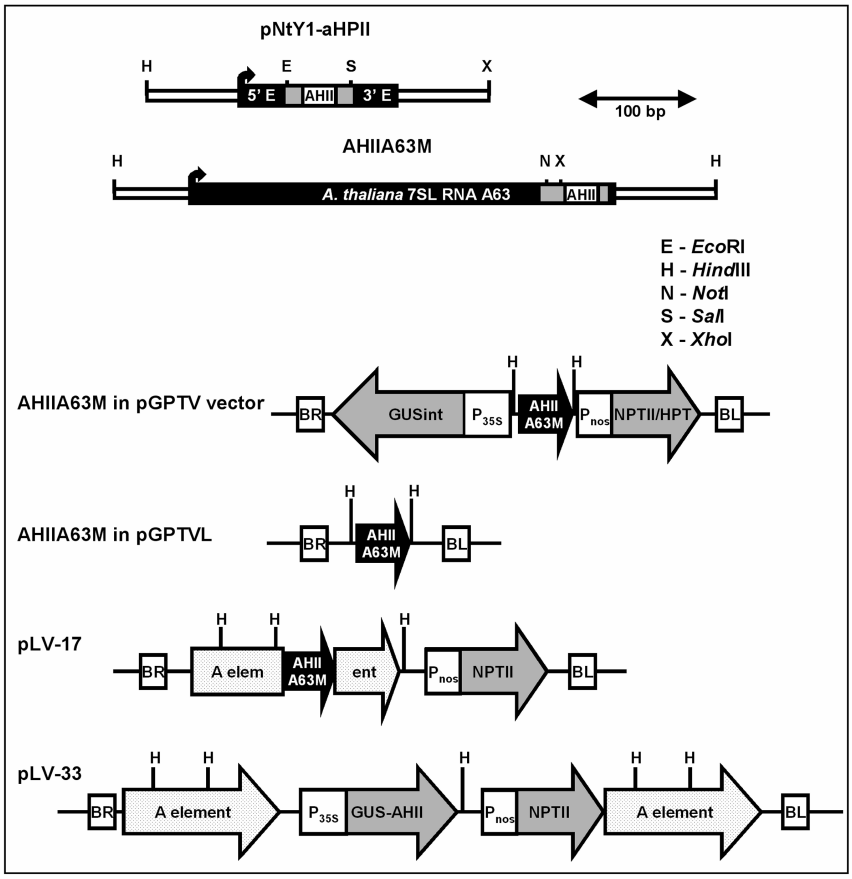

Fig. 1. Schematic drawing of polIII expression cassettes and plant transformation vectors. Upper part - modified $tRNA^{Tyr}$ (pNtY1-aHPII) and 7SL RNA (AHIIA63M) genes with an anti-hairpin II antisense sequence inserted are drawn in scale. Transcription starts are marked with arrows, original transcribed sequences are in black, modified sequences are in gray, while antisense sequence and 5' and 3' nontranscribed regions are in white. Bottom part - T-DNA parts of plant transformation vectors derived from the pGPTV and MAR vectors, not drawn in scale. The polIII cassette is in black, the chicken lysozyme A element is dotted, reporter genes are in gray, polII promoters and T-DNA border sequences are in white. Locations of certain restriction sites are marked also.

