ASSESSMENT OF THE FUNCTIONALITY OF THE DIRECT PCR-CLONED PUTATIVE PROMOTER REGIONS OF GENES FROM ARABIDOPSIS THALIANA

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ABSTRACT
The functionality of the direct PCR-cloned putative promoter regions of predicted genes from Arabidopsis thaliana was assessed. The regions located upstream of the four predicted open reading frames of genes from Arabidopsis thaliana were cloned directly in front of the gus reporter gene in plant transformation vectors. The GUS activity was analyzed in transgenic Nicotiana benthamiana plants carrying chimera promoter:gus genes, as well as after wounding and salicylic acid treatment of the tested plants. Two of the tested promoter regions, derived from putative peroxidase- and cysteine protease-coding Arabidopsis genes, drive strong near constitutive GUS expression in the transgenic lines. The third promoter region, derived from different cysteine protease coding Arabidopsis gene, directs strong salicylic acid inducible transgene expression. The efficiency of utilization of Arabidopsis bioinformation data for direct cloning of functional promoter regions is discussed.

Introduction
Bioinformatical analysis of the Arabidopsis genome led to the identification of more than 25 500 putative genes, (1). The performed annotation describes the putative exon/intron structures, open reading frames and corresponding amino acid sequences of the identified genes, as well as homology to already known genes and gene families. Now, this information is publicly available at several specialized databases, for example: http://ftp.ncbi.nih.gov/genbank/genomes/Arabidopsis_thaliana, http://mips.gsf.de/proj/thal/db (2), http://www.tigr.org/tdb/e2k1/ath1, http://mendel.cs.rhul.ac.uk/Arabidopsis.

Thus the sequence data of Arabidopsis genome become valuable source for identification, cloning and characterization of members of gene families and homologous genes. The prediction of the open reading frame positions allows additional localization of the corresponding putative promoter regions and their further direct PCR-cloning at high throughput manner. The recently developed bioinformation resources for identification of cis-acting elements, e.g. TRANSFAC, http://www.gene-regulation.com, (3,4); PlantCARE, http://intra.psbb.ugent.be:8080/PlantCARE, (5); PLACE, http://www.dna.affrc.go.jp/htdocs/PLACE/, (6), additionally facilitate the analyses of the putative promoter regions of the genes of interest. In spite of these exiting opportunities for high throughput analyses, the efficiency of direct cloning and characterization of promoter regions is still not well tested.

Here we report the direct cloning of putative promoter regions of four genes from Arabidopsis thaliana and assessment of their functionality through the characteri-
zation of the driven by them expression of GUS reporter gene in transgenic *Nicotiana benthamiana* plants carrying chimera promoter:gus gene. The efficiency of the direct cloning of functional promoter regions is discussed.

**Materials and Methods**

**Construction of promoter:gus fusions**

All used molecular biology techniques were performed according to *Sambrook et al.* (9). The positions of the four putative open reading frames At2g27420, At2g34080, At5g06730 and At4g17390 were identified using [http://ftp.ncbi.nih.gov/genbank/genomes/Arabidopsis_thaliana](http://ftp.ncbi.nih.gov/genbank/genomes/Arabidopsis_thaliana) Bioinformation resource. The region in average length of 1.2 kb located upstream of the pointed ATG start codon for each studied gene was considered as a putative promoter region. The selected DNA regions were PCR amplified from genomic DNA isolated from *Arabidopsis thaliana* var. Columbia, using specific primers, Table. Sequences of selected restriction sites, facilitating the direct cloning of the amplified DNA fragments, were inserted in all PCR primers. The obtained PCR products were restricted with corresponding enzyme set and cloned in front of the gus gene coding region in plant transformation vectors p1A4 and p1A8 (10), replacing 35S promoter region. p1A4 and p1A8 are binary vectors derived from *pCambia1300* and *pCambia1300*[http://www.cambia.org/main/r_et_camvec.htm](http://www.cambia.org/main/r_et_camvec.htm), through insertion of the 35S:gus:nos cassette from vector *pBI121* (Clontech, USA) at Hind III / EcoR I sites. The transformation with p1A4 and p1A8 vectors confers resistance to hygromycin and kanamycin in the obtained transgenic plants. The features of each cloning experiment are present in Table. The resulted plasmids: pAtPOX (pAt5g06730:gus:nos in p1A8), pAtCP1 (pAt2g27420:gus:nos in p1A4), pAtCP2 (pAt2g34080:gus:nos in p1A4), pAtL15B (pAt4g17390: gus:nos in p1A4) and p1A4 (used as positive control) were introduced into *Agrobacterium tumefaciens* LBA4404 via tri-parental mating.

