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Organ-specific gene expression in transgenic potato: the cloning a new promoter of a class I patatin gene

Using synthetic oligonucleotide probes homologous to conservative AT-rich motif of patatin genes class I of two different clones were isolated from a potato genomic library. One of two different genomic clones named λpat122 was subcloned and analysis 5'-region sequences. Using the chloramphenicol acetyltransferase (CAT) gene as a reporter it has been shown that a 1.8 kb promoter fragment of the class I patatin gene PAT122 provides all the information necessary for both tuber-specific and sucrose-induced expression in leaves in transgenic potato plants.

Introduction. Patatin is a family of glycoproteins that accounts for approximately 40 % of the soluble protein in potato tubers [1, 4]. The apparent molecular weights of all of the forms of patatin are approximately 40 kDa. Patatin as a rule is expressed when tuber stolon tips differentiate into a tuber [5], but it can be induced to accumulate in stems and petioles upon removal of tubers and stolons or in leaves of potato plantlets growing under axenic conditions on media supplied with high levels of sucrose [3, 6, 7, 11]. Patatin is encoded by a gene family with an estimated copy number per haploid genome of 10 to 18, depending on the cultivar [2]. The isoforms of patatin are immunologically identical both within a cultivar and between cultivars [1] and the sequences of different patatin-cDNA clones [2, 8] are highly conserved. Several cDNA and genomic clones have been isolated and the complete nucleotide sequences of both the promoter and the coding region has been determined for some of them [2—4, 6—9, 12]. Based on homology in the 5′ sequences, the patatin genes are divided into two classes, i. e. class I and class II. Up to position −87 from the transcription start site, the 5′ promoter sequences of both classes are more then 95 % conserved. The sequences further upstream from −87 differ between class I and class II genes, but are well conserved within the same class except for some deletion and insertion events [8]. The classification of these genes into two groups is based on the presence (class II) or absence (class I) of a 22-bp sequences within the 5′-untranslated region. Class I genes are strongly expressed in tuber and tuberized stolons and show very low expression in leaves, stems and roots [3, 6], while class II patatin genes are expressed only in certain cell types of tubers and root tips [10]. Thus, the proteins class I genes used as a biochemical marker for tuberization in potato.

Materials and Methods. *Plant material.* Potato (*Solanum tuberosum L.* cv. Zarevo and Nevsky) were obtained from Luzk Potato Selection Stations, Ukraine. Plants were grown under 20 h light at 20 °C and 8 h dark at 17 °C until flowering and were then shifted to induce tuberization. The shoots were frozen in liquid nitrogen and pulverize in mortar to a powder state and transfer into glass with 200 ml buffer A (1 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM mercaptoethanol). All procedure to conduct in the temperature 4 °C.

Genomic library construction. High-M, genomic DNA isolated from the tetraploid potato cultivar Zarevo was subjected to *Sau3A* partial digestion as

described by Maniatis et al. [15]. The digested DNA was size-fractionated by electrophoresis on a preparative 0.5 % agarose gel and DNA fragments 14—20 kb in lenth were purified by electroelution onto DEAE-paper. The size-selected DNA fragments were ligated to *BamHI+EcoRI* digested λ EMBL3 arms. The resulting λ phages were packaged *in vitro* and the packaging mix was adsorbed onto *E. coli* strain DP-50, plated onto 177 cm^2 plates and the resulting phage lawn was lifted onto nylon membrane filters. These filters were hybridized with labelled patatin class-I-specific oligonucleotide probe. Hybridizing plaques were subjected to an additional two to three rounds of plaque purification and DNA was prepared from liquid lysates [15].

Oligodeoxynucleotide labelling and hybridization. The class-I-specific oligos (5′-ATTATATAATACTAATAAAGA-3′ and 5′-TCTATTCTTTATTA-GTATT-3′) were annealed and the protruding ends were filled in by Klenow fragment in the presence of α-[^{32}P]dATP. The labelled oligos were purified by electrophoresis in PAAG and then used for library screening and Southern blot hybridizations.

