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EXPRESSION PATTERN OF *ARABIDOPSIS THALIANA* POLLEN- AND EMBRYO-SPECIFIC PROMOTER IN TRANSGENIC TOBACCO PLANTS

MARTIN JOPCIK, ILDIKO MATUSIKOVA, JANA MORAVCIKOVA, AND JANA LIBANTOVA*

Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademicka 2, P.O. Box 39A, 950 07 Nitra, Slovak Republic

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Current biotechnology research is focused on tissue-specific expression of genes of interest in plants. Promoters with specific spatial and temporal expression profiles in targeted organisms are in wide use for this. This study investigated whether the *Arabidopsis thaliana* seed- and pollen-specific promoter *MXL* maintains its specificity in transgenic tobacco plants. Histochemical analysis revealed that the *MXL* fusion promoter drives slightly different GUS expression in that heterologous organism. GUS staining was clearly detected in the bicellular stage of pollen development and later in germinating tobacco pollen grains. Unlike in *A. thaliana*, where the *MXL* promoter is active during the whole period of embryo development, in tobacco its activity was restricted to a short temporal and spatial window from late-heart to mid-torpedo stages, mainly in the apical part of the developing embryo. These results point to the need to test the expression profiles of heterologous promoters in targeted species before they are used in particular biotechnological programs.

Keywords: Embryogenesis, GUS, *Nicotiana tabacum*, specific promoter activity, transgenic plants.

INTRODUCTION

In flowering plants, reproductive processes occur within floral organs. Haploid gametophyte generation begins after meiosis, when microspores undergo mitosis and differentiate into pollen grains, and megaspores into embryo sacs. Diploid sporophyte generation begins when one sperm fuses with the egg cell to produce a zygote and initiate embryogenesis. This process is accompanied by modulation of the expression patterns of a number of genes (Goldberg et al., 1994). For example, almost 15,000 genes in *Arabidopsis thaliana* actively take part in pollen development (Wilson and Zhang, 2009); they are classified as early genes, expressed during microsporogenesis, and late genes, expressed during microgametogenesis. At least 10% of these genes appear to be active specifically in pollen. Arising proteins play both basic and specialized roles in pollen cell wall metabolism, cytoskeleton development, and signaling (Twell et al., 2006; Zhou et al., 2010).

Similarly, the embryo development process is accompanied by high genome activity, when almost 78% of *Arabidopsis* genes are transcribed (Xiang et al., 2011). Broadly and specifically expressed genes in globular, heart, torpedo and mature embryo stages play roles in the cell cycle, primary metabolism, storage reserve synthesis, auxin and abscisic acid biosynthesis, and signalling. The specificity of gene expression coincides with biologically distinct programs associated with different phases of embryo development such as initiation of the post-fertilzation sporophytic program, morphogenesis, deposition of storage reserves, and desiccation, leading to dormancy and the fully mature embryo (Xiang et al., 2011).

The *Arabidopsis thaliana MXL* promoter drives the expression of the *At5g38170* gene encoding the bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein, specifically during the processes of sexual reproduction. Microarray experiments revealed its transcriptional activity in sperm cells (Borges et al., 2008) and in the developing embryo (Casson et al., 2005), where the translated protein is involved in transport of lipids (Boutrot et al., 2008). The *At5g38170* gene shows strong co-expression with *At5g12460*, *At5g63240*, *At3g27785* and *At3g47190* genes mainly in siliques (Hanada et al., 2007).

Promoters, mainly those with specific expression profiles, have found application in plant genetic

*e-mail: jana.libantova@savba.sk

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engineering (Almasia et al., 2010). Before being used in biotechnology, their specificity in targeted organisms is often verified through junction of a 1–2 kb promoter sequence with the reporter gene. This junction often reflects the expression pattern of a native gene but there are also cases in which nonspecific expression of a transgene controlled by a specific promoter is observed (Huda et al., 2013). A transgenic system can also reveal a cell-type-specific pattern that would escape detection by microarray approaches (Xiao et al., 2010).