Plants and transformation: *Solanum tuberosum* L. cv. Kamýk plants were grown on solid Murashige and Skoog (1962; MS) medium in glass jars illuminated with fluorescent tubes (16-h photoperiod, irradiance of 70 μmol m^{-2} s^{-1} (PAR), and temperature of 22 °C). Nodal cuttings were transferred to fresh medium every two months. Internodes and leaves of these *in vitro* grown potato plants were cut into 5 - 10 mm pieces in a Petri dish containing 20 cm^3 of half-strength liquid MSmedium without sugars, to which 0.1 cm^3 of overnight grown *Agrobacterium* culture was added. The explants were incubated overnight at room temperature. The following day, explants were transferred to MS medium with 0.02 mg dm^{-3} NAA, 0.02 mg dm^{-3} GA_3, 1.0 mg dm^{-3} zeatin, 200 mg dm^{-3} timentin (*Duchefa*, Haarlem, The Netherlands) and 100 mg dm^{-3} kanamycin or 15 mg dm^{-3} hygromycin B (for the pGPTV-HPT-based vector) and kept at 22 °C and 16-h photoperiod. The medium was changed every three weeks. When shoots started to regenerate, they were cut and incubated in MS medium with 200 mg dm^{-3} timentin and 100 mg dm^{-3} kanamycin (or 15 mg dm^{-3} hygromycin B). Chimerical hairy roots expressing AHIIA63M were prepared by the application of *Agrobacterium* culture A4-24, bearing construct AHIIA63M in pGPTVL, onto *in vitro* grown potato plants with their apexes cut off. Hairy roots that started to appear after two weeks were further grown on MS medium with 200 mg dm^{-3} timentin.

Nucleic acid isolation: DNA was extracted from *in vitro* grown plants ground in liquid nitrogen and homogenized in a buffer containing 0.7 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 % CTAB, 1 % merkaptoethanol for 90 min at 60 °C. The mixture was extracted once in a chloroform:isoamyl alcohol (24:1, v/v) mixture, precipitated with isopropanol, spooled onto a glass rod, washed in 80 % ethanol, 10 mM LiCl, 10 mM Tris-HCl, pH 7.5, dried and dissolved in water. RNA was isolated using RNeasy Plant Mini Kit (*Qiagen*, Hilden, Germany) according to the manufacturer's manual.

Southern blotting: 30 µg DNA samples were digested with *Hind*III, separated on 1 % agarose gel overnight at 2 V.cm^{-1}, transferred by alkaline blotting solution (0.6 M NaOH, 0.4 M NaCl) onto a positively charged nylon membrane with a pore size of 0.45 µm (*Sigma-Aldrich*, St. Louis, USA), fixed by UV cross-linking and baked at 80 °C for 20 min. Pre-hybridization and an overnight hybridization with a ^{32}P-labeled DNA probe was carried out in a buffer containing 0.5 M Na_2HPO_4 pH 7.2, 7 % SDS, 1 mM EDTA at 65 °C. Final washing was performed for 20 min in 0.5 × SSC, 0.1 % SDS at 50 °C. The probe was prepared by random prime labeling of a 1.2 kbp PCR fragment of the right part of the chicken lysozyme A element.

RT-PCR and semiquantitative RT-PCR: 2 µg of RNA samples were treated with RNase-free DNaseI (*Promega*, Madison, USA) at 37 °C for 30 min, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, dried and dissolved in RNase-free water. RT-PCR was performed using the Titan One Tube RT-PCR System (*Roche Diagnostics*, Mannheim, Germany). A reaction mixture prepared according to the manufacturer's manual was used, except that the concentration of primers was increased to 1.5 µM. 0.025 cm^3 reaction mixture including 1 µg of template RNA was used per sample. The following program was used for RT-PCR: reverse transcription at 50 °C for 30 min, denaturation at 94 °C for 2 min followed by 30 cycles of PCR: 94 °C - 20 s, 50 °C - 30 s, 68 °C - 40 s. PCR products were separated on 1.5 % agarose gels in 1× TBE, 4 V cm^{-1} and stained with ethidium bromide.

When semiquantitative RT-PCR was performed, the reaction mixture contained additional [α-^{32}P] dCTP as described by Novák *et al*. (2003), however the final amount of ^{32}P was increased to 1.37 × 10^{-13} mol (46.25 kBq) per 0.025 cm^3 reaction. 0.2 µg of the template RNA was used per 0.025 cm^3 reaction, and 28 - 33 cycles of PCR were performed according to the abundance of RNA detected. 0.01 cm^3 of PCR products were separated on 6 % PAA gels, 1× TBE, 10 V cm^{-1}. The intensity of bands was determined by scanning on a *STORM PhosphoImager* and quantified using *Image-Quant* software (*Molecular Dynamics*, Sunnyvale, USA).

