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Genetic transformation of potato (*Solanum tuberosum L.*) using a binary *Agrobacterium tumefaciens* vector with patatin promoter class I

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Kanamycin resistant plants of S. tuberosum L. (in vitro-grown) cv. Zarevo were obtained from the cocultivated microtubers with A. tumefaciens. A disarmed binary vector systems containing the neomycin phosphotransferase (NPT II) gene as selectable marker and chloramphenicol acetyltransferase (CAT), as a reporter gene, under control of new patatin promoter class I were utilized. In vitro grown minitubers discs were used as sources of explants to produce transgenic plants on selective medium containing 100 μg/l kanamycin and CAT enzyme activities were detected.

Introduction There have lately been mayor advances in plant biology at the cellular and molecular levels. These include the biochemical dissection of developmental process, molecular assays of gene expression and the development of stably transformed plants. Potato, the most important non-cereal crop in the world, is proving to be one of the first beneficiaries of these technological advances. Thus, a considerable effort has been made to improve its qualities by genetic engineering methods such as Agrobacterium-mediated gene transfer. The first reports of transformed potato plants were from *in vitro* shoots of a tetraploid potato (cv. Maris Bard) infected with an oncogenic strain of *A. tumefaciens* [1]. Clones which produced opines could not give roots growth, strongly suggesting that these shoots contained the complete T-DNA sequence, since these encoded genes for opine synthesis and the plant growth regulators.

The use of perimedullary tuber tissue for transformation was first reported in 1987 [2]. Sheerman and Bevan (1988) refined the tuber disc systems and reported it to be a very rapid and prolific way to obtain kan^R plants carrying the NPT II gene. Shoots appeared on 6 —20 % of the discs within 6 weeks using cultivars, Desiree, and Golden Wonder. However, cv. Golden Wonder gave only a 30 % transformation efficiency [3, 12].

Using recombinant DNA and gene transfer technology, new approaches to

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understanding tuberization have recently become available. The study of tuberization at the molecular level requires some way to recognize a tuber biochemically. The primary biochemical marker that we have used in our work is the major tuber protein, patatin.

In plants that use somatic tissue such as roots, bulbs and tubers as major storage organs, abundant proteins are deposited for subsequent remobilization, along with other storage reservesants as starch [4]. Potato *(S. tuberosum)* tubers accumulate two classes of major proteins, as 40 kD patatin protein and 20 proteinase inhibitor [4, 6]. Patatin is a lipolytic acyl hydrolase, which may release fatty acids from membranes as part of a defense response [7]. Patatin is encoded by two classes of genes: type I genes are expressed at high levels of expression in roots and peripheral cells of the tuber [5—8]. S1 nuclease protection and primer extension experiments have shown that the class I patatin genes encode 98—99 % of the patatin mRNA in tubers.

The promoter of class I patatine genes is tuber-specific. Giving the important role of sucrose levels in determining gene expression, it is possible that the distribution of patatine in mature potato plants could be determined by the high concentration of sucrose [4, 15]. The class II patatin genes encode only 1 —2 % of the patatin mRNA in tubers, but unlike the class I genes, they are also normally expressed at low levels in roots. The patatin protein in roots is immunologically and electrophoretically distinct from that in tubers, but this may be due to differences in posttranslational proceeding. While the number of class II genes is equal to or greater than that of class I, many of them appear to be pseudogenes [4, 8].

To determine which part of the patatin genes are responsible for their tissue specific patterns of regulation, the 5′ flanking sequences from both class I and class II patatin genes was attached to the GUS reporter gene in the binary *Ti*-plasmid vector *pBI101.1* using the *DraI* site at position +10 of both classes. These constructs were then transferred into *A. tumefaciens* strain LBA4404 and were used to produce transgenic plants using the leaf disc transformation method [9].

Extracts from tubers from plants containing 2.5 kb of 5′ flanking sequence from the class I patatin gene, PS20, had high levels of GUS activity (≈3000-fold higher than those seen in stolon tips before tuberization). However, under normal conditions, the GUS reporter gene was not expressed at significant levels in roots, stems or leaves of either tuberizing or nontuberizing plants [4, 10].

The method we have developed allows the rapid recovery of many transformed potato shoots directly from Agrobacterium-infected tuber tissue. From regenerated shoots *in vitro* tuberization was induced in the solid propagation medium. In minitubers activity of reporter gene CAT (chloramphenicol acetyl transferase) was observed. This method will encourage the study of gene regulation and crop improvement in this important plant.