In a previous study we observed strong up-regulation of the *MXL* promoter in nontargeted vegetative tissues due to the influence of the adjoining CaMV 35S promoter. The *MXL* promoter could restore the tissue-specific character of its activity only when their mutual cloning distance on the binary vector was greater than 3 kb (Jopcik, unpublished). Our present study furnishes new data about the in silico and cell-specific expression profiles of the *MXL* promoter during pollen and embryo development in transgenic tobacco plants.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *Nicotiana tabacum* L. (cv. Petit Havana SR1) were surface-sterilized with 96% (v/v) ethanol, washed in 10% (v/v) sodium hypochlorite and 0.1% (v/v) Tween 20, rinsed five times in sterile distilled water, and placed on MS medium (Duchefa, The Netherlands) supplemented with 20 g l^{-1} sucrose and 7 g l^{-1} plant agar (pH 5.8), at 20°C under a 16 h photoperiod (50 μE m^{-2} s^{-1} light intensity). Following germination, two-week-old seedlings grown in Petri dishes were transplanted to screw-cap jars and regularly subcultured every five weeks. Leaf segments of the plants grown in vitro were used for transformation experiments.

VECTOR CONSTRUCT AND PLANT TRANSFORMATION

The *MXL* promoter of the *At5g38170* gene was isolated from *A. thaliana* as a PCR fragment 1.8 kb long and cloned into the plant binary vector pCAMBIA1304 (Roberts et al., 2002) by replacing the *CaMV 35S* promoter fused to the *gfp:gus* genes (Jopcik et al., 2013). To generate a 5 kb spacer, a 2 kb exon from the *A. thaliana At3g29060* gene was amplified by PCR using primers SpacFOR (5'-TTTAATCCCAAGAACAGTCTGCATCCA-3'), SpacREV (5'-TGAATCAATTGGCGTTTGCAAAGA-3') and cloned into pGEM-T Easy (Promega, USA) generating a pGEM2S construct. The construct p5SC*MXL* (Fig. 1) was prepared by cloning a 5 kb fragment generated by linearizing pGEM2S with Acc65I restriction enzyme and ligating with pC*MXL* (Jopcik et al., 2013), digested with Acc65I restrictase and treated with shrimp alkaline phosphatase (Thermo Fischer Scientific, USA). Then the p5SC*MXL* vector was introduced into *Agrobacterium tumefaciens* strain LBA 4404 by a freeze-thaw method (Höfgen and Willmitzer, 1988).

Leaf discs of four-week-old tobacco plants cultured in vitro were transformed using the *A. tumefaciens* method as described by Horsch et al. (1985). At first the leaf discs were co-cultivated in Petri dishes with overnight-grown *Agrobacterium* cells diluted to final optical density at 600 nm (OD_{600})=0.6 in liquid CIM (callus-inducing medium) consisting of MS medium, 30 g l^{-1} sucrose, 1 mg l^{-1} NAA (α-naphthalene acetic acid, Duchefa, The Netherlands) and 0.2 mg l^{-1} BAP (benzylaminopurine, Duchefa, The Netherlands), pH 5.7, in darkness for two days.

Callus formation on tobacco leaf discs proceeded on solid SIM (shoot-inducing medium) consisting of MS medium, 30 g l^{-1} sucrose, 0.1 mg l^{-1} NAA, 1 mg l^{-1} BAP, 8 g l^{-1} plant agar, 30 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime, pH 5.7. After several weeks the first shoot buds appeared on the surface of calli. When the shoots reached ~1 cm in length they were transferred onto solid MS medium supplemented with 10 g l^{-1} sucrose, 30 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime, and cultured until roots were induced. Later the individual regenerated transgenic plants were transferred to soil and cultivated in vivo.