Southern blot hybridization. Phage DNA contained patatin genomic clone (400—500 ng) were digested with restriction endonucleases, electrophoresed in 0.8 % agarose gels using standard procedures [14] and blotted onto Hybond™-N nylon membranes according to the manufacturers instructions. Some blots were hybridized to α-[^{32}P]dATP labelled patatin-specific oligos. Hybridization conditions were as described by Burrell et al. [20], except that formamide was omitted from the hybridization solution and the temperature was raised to 65 °C. Blots were washed three times in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Sodium-citrate), 0,1 % SDS at room temperature for 15 min each, followed by three washes in (0.1—0.3) × SSC, 0,1 % SDS at 65 °C each for 30 min, before exposure to X-ray film for 16—24 h at −70 °C.

DNA sequencing and computer analysis. Nucleotide sequence analysis was performed using the dideoxy chain-termination method [17]. Sequence reactions were performed with T7 DNA polymerase according to the instructions of the supplier ("Fermentas", Vilnius). Computer analysis and comparison of nucleotide sequences was performed using the PC-GENE DNA analysis software package.

Plant transformation and regeneration. Virus-free tubers of *S. tuberosum* cv. Zarevo and Nevsky was used. Shoot cultures were maintained by subculture of nodal cutting on a sterile medium containing Murashige and Skoog (MS) salts [18], vitamins, 20 g/l sucrose, solidified 0.8 % agar Difco, pH 5.8. Potato shoots were maintained at a temperature of 22—24 °C and exposed 16 h daily to 2500 lux illumination. 100 μl of the recombinant strain *pp1-CAT* were added and mixed for 5 min and then transferred to the upper cut surface of the tuber discs. The Petri plates were placed in an incubator maintained at 24 °C. After two days of cocultivation, the infected microtubers were transferred to fresh modified MS medium with addition of 500 mg/l cefotaxime ("Roussel"). Every three weeks the explants were transferred to fresh modified medium, wich consisted of MS salts, 3 % sucrose, vitamins, 0,2 mg/l benzyladenine (BA), 0.1 mg/l zeatin, 0.02 mg/l NAA, and 0,7 % agar Difco. The first shoots from tuber discs were initiated after 10—12 days in modified MS medium. Plants that developed from transformed minitubers were used for *in vitro* tuberization and expression assay.

Sucrose induction of leaf discs. Leaves were floated on MS medium containing 10 % sucrose under long day conditions (16 h light/8 h dark) for 2 days.

CAT assays. CAT-activity of protein extracts was determined as described by Gorman et al. [19] using [^{14}C]-chloramphenicol as substrate and using 0.25 M Tris-HCl (pH 7.5) as buffer throughout. The concentrations of total protein in plant extracts for each points was identical for all cases (protein