The following primers were used for RT-PCR reactions: AHIIA63M - primers A63startRT (GTCGAGCTAAGTAACAGTAGCTTG) and SHII-RT (AGCGACAGCGCAAAGG); wild type 7SL RNA - primers α (TGTAACCCAAGTGGGGG) and anti-β (GCACCGGCCCGTTATCC); NPTII transcript - primers NPTII5' (CTGTCATCTCACCTTGCTCC) and NPTII3'-RT (ATAGCGGTCCGCCACAC); GUS-AHII fusion transcript - primers GUS-RT5' (CGGGCTGCACTCAATG) × SHII-RT; GUS-SHII fusion transcript - primers GUS-RT5' × AHII-RT (CCTACTCGCTCCCTTTGC); actin - primers ActPot5' (GGTATTGTGCTGGATTCTGG) and ActPot3' (TCCAGCAGCTTCCATTCC); A element - primers MAR-F (CTTTGATCCCAATGAAATCG) and MAR-R (CATTACATGGTATGAATAGGCGG).

Results

Modification of the *A. thaliana* 7SL RNA gene A63, resulting in a chimerical gene designated AHIIA63M, and its *in vitro* transcription in HeLa cell extract was published earlier (Matoušek *et al.* 2000). The transcription of A63M-based constructs in plant extracts from tobacco cells was also demonstrated earlier by Yukawa *et al.* (2002). To verify whether the particular construct, AHIIA63M (Fig 1), is also transcribed in plants, *in vitro* transcription of this gene in plant extract from tobacco cells was performed (Fig. 2). These experiments showed transcription levels comparable to the wild type sequences of the 7SL RNA gene Hl7SL-1 from hop used as a positive control (Fig. 2).

For potato transformation, an AHIIA63M expression cassette was cloned into both the pGPTV-KAN and pGPTV-HPT binary vectors (Fig 1). However, despite the high number of explants used, no potato regenerants with significant rooting were obtained using either kanamycin or hygromycin selection (Fig 3), suggesting that the transformation was inefficient. Similar results were obtained with other polIII expression cassettes based on A63 or pNtY1 (tRNA^{Tyr} gene from tobacco)

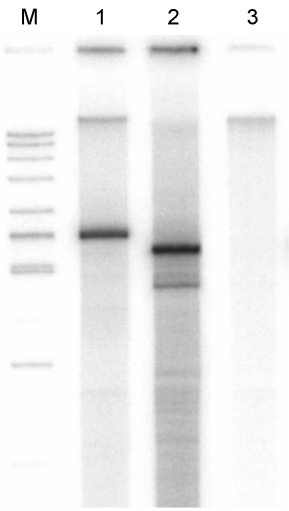

Fig. 2. *In vitro* transcription of AHIIA63M in plant extract prepared from tobacco. M - marker (*Hinc*II digested ΦX174); 1 - AHIIA63M; 2 - Hl7SL-1 - wild type 7SL RNA gene from hop (positive control); 3 - pBSKS+ - empty vector.

targeted against either PVS (potato virus S) or PSTVd (data not shown).

The AHIIA63M gene was cloned into a modified binary vector, pGPTVL, containing only T-DNA borders (Fig 1). This step eliminates a potential interaction of sequence elements within the AHIIA63M and pGPTV vector which could lead to low or no expression of the selection gene. The construct AHIIA63M in pGPTVL was introduced into a modified *A. tumefaciens* strain bearing the Ri plasmid pA4. This system allowed the expression of AHIIA63M *in vivo* using co-transformation. Chimerical hairy-roots were induced on *in vitro* grown potato plants using this *Agrobacterium* strain. The AHIIA63M specific transcript was unambiguously detected by RT-PCR, using RNA from these chimerical hairy-roots cultures (Fig. 4), suggesting expression of the modified 7SL RNA gene in transformed potato tissue.