PCR ANALYSIS

To verify the presence of transgenes, putative transgenic plants were screened by PCR. Total genomic DNA was isolated from all hygromycin-resistant plants using the DNeasy Plant Mini kit (Qiagen, Germany). PCR amplification was performed in a reaction volume of 25 μl containing 200–300 ng plant genomic DNA, 1 x PCR buffer, 0.2 mmol l^{-1} dNTPs, 10 pmol of each forward and reverse primer and 1U FIREPol DNA polymerase (Solis BioDyne, Estonia). PCR primer pairs were as follows: P1 (5'-TACATTGACACACCATGCATTCTCC-3') and P2 (5'-TTGGGACAACTCCAGTGAAAAGTTC-3') for confirmation of *MXL* promoter; and P3 (5'-TTACCAATGCTTAATCAGTGAGGCA-3') and P4 (5'-CAACAACGTTGCGCAAACTATTAAC-3') for confirmation of the 5S spacer sequence in 5SC*MXL* transformants (Fig. 1).

HISTOCHEMICAL LOCALIZATION OF GUS ACTIVITY

Leaves, stems and roots of all primary transformants were subjected to histochemical analysis to detect GUS activity. Callus material for this was

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Fig. 1. Scheme of the p5SCMXL plant binary construct used for genetic transformation of tobacco explants. The 1.8 kb fragment carrying the *MXL* promoter was fused in the frame to the gfp:gus reporter genes. To obviate the influence of the CaMV35S enhancer on the tested tissue-specific promoter, which might result in ectopic activity between the two promoters, a 5 kb spacer was cloned. *hpt* – hygromycin resistance; 35S – Cauliflower Mosaic Virus promoter; *mgfp5* – gene for modified green fluorescence protein; *gusA* – *E. coli* glucuronidase gene (*gusA*), *MXL* – *A. thaliana* fragment harboring promoter encoding bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein.

obtained by cultivating leaf discs of individual transgenic plants on Petri dishes containing solid CIM medium supplemented with 30 mg l^{-1} hygromycin. Pollen grains and anthers were analyzed when flowers of primary transformants growing in pots reached developmental stage 12 according to Koltunow et al. (1990). For analysis of embryos, squash preparations of seeds of individual self-pollinated primary transformants in different stages of development were prepared.

Except for seeds, all plant tissue samples from individual transgenic lines were incubated in GUS solution [2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide [(X-Gluc, Duchefa, The Netherlands), 50 mmol l^{-1} phosphate buffer (pH 7.0)] at 37°C in the dark overnight (Jefferson et al., 1987). Before GUS detection the chlorophyll had been removed in a series of 70% (v/v) ethanol washes.

For seeds, following incubation in GUS solution at 37°C for 24 h they were immersed in an ethanol-glacial acetic acid mixture (1:1) for 4 h. This mixture was replaced by Hoyer's solution (Stangeland and Salehian, 2002). GUS activity was observed under an Axioplant II microscope (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

The majority of approved genetically modified (GM) crops show constitutive transgene expression. However, the recently approved Golden Rice(s) and High-Lysine Corn have the transgenes expressed solely in rice endosperm and maize germ respectively. Temporally and/or spatially controlled transgene expression has several advantages, such as limiting the occasional transgene interference with normal growth and developmental processes in plants, and increased public acceptance (Moravcikova et al., 2007; Corrado and Karali 2009; Zhang et al., 2010; Lim et al., 2012). This is why in recent years there is a growing trend to use promoters with desired specific expression profiles.

The main aim of this research was to characterize the *Arabidopsis MXL* promoter in silico and determine its cell-specific expression profile in transgenic tobacco plants. For this, the 1846 bp

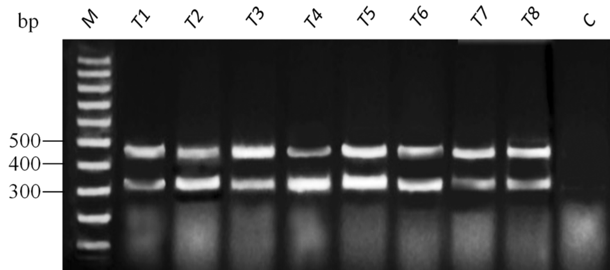

Fig. 2. Representative PCR verification of 5SCMXL transformants. PCR reaction was carried out on genomic DNA (isolated from 5SC*MXL* transgenic plants) with P1-P2 primers and P3-P4 primers yielding amplicons 450 bp long and 308 bp long respectively. Non-transgenic control (C) did not yield any PCR product.