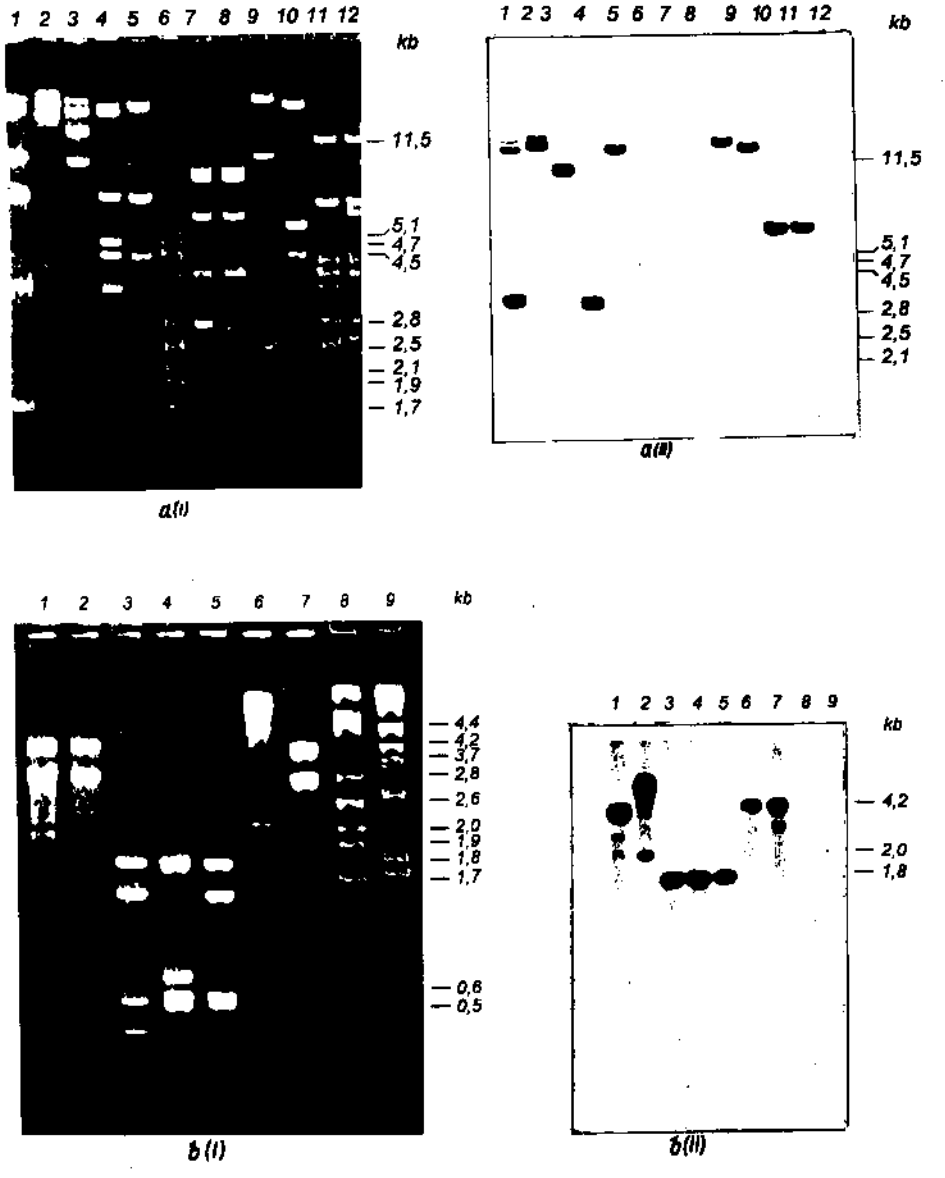


Fig. 1. Southern blot analysis of patatin genomic clone *λpat122* and plasmid DNA contained subcloned patatin promoter using synthetic oligonucleotide probe specific for patatin promoter: *a* — Gel electrophoresis in 0,7 % agarose *λpat122* DNA digested with different restriction endonucleases (I) and autoradiograph obtained during hybridization of the corresponding filter with ^{32}P-labelled patatin-specific oligos (II); lanes: *1* — *XbaI*; *2* — *BamHI*; *3* — *SalGI*; *4* — *SalGI + HindIII*; *5* — *HindIII*; *6* — *λDNA + PstI*; *7* — *DraI*; *8* — *DraI + EcoRI*; *9* — *EcoRI*; *10* — *HindIII + EcoRI*; *11* — *ClaI + EcoRI*; *12* — *ClaI*; *b* — Gel electrophoresis in 0,9 % agarose *pSK* (*Sal-HindIII*-fragment) plasmid DNA (I) and autoradiograph obtained during hybridization of the corresponding filter with ^{32}P-labelled patatin-specific oligos (II); lanes: *1* — *PstI*; *2* — *SalGI + PstI*; *3* — *DraI + SalGI*; *4* — *DraI + HindIII*; *5* — *DraI*; *6* — *KpnI*; *7* — *KpnI + HindIII*; *8* — *pDNt23* plasmid *DNA + PstI*; *9* — *λDNA + PstI*