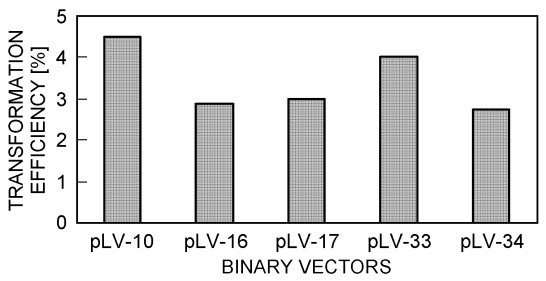

Fig. 3. Transformation efficiency of particular binary vectors. Percentage of explants transformed to vigorously rooting regenerated plants. Approximately 400 explants were used per variant. No rooting regenerants were obtained using pGPTV-KAN and pGPTV-HPT based binary vectors.

Furthermore, a new series of plant binary vectors for the expression of polIII-based antisense genes in intact transgenic plants were prepared. A MAR element, which is known as an expression stabilizer, was used to separate the polIII promoters of expression cassettes from the polII promoters of selection genes. In the first group of MAR vectors, the polIII cassette was cloned into an AT-rich A element. These vectors include pLV-10 (a control without a polIII cassette), pLV-16 with a modified $tRNA^{Tyr}$ and also pLV-17 with AHIIA63M (Fig. 1). Furthermore, vectors pLV-33 (Fig. 1) and pLV-34 were used as controls with our polII driven chimerical genes. These two vectors were based on pLV-25 - a plant binary vector with a multiple cloning site capable of blue-white selection and an *NPTII* selectable marker surrounded by two A elements. Vectors pLV-10, 16, 17, 33 and 34 were used for potato transformation. Satisfactory numbers of vigorously rooting plants were obtained after transformation (Fig 3).

Southern blots from selected transgenic clones revealed that the *Hind*III fragment of the A element, including the inserted polIII cassette, is intact. The bands

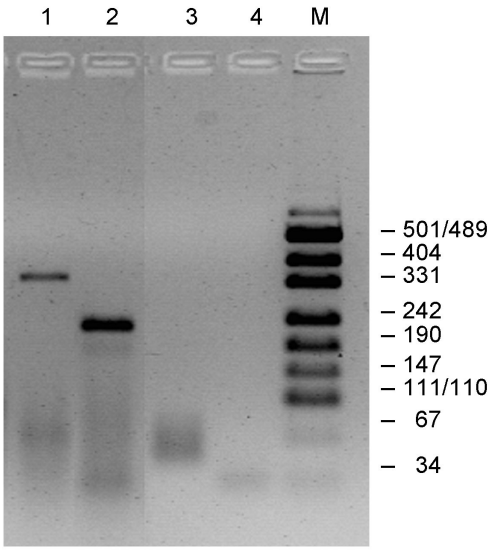

Fig. 4. An example of RT-PCR analysis of RNA isolated from chimerical hairy roots culture co-transformed with AHIIA63M. 1 - product with primers A63startRT × SHII-RT specific for AHIIA63M, 2 - positive control of RNA integrity with wild type 7SL RNA specific primers α and anti-β, 3 and 4 - negative PCR controls, M - marker (*Msp*I digested pUC19). Negative of ethidium bromide-stained agarose gel.