**Plant material and transformation procedure**

*Nicotiana benthamiana* plants, grown in vitro on MS basal media at standard cultivation conditions, were used. The transformation of *N. benthamiana* plants was performed using co-cultivation of leaf explants with *Agrobacterium*, (11). The transgenic shoots were regenerated after cultivation on selective MS, 2mg/l BAP, 0.2 mg/l NAA media. The obtained kanamycin and hygromycin resistant plantlets were transferred to soil and grown in the greenhouse. The incorporation of the transgene was verified through PCR amplification from genomic DNA with PCR primers specific to the promoter and gus sequences (GUS-R: 5’-gggagctccacatcaccacgcttg). The “PCR-positive” plants were self pollinated. The harvested seeds, after surface sterilization with 0.1% HgCl2, were grown on selective MS media. For the pAtPOX transgenic seeds the media was supplemented with 100 mg/l kanamycin and for the pAtCP1, pAtCP2, pAtL15B and p1A4 transgenic seeds – with 20 mg/l hygromycin.

**Analyses of GUS activity**

The histochemical assay of tissue expression of the GUS gene in the obtained transgenic F1 *N. benthamiana* plants was conducted according to Jefferson et al. (12).

Quantitative analysis of GUS expression levels was performed through fluorometric analyses of the GUS enzyme activity. The tissues from the transgenic plants were frozen immediately after collecting in liquid nitrogen and homogenized in extraction buffer (50mM NaHPO4, 10mM Na2EDTA, 10mM DTT, 0.1% Triton X-100, 0.1% Sarcosyl). After centrifugation for 3 min at 13000 rpm, the supernatant was collected and the total protein concentration was determined via Bradford Protein Assay (13).
details on direct PCR cloning of putative promoter regions from Arabidopsis and construction of plant transformation vectors. The restriction sites for cloning are present in bold

<table>
<thead>
<tr>
<th>Gene acc. No / Designation</th>
<th>PCR primers</th>
<th>Length of the amplified region</th>
<th>Vector and cloning sites</th>
<th>Obtained promoter:gus fusion vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g06730 AtPOX</td>
<td>V2f-2 5’ ggaacttagcgttgactgcacatt V2R 5’ gtctagattgtagctgctttaaatagg</td>
<td>1297 bp</td>
<td>pIA8 Hind III Xba I</td>
<td>pAtPOX</td>
</tr>
<tr>
<td>At2g27420 AtCP1</td>
<td>P1f-2 5’ gctgcagtggtgggagaggtttaaattg P1r-1 5’ eggatcgaagagcggtgtgtcataa</td>
<td>1195 bp</td>
<td>pIA4 Pst I Bam HI</td>
<td>pAtCP1</td>
</tr>
<tr>
<td>At2g34080 AtCP2</td>
<td>P2f-2 5’ ggaactgtgtgcttttcatcbeaa P2r-1 5’ gtctagaaagtttgaataggagaa</td>
<td>1133 bp</td>
<td>pIA4 Hind III Xba I</td>
<td>pAtCP2</td>
</tr>
<tr>
<td>At4g17390 AtL15B</td>
<td>R2f-2 5’ gctgcagtgcttcatcagatattc R2r-1 5’ eggatcgaagctctgctgggaattagg</td>
<td>1384 bp</td>
<td>pIA4 Pst I Bam HI</td>
<td>pAtL15B</td>
</tr>
</tbody>
</table>

The concentrations of the total proteins in the tested samples were normalized through dilutions with the extraction buffer. Portions of 30µl from the crude extract were mixed with 0,3 ml of GUS assay buffer (2mM 4-methylumbelliferyl glucuronide in 50mM NaHPO₄ pH 7.0, 10mM EDTA, 0,1% (v/v) Triton X-100, 0,1% Sarcosyl, 10mM β-mercaptoethanol, 20% (v/v) methanol) and incubated at 37°C. Aliquots of 0,2 ml were taken after 0, 5, 15 and 20 min and the reaction was stopped by adding 1,8ml 0,2 M NaCO₃. Fluorescence was measured at fluorometer Hoefer DyNA Quan™ 200 (Amersham), calibrated according to the manufacturer instructions. The GUS activity was present in units (U)/ minute/ mg protein, as 1000U correspond to formation of 100nM 4MU (4 methylumbelliferyl). Two control samples were used in all the experiments: (1) positive – N. benthamiana plants transformed with pIA8, carrying p35S/gus/nos expression cassette and (2) negative – wild type N. benthamiana plants.

**Treatment with salicylic acid and wounding**

Greenhouse grown transgenic plants, at stage of formation of the first flower buds, were sprayed with 1mM salicylic acid. Leaf samples were taken 48 hours after the induction, immediately frozen in liquid nitrogen and stored at –70°C for further fluorometric analyses.

For wound induction, detached leaves from the analyzed transgenic plants were placed on wet filter paper and cut with razor blade at 3 mm strips. Samples were taken after 1 and 24 hours, frozen in liquid nitrogen and store at –70 °C.