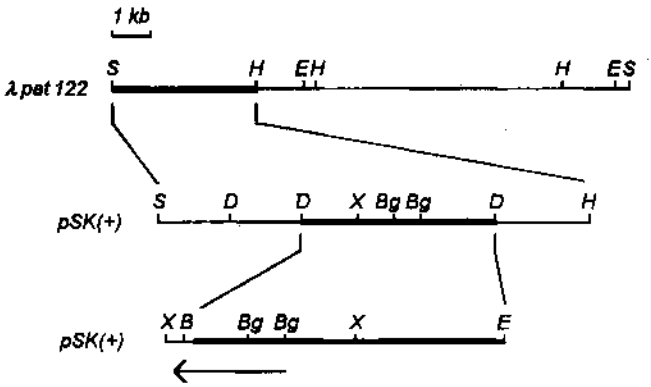


Fig. 2. Restriction maps of the genomic clone *λpat122* and the regions which contain the patatin promoter. Heavy lines denote restriction fragments that hybridize to labelled oligos. The arrow indicate the promoter orientation. Restriction endonuclease sites are indicated as single letter abbreviation: *B — BamHI; Bg — BglII; D — DraI; E — EcoRI; H — HindIII; S — SalGI; X — XbaI*

concentration was determined according to Bradford [21].

Results and Discussion. *Isolation of patatin genomic clones.* Previously it has been shown that one of the patatin genes named *B33* differed from the other class I genes by the presence of the 208 bp direct repeat and a 37 bp AT-rich motif occurring three times in the promoter region [3]. The *B33* promoter can drive the expression of the reporter genes in transgenic plant and has the fivefold higher expression as compared to another class I patatin gene named *B24* [12]. Three different *cis*-acting positive elements influencing expression in tubers were identified within the *B33* promoter sequences [11].

In order to understand the molecular mechanisms underlying the complex control of patatin expression and to reveal the role of the 37 bp AT-rich motif in these mechanisms we decided to isolate the similar promoter(s) from potato and to study its structure and expression in transgenic plants. Two complementary partially overlapping oligos homologous to 37 bp AT-rich motif were synthesized and used for library screening. A total of 300 000 recombinant plaques were screened with ^{32}P-labelled synthetic oligonucleotides. Two recombinant λ clones with greatly different intensity of hybridization to labelled oligos were identified (data not shown). Since clone *λpat122* had a very strong hybridization signal and therefore could contain in its promoter region several sequences homologous to oligos it was chosen for further analysis.

Fig. 1 shows results Southern analysis purified λ DNA and plasmids contain subcloned fragments digested different restriction endonucleases.

Fig. 2 shows the restriction maps of *λpat122* clone along with the fragments subcloned in *pSK*(+) that hybridized to the labelled oligos. As can be seen from Fig. 1 and Fig. 2 the labelled oligos hybridize to a 3.5 kb *SalGI-HindIII* fragment adjacent to the left arm of the recombinant λ phage. This fragment subsequently was cloned in *pSK*(+) plasmid between the corresponding restriction sites and then the 1.8 kb fragment was excised with *DraI*. We used the restriction endonuclease *DraI* for excising the promoter region because it cut most of patatin genes between the transcription initiation site and the initiation codon of the coding sequences. In order to make a useful cloning sites for the next promoter fusion constructions this fragment was cloned in *SmaI* site of *pSK*(+) plasmid.

Since the orientation of patatin promoter in *λpat122* clone could not be determined by the restriction analysis we have sequenced the flanking regions of the cloned *DraI* fragment. These results are presented in Fig. 3. Inspection