long *A. thaliana* 5'-flanking regulatory region upstream of the translation start site of the gene encoding a bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein was cloned into the derivative of the pCAMBIA binary vector (Fig. 1). To eliminate any influence of the *CaMV 35S* promoter on alteration of promoter tissue specificity (Zheng et al., 2007), a 5 kb long spacer sequence was cloned between the two regulatory sequences (Fig. 1).

Following transformation and PCR verification of the regenerated transformants (Fig. 2), 15.8% of the plants still showed weak ectopic GUS expression in some of the analyzed vegetative tissues. This phenomenon occurs with some frequency and is considered to be a result of the influence of the plant genome at the site of T-DNA insertion (Yoo et al., 2005). For this reason we excluded the plants with ectopic GUS expression from further experiments.

To search for a developmental-specific expression profile of the *MXL* promoter, pollen and developing embryos of 16 tobacco plants with no GUS activity in roots, stems, leaves and calli (Fig. 3a–d) were subjected to detailed GUS assays. In transgenic tobacco plants we observed *MXL* promoter activity in late stages of pollen development. Strong GUS activity of the reporter gene was seen in the bicellular stage of pollen development and later in germinating pollen grains (Fig. 3h,i). Potential pollen-spe-

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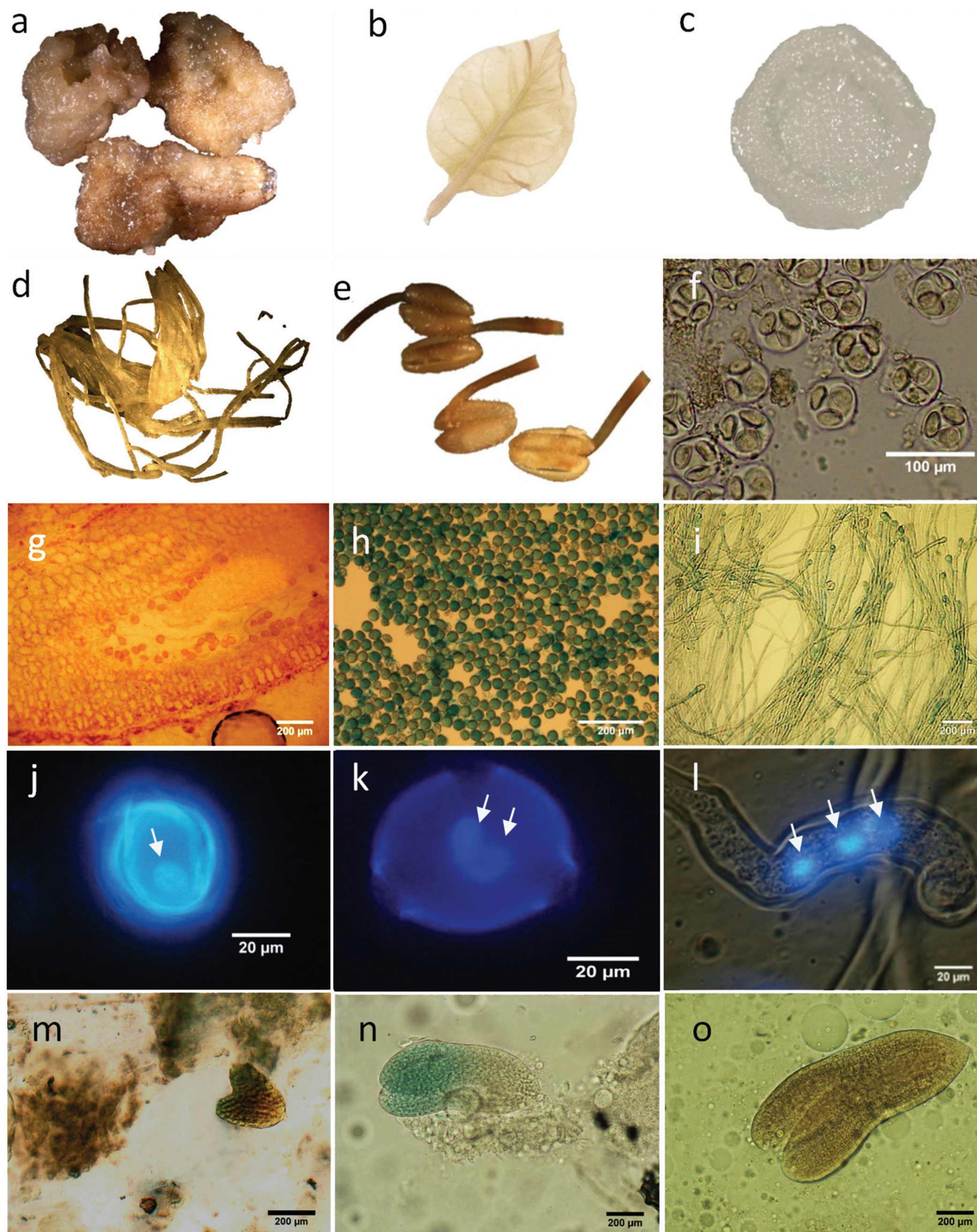