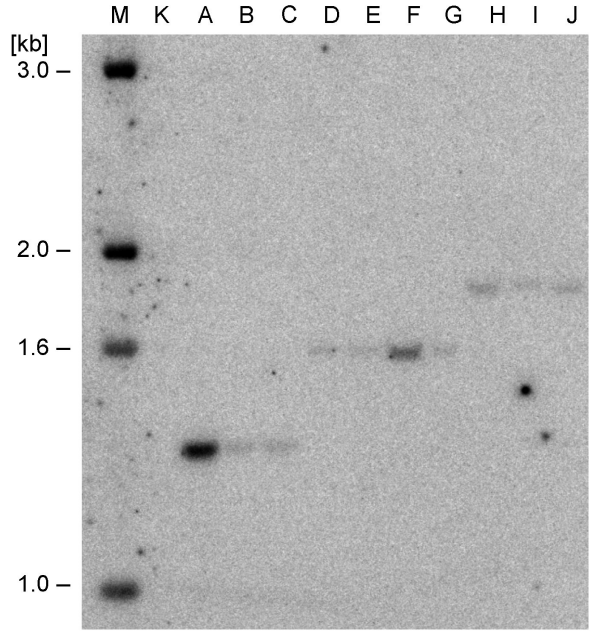

Fig. 5. Southern blot from plants transformed with MAR vectors. *Hind*III digested genomic DNA hybridized with a probe specific for the right part of the chicken lysozyme A element where polIII expression cassettes are inserted in vectors pLV-16 and pLV-17. M - 1 kb ladder, K - Kamýk (wild type control), A - C - clones transformed with pLV-10, D - G - clones transformed with pLV-16, H - J - clones transformed with pLV-17 (H - clone 2, I - clone 4).

of appropriate sizes appeared after hybridization with a probe specific for this part of the A element (Fig. 5). RT-PCR screening was used to select transformants that express the AHIIA63M transgene. Four plants, numbered 2, 3, 4 and 5, out of six independent transformants tested, were shown to contain AHIIA63M specific RNA, as could be judged from the presence or absence of an RT-PCR product (data not shown).

Northern blot analysis was performed using extracts from transformed plants to evaluate the level of expression. However, there was no AHIIA63M-specific signal on a Northern blot from Southern blot positive clones, suggesting a very low level of transcribed RNA (data not shown). Therefore, subsequent evaluations of the level of chimerical RNA were performed by semi-quantitative RT-PCR.

Transgenic clones 2 and 4 were chosen for further analysis. At first, the levels of AHIIA63M transcript were compared to the levels of the P-nos-driven *NPTII* transgene within the corresponding transformants. In addition, AHIIA63M transcript levels were compared to the RNA levels of the P-35S-driven GUS-AHII and GUS-SHII genes in transgenic potato clones transformed with pLV-33 and 34. The RNA samples were taken from leaves and stems of *in vitro* grown transgenic plants. The level of AHIIA63M RNA detected was 20 times lower than the level of *NPTII* mRNA and approximately 70 times lower than the levels of GUS-AHII and GUS-SHII fusion mRNA (Fig. 6*a*).