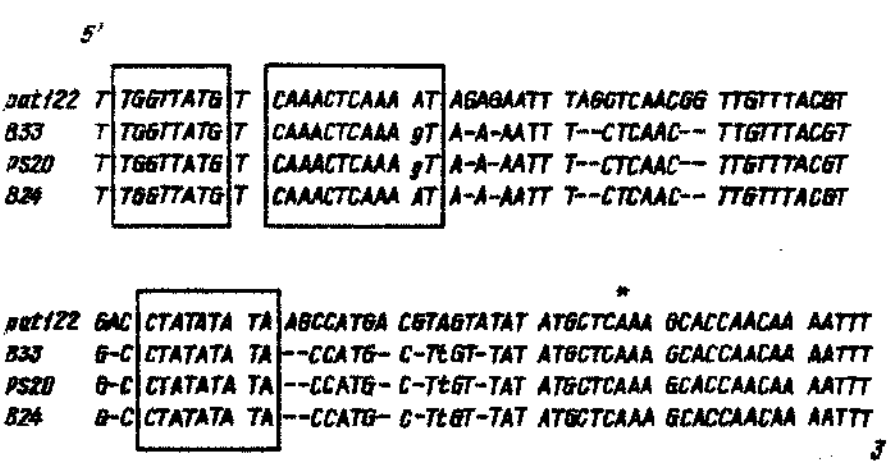
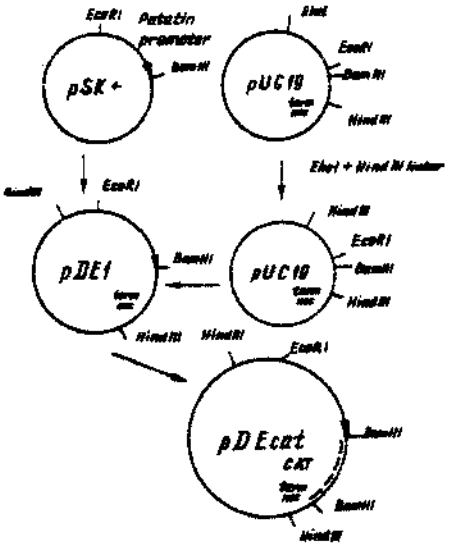


Fig. 3. DNA sequences of the 5' flanking regions patatin genomic clone *λpat122* and alignment of the 5' sequences of different class I patatin genes. Sequences homologous to the core enhancer, CAAT and TATA elements are boxed. The transcription start point is indicated by symbol *

of DNA sequence of the isolated patatin promoter shows that it contains regions homologous to the TATA and CAAT consensus sequences at 36 and 80 bp upstream of the transcription start point, respectively. Computer comparison of sequence homology between the sequenced fragment and some patatin sequences from the EMBL database allowed us to located the putative promoter regulatory elements and to determine the patatin promoter orientation in *λpat122* clone. These results are presented in Fig. 3. Thus, it was found that *λpat122* clone contains the 5'-upstream regulatory region and only a part of the patatin coding sequence. As can be seen in Fig. 3, the differences between the sequenced fragment and 5'-regions of the other class I promoter fragments are mainly due to point insertions in *λpat122* sequence. We could not find the analogous sequence among the patatin genes available frome EMBL database. It was concluded that we cloned one of the unexplored patatin gene. It should be noted that we did not determine the nucleotide sequence of the 5'-untransiated region of the *λpat122* and therefore had no information whether it contained a 22 bp insert. However, using a computer search program, we found that sequences homologous to the oligos choosed for hybridization could be found only in the 5'-region of the class I patatin genes.

Construction of a chimeric gene containing the 5'-upstream region of the patatin gene PAT122 fused to the coding region of the chloramphenicol acetyltransferase (CAT) gene and transfer into potato. In order to analyse whether the upstream region of gene *PAT122* is sufficient to obtain a controlled expression in transgenic potato plants, a *DraI* fragment extending from position +10, −1.8 kb into the upstream region, was fused to the *CAT* gene from *Ecsherichia coli* and the poly(A) site of the nopaline synthetase gene added to the 3'-end of this gene. The scheme construction presented in Fig. 4. A 1.8 kb *DraI* fragment, as indicated in Fig. 1, was cloned in *SmaI* site of plasmid vectors *pSK*bluescript(+) (Stratagene Inc., San Diego, CA) [13]. The 1.8 kb *EcoRI-BamHI* promoter fragment was subcloned into *pUC19* containing recloned into *BamHI-HindIII* nopaline synthetase terminator (nos 3'). *pUC19* digested *EheI* and fused *HindIII* linker to yield *pUC19*/patatin promoter/term nos/*HindIII* flanking construct. This construct named *pDE1* containing *BamHI* site between patatin promoter and NOS terminator. Into *BamHI* site was fused coding sequences *CAT* gene (Fig. 4) to yield the plasmid *pDE/CAT/HindIII* flanking construct. This was digested with *HindIII* and cloned into *HindIII*-digested *pBin19* [14] to yield the final construct, a *pBin19*-based binary vector