Fig. 3. Histochemical detection of GUS activity in 5SCMXL transgenic tobacco plants. No GUS activity in calli (**a**), leaves (**b**), stems (**c**), roots (**d**), outer layers of anthers (**e**), tapetal cells and tetrads (**f**) and microspores (**g**). Specific pattern of GUS activity in bicellular pollen (**h**) and in germinating pollen grains (**i**). DAPI counterstaining for developmental stage of unicellular microspores (**j**), bicellular pollen (**k**), and germinating pollen grains (**l**); white arrows show localization and number of nuclei. During embryogenesis, GUS activity in late-heart (**m**) and mid-torpedo stages, mainly in apical part of developing embryo (**n**). No GUS activity in late-torpedo stage of developing embryo (**o**).

cific expression was also confirmed in silico using the PLACE program (Higo et al., 1999). We identified 10 AGAAA motifs, 13 GTGA motifs and 4 AAATGA motifs within the fragment carrying the *MXL* promoter. These *cis*-elements have also been found in pollen-specific promoters of other plant species. For example, the *PSG076* promoter, directing specific expression at late stages of pollen development in wheat, contains multiple copies of AGAAA, GTGA and AAATGA motifs (Chen et al., 2012). Similarly,

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the *Solanum tuberosum* pollen-specific *SBgLR* promoter contained 10 copies of AGAAA and 1 copy of GTGA (Zhou et al., 2010). Geng et al. (2009) demonstrated that both the GTGA and AGAAA motifs were essential for high-level anther-preferred expression from a *bnfs* promoter in plants. This indicates that not only the occurrence but also the mutual cooperation of several organ-specific *cis*-regulatory elements enable organ-specific gene expression (Taha et al., 2012). Depending on the individual plant species, a number of other factors may influence the resulting promoter activity pattern.

We also performed histochemical GUS detection at individual stages of the developing embryo. Based on results from microarray experiments done by Casson et al. (2005), we expected to see *MXL* promoter activity during the whole course of embryo development, reaching maximum at globular stage. We observed GUS staining in transgenic tobacco in a short temporal and spatial window from late-heart to mid-torpedo stages, mainly in the apical part of the developing embryo, in the shoot meristematic region (Fig. 3 m–o). Unlike the microarray expression data, the transgenic system was able to reveal that the GUS signal was not evenly distributed over the whole embryo. These findings are important for some biotechnological applications. For example, selectable marker plants can be generated using a self-excision Cre/*lox*P strategy when excision of both selectable marker and *cre* recombinase genes is directed by the embryo-specific promoter (Moravcikova et al., 2008). If the activity of the chosen promoter is located only in the shoot meristem region of the developing embryo, the progeny will be chimeric – with the excised selectable marker gene in shoot tissues and without it in root tissues. Completely marker-free plants might be obtained only in the T2 generation, since reproductive organs differentiate from the shoot apical meristem (Goldberg et al., 1994).