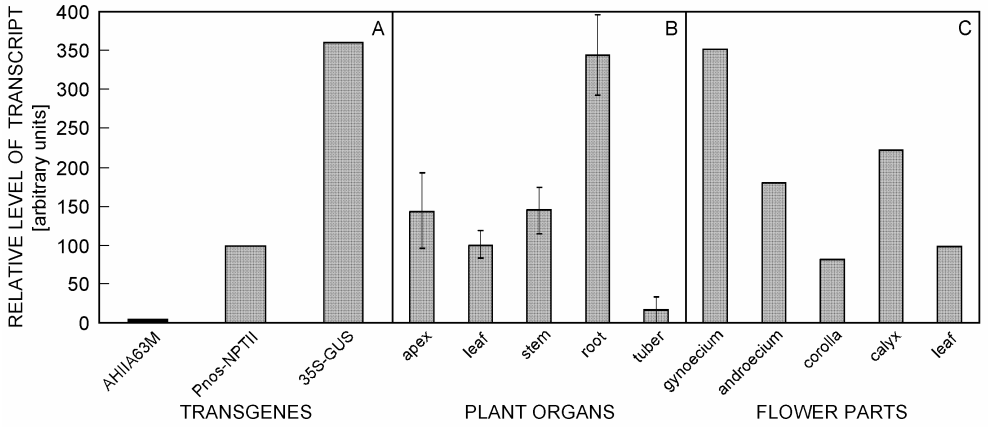

Fig. 6. The relative levels of RNA detected by semiquantitative RT-PCR. Comparison of different transgenes relative to P-nos-*NPTII* in a particular clone (*A*), comparison of AHIIA63M levels in plant organs - mean of three measurements from two independent transgenic clones 2 and 4 (*B*) and comparison of AHIIA63M levels in flower parts from transgenic clone 2 only (*C*).

The expression could differ depending on the types of plant cells and in particular tissues. Therefore, we evaluated transcript levels in apexes, leaves, stems, roots and tubers of transgenic plants. In these experiments, semiquantitative RT-PCR was performed using primers specific for AHIIA63M on RNA samples from different organs of clones 2 and 4 grown in a greenhouse. To assess the amounts of RNA in individual samples, RT-PCR reactions using the actin-specific primers ActPot5' and ActPot3' were performed in parallel as an internal control. The variability of the actin signal obtained from different tissues was within 10 % (data not shown). Differences of AHIIA63M transcript level varied up to 20 fold (Fig. 6*b*). The organ with the highest level of AHIIA63M detected were the roots, with more than 3 times higher levels of transcript relative to leaves (Fig. 6*b*). The levels of transcripts in apexes and stems were found to be only slightly higher than in leaves (143.2 and 144.3 % respectively, Fig. 6*b*). The lowest level of AHIIA63M transcript, reaching 16.1 % of that in leaves, was found in tubers (Fig. 6*b*).

Next, experiments were performed using RNA

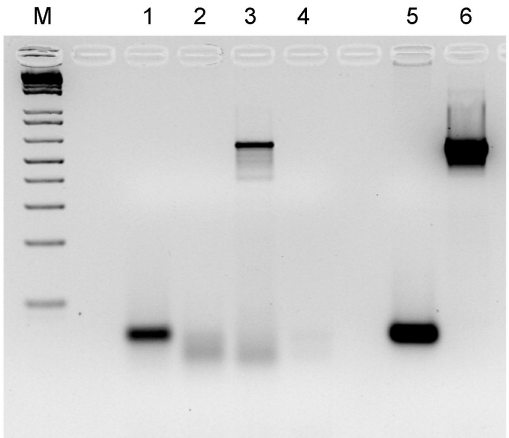

Fig. 7. RT-PCR with primers MAR-F and MAR-R specific for the part of the MAR element where the AHIIA63M gene was inserted. M - 1 kb ladder, 1 and 3 RT-PCR from roots of plants transformed with the MAR element alone (pLV-10) and MAR element with inserted AHIIA63M (pLV-17 clone 2) respectively, 2 and 4 negative PCR controls, 5 and 6 positive controls from plasmids pLV-10 and pLV-17 respectively. 35 cycles of PCR was performed. Negative of ethidium bromide-stained agarose gel.

samples from flower parts of clone 2 grown in a greenhouse. Again, actin was used as a control (data not shown). AHIIA63M RNA was most abundant in gynoecium - 353.7 % of that in leaves (Fig. 6*C*). The level of transcript in androecium and calyx was approximately 2-fold higher than that found in leaves, 180.6 and 223.2 % respectively. Finally, a level comparable to that in leaves (81.8 %, Fig. 6*C*) was found in corolla.