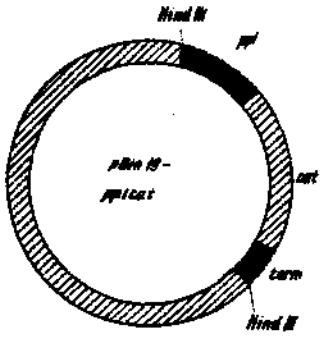


Fig. 4. Construction vectors molecule for tuber-specific gene expression and chimeric gene containing the RNA-coding region of the chloramphenicol acethyltransferase gene and the 5′ upstream region of the patatin class I gene *λpat122*

Fig. 5. The structure a binary vector containing coding region of chloramphenicol acethyltransferase gene under control of the *λpat122* patatin promoter

(*pBin19*/patatin promoter/*CAT*/term nos) with the transferred DNA containing the *npt*II-selectable marker, and a *CAT* reporter gene under the control of a patatin class I promoter (Fig. 5). The recombinant vector was transformed into the *Agrobacterium tumefaciens* strain *pGV3850* Km^r. By reisolating the plasmid DNA and digestion with appropriate enzymes (data not shown) the correct structure of the chimeric gene in *Agrobacterium* was proven.

Subsequently tuber discs of potato plants cv. Zarevo were infected and transformed plants (see "Materials and Methods") was use for analysis *CAT* activity.

The upstream region of the patatin gene PAT122 directs tuber-specific expression of the fused CAT gene. Because of the high variability in the level of expression of genes in plants independently transformed with the same construct, we decided to analyse a statistically significant number of transformed potato plants for expression of the chimeric patatin gene. Thus in the first screening leaves and tubers of 15 independently derived transgenic potato plants were assayed for *CAT* activity using the method Gorman et al. [19]. As expected the *CAT* activity varied from plant to plant however mid activity and the same in tested plants. Southern analysis of transformed plants showed in all cases analysed the presence of intact nonrearranged chimeric genes. The copy number varied between 3 and 10, in a few cases more than one integration site was seen. Is be showed in [3] no correlation between the level of expression and copy number integrated was observed. The mean value for the *CAT* activity in tubers analysed transgenic plants was 19 500 $imp \cdot min^{-1} \cdot mg^{-1}$ protein as compared to a value 89 $imp \cdot min^{-1} \cdot mg^{-1}$ protein in leaves. In order to futher analyse the organ specificity of the expression of the transferred gene, 10 plants exhibiting a high level of expression in tubers were also analysed for *CAT* activity in stem and root tissue. As showed in Fig. 6 (middle typical result) in all cases, the expression was highest in tubers followed by stems and roots and lowest in leaves. The mean value in tubers from all tested transgenic plants was ~100-fold higher when compared to stem, ~150-fold higher than in roots and 1500-fold higher than in leaves. The discrepancy between the average value for the activity in the leaves of the 15 plants described above and data for the 10