**Results and Discussion**

The genomic regions, in average length of 1.2 kb and located upstream of the starts of the open reading frames of four putative *Arabidopsis* genes, were PCR amplified using specific primers with inserted appropriate restriction sites, Table. The predicted amino acid sequences possess higher homology to known genes, as follow: (1) *At5g06730* (designated further as *AtPOX*) to anionic peroxidase from *Nicotiana tabacum* (7; acc. No P11965); (2) *At2g27420* (designated further as *AtCP1*) and *At2g27480* (designated further as *AtCP2*) to cysteine protease from sweet potato *Ipomoea batatas* (8; acc. No GI:13491750) and (3) *At4g17390* (designig-
nated further as **AtL15B** to 60S ribosomal protein L15 (RPL15B). The amplified DNA fragments were readily digested with the enzyme set corresponding to the primer sequences and directly cloned in front of the *gus* reporter gene in *p1A4* or *p1A8* vectors, Table. At least ten independent transgenic lines were generated after *Agrobacterium* mediated transformation of *N. benthamiana* plants with each constructed vector: *pAtPOX, pAtCP1, pAtCP2, pAtL15B*. The histochemical assay of GUS activity in the obtained T₀ transgenic lines and their T₁ seed progeny showed presence of strong GUS enzyme activity in all aerial parts of *pAtPOX* and *pAtCP2* plants, weak GUS activity in leaves of *pAtCP1* plants and no visible GUS staining in *pAtL15B* plants. Further fluorometric analysis confirmed the absence of GUS expression in all organs of the tested *pAtL15B* plants, as well as in two-week old seedlings, (data not shown). Such lack of functionality of the tested putative promoter region *pAtL15B* could be result from improper determination of its length and positions or eventuality based on species-specific differences of the transcription apparatus between *A. thaliana* and *N. benthamiana*. The comparison of the level of expression of GUS reporter gene in the leaf tissues of the T₁ transgenic lines with the level of expression of GUS gene in positive control *p1A4* plants possessing **35S:gus:nos** expression cassette demonstrated that: (1) the levels of GUS expression in *pAtPOX* and *pAtCP2* plants are comparable to those of the positive control and (2) the GUS expression in *pAtCP1* plants is 3.5 times lower than in *p1A4* plants, Fig. 1.

The characterization of features of the identified three functionally active promoter regions *pAtPOX, pAtCP1* and *pAtCP2* was further extended through analysis of the GUS expression in the leaf tissues of the tested plants after wounding and salicylic acid treatment. The obtained data for *pAtPOX* plants demonstrated no

**Fig. 1.** Fluorometric analyses of the GUS enzyme activity in transgenic *Nicotiana benthamiana* plants. The activities were measured for expression of the gus gene under the control of a. *pAt5g06730*; b. *pAt2g27420*; c. *pAt2g34080* promoter. **35S:gus** – positive control *Nicotiana benthamiana* plants expressing *gus* gene under the control of 35S promoter; As a negative control were used wild type *Nicotiana benthamiana* plants.
induction of the AtPOX promoter from wounding Fig. 2a, and weak increase of GUS activity after treatment with salicylic acid, Fig. 3a. These results correspond to the expression profile of tobacco peroxidase-coding gene (acc. № P11965, 7, 18) possessing high homology to AtPOX. This gene is highly expressed in the whole plant with stronger expression in the upper parts (7) and is not induced by wounding (18). The expression driven by the analyzed AtPOX promoter region differs from the expression pattern of other peroxidase genes from rice (14), tobacco (15), sweet potato (16) and tomato (17), showing strong wound inducibility.

The analyses of the other two type of transgenic lines, showed relatively small increase of GUS expression after wounding, 1.5 times in pAtCP1 and 2.5 times in pAtCP2 plants Fig. 2bc, demonstrating weak wounding inducibility of the studied promoter regions. Further studies of salicylic acid-treated plants demonstrated strong, more than eight times, increased GUS activity in pAtCP1 transgenic plants Fig. 3bc, possibly related with the involvement of the AtCP1 gene in salicylic acid mediated stress response.

In conclusion, the results from present study demonstrate that, the information on predicted genes, location of the corresponding open reading frames and sequence data of the Arabidopsis genome could be efficiently used for direct PCR cloning of functional promoter regions. Thus, the promoters with desired features could be easily and timely identified after expression in heterologos system of interest. The identified in this work, functional promoter regions will be further used for providing strong constitutive (pAtPOX and pAtCP1 promoters) and salicylic acid inducible (pAtCP2 promoter) expression.
Fig. 3. Fluorometric analyses of the GUS enzyme activity after treatment with 1mM SA in transgenic *Nicotiana benthamiana* plants. The activities were measured for expression of the gus gene under the control of a. *pAt5g06730*; b. *pAt2g4080*; c. *pAt2g27420* promoter. As non-induced are marked basic enzyme activity values, contrary to those measured after wound-induction. As a positive control were used *Nicotiana benthamiana* plants expressing gus gene under the control of 35S promoter. As a negative – wild type *Nicotiana benthamiana* plants.
REFERENCES