Material and Methods. *Plant material.* Virus-free tubers of *S. tuberosum* var. Zarevo and Nevsky were obtained from Potato Selection Station (Lutsk, UA). Shoot cultures were maintained by subculturing of nodal cuts on a sterile medium containing Murashige and Skoog (MS) salts [13], vitamins, 20 g/l sucrose, solidified with 0.8 % agar (Difco), pH 5.8. Potato shoots culture were maintained at a temperature of 22—24 °C and exposed for 16 h daily at 2500 lux illumination. Two different media for *in vitro* tuberization were compared: 1) a solid modified MS containing 7 % sucrose, 4.5 mg/l benzyladenine (BA); 2) modified MS medium containingm 10 % sucrose, 10.0 mg/l BA and 1.3 ml/l CCC (chlorocholinechloride), supplemented with 2 g/l Gelrite (Sigma). The medium was distributed as aliquots of 10 ml culture test tubes, capped with foil closures and sterilized by autoclaving for 15 min at 1.05 kg/cm^2.

Bacterial strain and growth conditions. A. tumefaciens strain GV 3101, a nopaline strain, C58 type chromosome, carrying the binary vector plasmid system *pBin* — *λpat122* CAT, a modified *Bin19* vector (provided by M. Bevan, AFRC, U. K.) and *pGV3850* (provided by J. Schell, Max Planck Inst., Koln) was used. *pGV3850* is the helper plasmid, and *pBin* — *λpat112* contains the coding sequence of the bacterial neomycin phosphotransferase II (NPT II) gene under the control of the nopaline synthase promoter and the bacterial CAT gene under the control of the *λpat122* patatin promoter class I, which was early obtained in our laboratory [11, 15].

The strain was grown at 28 °C in LB medium: (Bacto-tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l) using rotary shaker to the late logarithmic stage. The bacteria were centrifuged and pellet wash resuspended to the same density in MS medium containing 2 % sucrose and used for the cocultivation procedure.

Transformation and regeneration procedure. In vitro-grown potato microtubes, less than 5 month old, were freshly harvested and cut into 1-mm slices. The discs were placed with a cut surface down in petri plates containing a medium containing the MS salts, 2 % sucrose, vitamins, and solidified with 0.8 % agar (Difco). A small amount of Agrobacterium was picked off bacterial lawn using a bacterial loop and transferred to the upper cut surface on the tuber discs. The petri plates were placed in an incubator maintained at 24 °C.

After two days of cocultivation, the inoculated slices were transferred to fresh modified MS medium with 500 mg/l cefotaxime (Roussel) added. Every three weeks the explants were transferred to fresh modified medium, which contained MS salts, 3 % sucrose, vitamins, 0.2 mg/l BA, 0.1 mg/l zeatin, 0.02 mg/l naphtalenacetic acid (NAA), and 0.7 % agar (Difco). The first shoots from tuber discs were initiated after 10—12 days in modified MS medium.

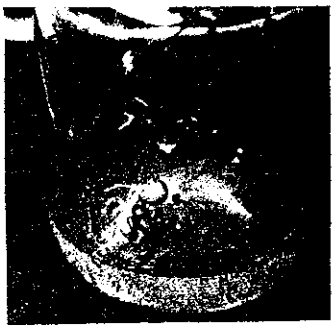
Plants that developed from transformed minitubers were used for *in vitro* tuberization and expression assay.

CAT expression assay. CAT enzyme activities were analyzed in young tubers, which were initiated from transformed plants. For this purpose Gorman et al. method was used [14].

Results and Discussion. Currently, transformation work in potato has been done with *A. tumefaciens*, because the *Solanaceae* species, is very sensitive to Agrobacterium infection [10]. The experiments described here, showed that the method used for potato transformation has several advantages compared with other methods. So far the advantage is that a medium has been found that allows the *in vitro* tuberization and using tuber discs for transformation procedures. The second advantage is the possibility of using *in vitro* induced tubers obtained from transformed plants which allow rapid study of a class I patatin promoter activity and CAT reporter gene expression at significant levels in tuberizing transgenic plants.

The combination of phytohormones in modified MS medium caused to the tuberization process. Optimal concentration of the plant growth regulators in nutrient medium as: BA and CCC were doubled compared with the high sucrose concentration (10 %), and low concentration of nitrogen in nutrient medium, could easily be induced tubers *in vitro* techniques. The situation with BA is more subtle. BA does not facilitate the sucrose induction of patatine gene expression even through it promotes tuberization *in vitro* and the level of cytokinin has been shown to increase when plants are placed under conditions that favor tuberization.