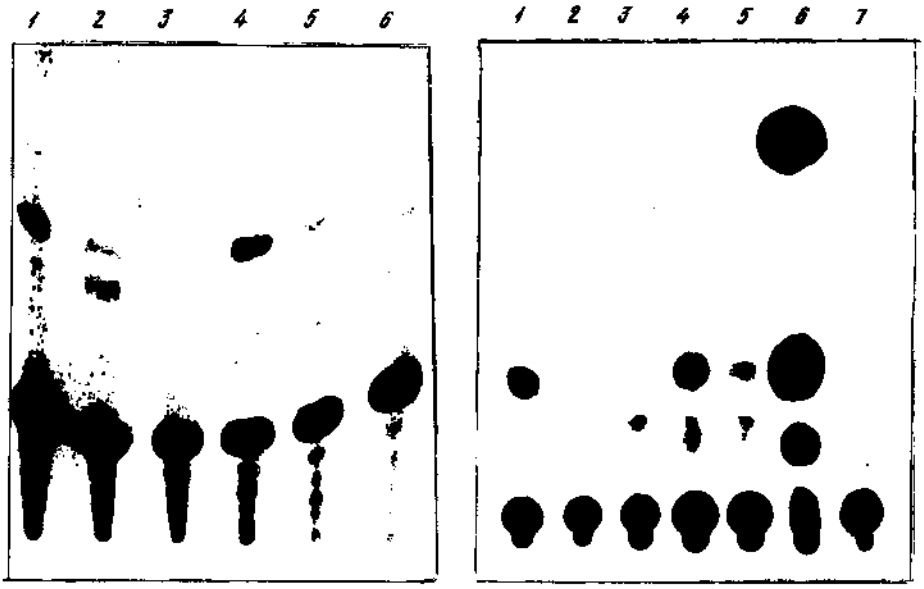


Fig. 6. *CAT* assays of leaf, tubers, stems extracts obtained transgenic potato plants transformed with either the *λpat122-CAT*. Extracts assayed for *CAT* activity were adjusted to same amounts of protein: *1*, *4* — tubers; *2* — extract *E. coli* with *pBR325*; *3* — untransformed plants (tuber); *5* — stems and leaves; *6* — roots

Fig. 7. Comparison activities *CAT* in plants which transformed of plasmids *pDEcat* and *pCaMVcat*: *1* — *pCaMVcat* (tuber); *2*, *3* — *pDEcat* (leaves); *4*, *5* — *pDEcat* (tuber); *6* — *E. coli* (*pBR325*); *7* — C^{14}-chloramphenicol

plants given here (which subset of the 15 plants) will be discussed later.

The *CAT* expression with patatin promoter we compared with activity in plant transformed *pCaMVcat* plasmids, these results presented in Fig. 7. As shown at the chromatogram picture, the strenth patatin promoter stronger than *CaMV* promoter. However, the level of activity observed in tubers with the *B33* patatin promoter [3, 5] is within the same range as the one observed with other strong promoters such as the 35S promoter of the *CaMV* and the photosynthetic *ST-LS1* gene [11].

The data presented here show that a class I patatin gene (*λpat122*) carries a *5'*-upstream promoter region of 1.8 kb which on average is 100- to 1000-fold more active in tubers as compared to stems, roots or leaves. Futhermore the *λpat122* as other promoters class I [3, 5, 7] also carry a *cis*-acting elements that react to metabolic signals. One and the same member of the gene family can therefore mediate patatin expression in tubers but also in other organs under defined metabolic conditions (high concentration sucrose).

Were a theory advanced according to availability of starch or one of its precursors is a signal not only initiate the morphological changes typical for tuberization but also for the activation of "tuber-specific" genes. If this hypothesis would turn out to be correct the *λpat122* promoter with different reporter genes would provide a convenient marker to study the factors involved in the switching of a somatic tissue into a storage tissue.

We are also planning to obtain constructions which contain coding regions homoserine kinase and glucoronidase genes under control of the *λpat122* patatin promoter. Then these constructions planning to introduce into sugar beet plants.

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ОРГАНОСПЕЦИФІЧНА ЕКСПРЕСІЯ У ТРАНСГЕННИХ РОСЛИНАХ КАРТОПЛІ — КЛОНУВАННЯ НОВОГО ПРОМОТОРА ГЕНА ПАТАТИНУ КЛАСУ І

Резюме

З застосуванням синтетичних олігонуклеотидних зондів, гомологічних консервативному АТ-багатому мотиву пататинових генів класу I, з геномної бібліотеки генів картоплі виділено два клони. Один клон, названий *λpat122*, було субклоновано і визначено нуклеотидну послідовність 5'-кінцевої ділянки. Використовуючи ген хлорамфеніколацетилтрансферази як репортерний, було показано, що 1.8 kb фрагмент промотора гена пататину *λpat122* несе в собі всю інформацію, необхідну для бульбо-специфічної і цукрозо-регульованої експресії у трансгенних рослинах картоплі.

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