The specificity of the AHIIA63M transcript was tested by RT-PCR using A element-specific primers MAR-F and MAR-R located upstream and downstream of AHIIA63M. A PCR product of notional size was detected (Fig. 7). These primers produced a shorter product also when RNA from a plant transformed with the A element alone was used as a template (Fig. 7). According to additional two-step RT-PCR reactions (data not shown) the RNA detected was the sense strand, *i.e*., the same strand as the AHIIA63M-specific transcript. These results indicate that the transcription is directed not only from the 7SL RNA-specific promoter and terminator elements, but a certain level of transcription also results from the A element alone.

Discussion

No rooting transformants were obtained using pGPTV-based binary vectors for transfer of modified polIII genes into plants. PolIII expression cassettes were inserted just between two divergently oriented promoters for RNA polymerase II in these vectors (Fig. 1). PGPTV-based vectors, together with the *Agrobacterium* strain EHA 105, were used in our previous experiments for the transfer of P-35S-driven transgenes into potato plants without any problems. We propose that the close proximity of the polIII promoter could somehow inhibit the transcription from adjacent polII promoters. Inhibition of transcription from the polII promoter by the presence of polIII-driven tRNA genes was observed in *Saccharomyces cerevisiae* (Hull *et al.* 1994, Bolton and Boeke 2003). Transcription factors binding to the polIII promoter could, for example, inhibit the binding of factors necessary for polII transcription. Localization of the polIII-specific transgene in a special nuclear compartment, which is not suitable for polII expression, is another possibility. This kind of transcriptional silencing of the polII gene by nucleolar localization, due to the presence of polIII gene, was reported by Kendall *et al.* (2000) in budding yeast. When a mutant yeast strain, where pre-tRNAs lost their nucleolar localization was used in their experiments, transcriptional silencing of the polII gene did not occur. *Alu* repeats in the human genome showing homology to 7SL RNA are transcribed by polIII, and have been shown to contain a transcriptional silencer (Tomilin *et al.* 1990). On the other hand, there are data reporting naturally occurring, closely linked polII and polIII genes. The bidirectional promoter connects, for example, the polII-driven gene PARP-2 to polIII-driven RNase P RNA gene. These genes are oriented head to head and there is only a region of 113 bp between the transcription start sites in mice and 152 bp in humans (Amé *et al.* 2001).

Transient expression of tRNA-based cassettes in plants, where no selection is necessary, was reported by Bourque and Folk (1992) and Perriman *et al.* (1995). Transgenic plants expressing the tRNA-based antisense RNA gene were obtained by Murfett *et al*. (1995). However, a binary vector with a longer distance between the polII and polIII promoters than in our pGPTV-based vectors was used (Murfett *et al.* 1995). Consequently, we were able to obtain chimerical hairy roots unambiguously expressing polIII cassettes by co-transformation of binary vectors without any selectable marker, together with the wild type Ri plasmid. This clearly suggest the activity of 7SL RNA-specific promoter elements *in vivo,* and this finding corresponds to positive results published for *in vitro* transcription of modified 7SL RNA in tobacco extracts (Yukawa *et al.* 2002).

However, we were able to select for transformants when the MAR vectors, where the P-nos promoter was insulated by 1.2 kbp of the A element from the polIII expression cassette, were used (Fig. 3). We obtained kanamycin resistant transgenic plants expressing either modified 7SL RNA or tRNA genes. It can be concluded from these results that most probably, the polII-driven P-nos of *NPTII* selection gene was somehow repressed by the polIII gene in pGPTV-based vectors, and therefore, no rooting transformants were obtained. The exact mechanism is not known, but it is advisable to use some insulation of polII and polIII promoters for the application of polIII promoters within complex artificial T-DNA constructs.

Surprisingly, a low level of modified 7SL RNA, relative to polII-driven transgenes, was detected in our transgenic potato. In previous reports, there was shown to be a high level of expression of modified tRNA in transient expression systems (Bourque and Folk 1992, Perriman *et al.* 1995). The antisense RNA transcribed transiently under the control of the $tRNA^{Met}$ promoter in carrot protoplasts was 5 times more efficient than similar antisense RNA driven by P-35S (Bourque and Folk 1992). However, the tRNA-based antisense RNA was less efficient than P-35S driven antisense RNA in transgenic plants (Murfett *et al.* 1995). There are multiple DNA template copies per cell in transient expression systems, whereas a single or only few transgene copies per cell are present in transgenic plants. This fact could be one of the reasons for the relatively low level of

modified 7SL RNA detected in our experiments.