Fig. 1 shows *in vitro*-grown tubers in potato plants after 5 weeks of cultivation in modified MS medium. We have thus demonstrated Agrobacterium-mediated genetic transformation of cells of *in vitro*-grown microtuber discs of Zarevo potato cultivars. The success with production of transgenic plants from inoculated tubers using Agrobacterium-mediated gene transfer depends



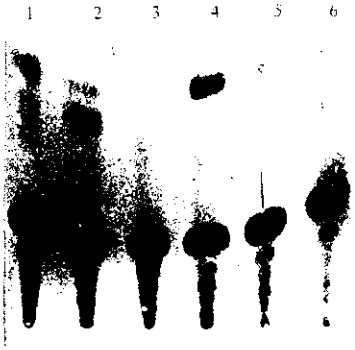


Fig. 1. *In vitro*-grown tubers in potato transformed plants after 5 weeks cultivation in modified MS medium

Fig. 2. Comparison of the CAT activity in different plant organs of the transgenic potato plants: *1*, *4* — tubers; *2* — *E. coli* with *pBR325*; *3* — nontransformed plants; *5* — stems; *6* — leaves

primarily on the availability of high frequency shoot regeneration from tuber tissues. Apparently the microtuber discs transfer on modified medium supplied with breaks bud dormancy and active growth begins. After the primary shoots were excised from Agrobacterium-infected tuber discs, new shoots appeared from the same sites. After 2.5—3 months, these new secondary shoots were placed on modified MS medium for tuberization. Both a solid induction medium and a liquid medium which generally give larger using different phytohormone concentrations showed which roots did not develop due to the high concentration of BA more than 1 mg/l in modified MS medium. However, full tuberization was achieved by simply overlayering the modified MS medium when plants had a developed good root system. The tubers produced by this method were about 3 times larger.

The results presented in Fig. 2 show the CAT activity in different organs of transgenic potato plants. This experimentation has shown the importance of promoter selection in obtaining transient CAT expression in transgenic potato plants. To determine what factors are responsible for the induction of class I patatin promoter expression, we performed a series of experiments in which segments from transgenic plantlets containing the class I patatin promoter/CAT gene. Using the CAT as a reporter gene it has been shown that a 1.7 kb fragment of the plasmid containing promoter of the class I patatin gene *PAT122* provides necessary for both tuber-specific and sucrose-induced expression in leaves in transgenic potato plants. The use of binary, disarmed Agrobacterium vectors, which may be easier for genetic manipulation with potato plants cv. Zarevo and Nevsky potato species.

Our work with patatin has led to the discovery that the differentiation of transformed segments to tubers and some cultural factors are important determinants of morphogenesis in the potato species.

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Генетична трансформація картоплі (*Solanum tuberosum L.*) за допомогою бінарного вектора *Agrobacterium tumefaciens* з промотором пататину класу I

Резюме

Стійкі до канаміцину рослини-ресенеранти картоплі S. tuberosum L. (сорту Зарево) було отримано шляхом культивування мінібульб зі штамом A. tumefaciens. Використано бінарну векторну систему, яка містила ген неоміцинфосфотрансферази (НФТ II) як селективний

маркер та репортерний ген хлорамфеніколацетилтрансферази (CAT) під контролем промотора пататину класу I. Трансгенні рослини було одержано з трансформованих мінібульб картоплі на селективному поживному середовищі з концентрацією канаміцину 100 мл/л. Було підтверджено активність CAT.

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Генетическая трансформация картофеля *(Solanum tuberosum L.)* с помощью бинарного вектора *Agrobacterium tumefaciens* с промотором патагина класса I

Резюме

Устойчивые к канамицину растения-регенеранты картофеля S. tuberosum L. (сорта Зарево) получены культивированием миниклубней со штаммом A. tumefaciens. Использована векторная система, содержащая ген неомицинфосфотрансферазы (НФТ II) как селективный маркер и репортерный ген хлорамфениколацетилтрансферазы (CAT) под контролем промотора пататина класса I. Трансгенные растения получены из трансформированных миниклубней картофеля на селективной питательной среде с концентрацией канамицина 100 мл/л. Была экспериментально подтверждена активность CAT.

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