The activity of the *MXL* promoter during embryo development was also investigated in silico. The analyzed promoter sequence contained multiple *cis*-elements involved in transcriptional control of genes during seed development. We identified 2 ACGTG motifs, 4 ACGT motifs, 3 CANNTG motifs, 1 TACGTA motif, 7 RTTTTTR motifs, 3 ATATTTAWW motifs, 4 CATGCATG motifs, 1 TACACAT motif, 1 CAAACACC motif, 1 CNAACAC motif, 1 CCTTT motif, 3 TAACARA motifs, 2 TAACAAR motifs, 3 WAACCA motifs, 3 YAACKG motifs, 16 CAAT motifs and 1 CAAACAC motif.

Among these, the ACGT, ACGTG and CANNTG motifs were found to be necessary for *erd*1 promoter activity in dehydrated seeds of *A. thaliana* (Simpson et al., 2003). The RY motif, present in the analyzed sequence, is among the essential *cis*-regulatory elements for seed-specific transcriptional activation (Ezcurra et al., 1999). A decrease of RY motif number in the promoter sequence of the β-conglycinin gene resulted in a clear decrease of gene expression in *Arabidopsis* seeds (Yoshino et al., 2006).

Next, we identified two different embryo factor binding sites (ATATTTAWW for SF1 and RTTTTTR for SF4) within the *MXL* fragment. Initially they were found in the soybean β-conglycinin promoter driving embryo-specific expression (Lessard et al., 1991), but later studies did not confirm the need for these elements for seed-specific gene expression (Fujiwara and Beachy, 1994). The *MXL* fragment also contains the TACACAT motif, shown to be responsible for activating seed-storage protein expression in rapeseed and soybean seeds (Ericson et al., 1991; Caiyin et al., 2007). The CAAACAC and CNAACAC sequences [(CA)n elements] were found to be important for embryo- and endosperm-specific transcription of the *Brassica napus napA* gene (Ellerstrom et al., 1996). The (CA)n motif was also found in the E8 promoter of tomato (Zhao et al., 2009). Finally, the pyrimidine box (CCTTT), AMY1 box (TAACARA) and GAREAT sequence (TAACAAR), motifs occurring in the investigated fragment, are closely related to gibberellin-response elements (Huang et al., 1990; Chavez-Barcenas et al., 2000). Promoters of the above-mentioned motifs are important for temporally and spatially regulated (trans)genes, but other unknown repressors and activators probably determine the resulting expression profile in specific plant tissues (Zhou et al., 2010).

As mentioned in the introduction, the transgenic system can verify promoter specificity in heterologous organisms. Here we reported evidence that the *MXL* promoter can retain the pollen- and embryo-specific character of its activity in transgenic tobacco plants if it is sufficiently distant from the 35S enhancer within the same T-DNA. However, in the tested heterologous organism it had a different temporal expression profile during embryo development. When other organ-specific promoters were tested to determine if they would drive reporter gene expression in heterologous organisms, some of them retained it, and other ones showed different or no activity profiles (Furtado et al., 2007; Huang et al., 2011; David-Schwartz et al., 2013). Our experiments and those of others suggest that the expression profile of a heterologous promoter should be tested in the target species before directing a transgene of interest into a heterologous organism.

AUTHORS' CONTRIBUTION

JL and IM designed the entire experiments; MJ and JM carried out the experiments; JL wrote and edited the manuscript. All authors read and approved the final manuscript. The authors declare that there are no conflicts of interests.

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