It is not known whether or not the modification of 7SL RNA gene can decrease the efficiency of its transcription *in vivo*. The stability of the modified 7SL RNA transcript in the cellular environment, in reality the aberrant RNA, could be lower than in the case of native 7SL RNA. The insertion of unrelated sequence into 7SL RNA however allows for its specific detection by RT-PCR, so it could be used not only as a potential antisense system, but also as a specific tag to differentiate it from the host 7SL RNA transcripts that share a high degree of homology.

We have detected variability in the levels of AHIIA63M in separate plant organs. The cause of this variability could be a tissue-specific transcription of the modified 7SL RNA gene. The tissue-specific transcription of 7SL RNA genes could be anticipated because multiple 7SL RNA gene variants were found in plants (Campos *et al.* 1989, Heard *et al.* 1995, Riedel *et al.* 1995, Matoušek *et al.* 1999). These different 7SL RNAs could interact with different isoforms of SRPs and be involved in the translocation of different proteins. However, a tissue-specific expression of plant 7SL RNA variants has not been studied in detail. No variability of 7SL RNA expression was reported by Riedel *et al.* (1995, 1996) in different tissues of the tomato using molecular hybridization. However, it is possible that the hybridization technique was unable to distinguish low expression differences among 7SL RNA gene variants due to their high homology, in contrast to the specific detection method used in this study.

The detected variability in the level of modified 7SL RNA could be also the result of variations in the stability of the transcript in different tissues. The turnover of unusual 7SL RNA which probably does not form a complex with SRP proteins in the cell could be much faster than in the case of native 7SL RNA. Lastly, this aberrant RNA species could serve as a template for RdRP (RNA dependent RNA polymerase) resulting in an RNA silencing mechanism (*e.g.* Ahlquist 2002). However, silencing-specific small RNAs, homologous to AHIIA63M, were not detected on Northern blot (data not shown).

The tissue-specific expression was similar in both independently obtained transformants tested, therefore we can exclude the possibility of tissue-specific expression due to a position effect. The A63 gene itself was isolated from the genomic DNA of arabidopsis (Yukawa *et al.* 2002) but its *in vivo* expression in *A. thaliana* has not yet been studied. The effect of a heterologous MAR element from chicken for the transcription of plant polIII genes has not been studied and its presence could be one of the reasons for the organ-specific level of AHIIA63M RNA.

Finally, the relatively low level of AHIIA63M transcript *in vivo*, in comparison with *in vitro* experiments, could be due to the DNA topology of the transgene region and the presence of chromatin in the nucleus in comparison with the naked DNA template used for *in vitro* studies. We expected that expression of the AHIIA63M transgene from an AT-rich region of the A element (Fig 1) could facilitate its transcription, as polIII genes are often situated within AT-rich regions (Arnold *et al.* 1986, Matoušek *et al.* 1999). However, the binding of the nuclear matrix to the A element could interfere with the binding of transcription factors, which are necessary for efficient polIII-mediated cassette transcription. We showed that some of AHIIA63M-specific RNA was produced due to the previously unknown promoter activity of the A element alone. Other constructs with AHIIA63M inserted outside the A element have to be transformed into plants and their transcription must be evaluated to address this issue. The A element has been shown to have insulator and enhancer functions (Phi-Van and Strätling 1996), and according to a recent report, it can, in certain situations, also prevent posttranscriptional gene silencing of neighboring polII genes (Mlynárová *et al.* 2003). Our results indicate, that the A element also has weak promoter activity.

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