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Effect of *Rol* Transgenes, IAA, and Kinetin on Starch Content and the Size of Starch Granules in Tubers of *In Vitro* Potato Plants

I. A. Gukasyan, S. A. Golyanovskaya, E. V. Grishunina, T. N. Konstantinova, N. P. Aksenova, and G. A. Romanov

Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya ul. 35, Moscow, 127276 Russia; fax: 7 (095) 977-8018; e-mail: romanov@ippras.ru

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Abstract—Stem cuttings were produced from *Solanum tuberosum* L., cv. Désirée, plants and their transgenic forms harboring *rolB* and *rolC* genes from *Agrobacterium rhizogenes.* Plants were cultured on hormone-free Murashige and Skoog nutrient medium (MS) and on MS supplemented with IAA or kinetin. In microtubers developed on these cuttings, we estimated the content of starch and the number and size of starch granules. Expression of *rol* genes changed these indices: in tubers of *rolC* transformants, a greater number of small granules were produced, whereas in tubers of *rolB* transformants, a fewer number of large granules were developed as compared with wild-type plants. Expression of *rol* genes did not affect starch content during the first three weeks of cutting culturing but increased it by 15–30% in five-week-old tubers. IAA addition to MS medium increased starch content and the size of starch granules in control plants and *rolB* tubers by 10–30%, whereas kinetin did not exert any significant influence. The effects of *rol* transgenes on the initiation and termination of starch granule development are discussed.

Key words: Solanum tuberosum - rol transgenes - tuberization - starch granules - auxin - kinetin

INTRODUCTION

Potato tuberization is characterized by active starch biosynthesis and its deposition in starch granules of amyloplasts. During this process, soluble sugars are converted into an osmotically inert storage compound, and this facilitates assimilate inflow to developing tubers [1]. In addition, starch content and quality determine potato nutritional value and its suitability for technical treatments [2]. Currently, the pathway from sucrose uptake by tuber cells to the synthesis of phosphorylated sugars and their penetration into amyloplasts are well studied; a crucial role of ADP glucose pyrophosphorylase in starch biosynthesis in potato plants is also elucidated [3]. Functioning of specific enzymes determining amylose and amylopectin structures and their packing in starch granules are studied as well [4]. However, processes determining the number and sizes of produced starch granules are still obscure.

Transgenic plants can be used for investigation of potato carbohydrate metabolism. Earlier it was shown that *rolB* and *rolC* genes of *Agrobacterium rhizogenes* inserted into the plant genome could change various characteristics of tuberization [5, 6] including dependence of tuber initiation on sucrose concentration in nutrient medium and the size of starch granules. Frequently, *rolB* and *rolC* genes exerted an opposite action. Therefore, further investigations of transformants harboring these genes are rational in order to better understand the regulation of starch accumulation and deposition in tubers and to elucidate the possibility for the control of this process.

The insertion of *rol* genes is known to evoke, in some cases, morphological changes similar to phytohormonal effects, i.e., expression of the *rolB* gene resembles auxin action, whereas expression of the *rolC* gene resembles cytokinin action [7]. However, inspite of numerous suggestions [8] the mechanisms of transgene action remain unstudied. Some reports appeared about substantial effects of exogenous auxins and cytokinins on particular reactions of carbohydrate metabolism in potato tubers and about the enzymes involved. Thus, kinetin-stimulated tuberization of cultured stolons was accompanied by an activation of some enzymes of starch metabolism [9]. Auxin treatment of potato plants in soil culture enhanced ^{14}C-glucose flow to tubers [10], whereas kinetin and IAA treatments of potato tuber disks activated ^{14}C-glucose incorporation into starch at the early stages of tuber development but did not affect this process in mature tubers [11]. In some plants, cytokinins stimulated the synthesis of invertase and simultaneously activated hexose transport

Abbreviations: C_{wt}—wild-type control plants; MS—Murashige and Skoog nutrient medium.

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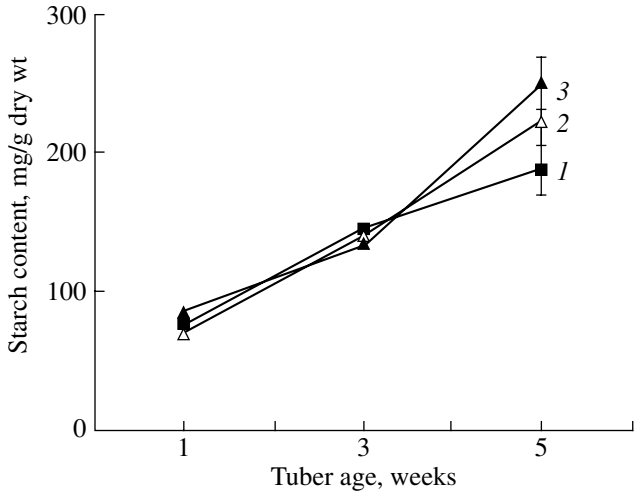

Fig. 1. Starch content in potato tubers of transformed and wild-type plants as dependent on tuber age.
(*1*) C_{wt}; (*2*) *rolC*; (*3*) *rolB*.

to the cells of sink tissues [12]. However, the mode of cytokinin and auxin action on processes related to starch granule formation and starch deposition in potato tuber amyloplasts remains to be studied.

The objective of this work was to evaluate the effects of *rol* genes on starch content in potato tubers and on the number and size of starch granules in them, and to compare the effects of *rol* genes with those of exogenous phytohormones, IAA and kinetin.

MATERIALS AND METHODS

Experiments were performed with potato (*Solanum tuberosum* L., cv. Désirée) plants and their transgenic forms harboring the *rolB* and *rolC* genes under the control of the *B33* class I promoter, which provided for their predominant expression in developing tubers. The *rol* genes were isolated from *A. rhizogenes* strain A-4. Plant transformation and characterization of transgene expression were performed in the Institute of Genetic and Biological Investigations (Berlin-Golm, Germany) and described earlier [13].

Wild-type (C_{wt}) and transformed (*rolB* and *rolC*) plants were cultured on agar-solidified MS medium supplemented with 60 mg/l *myo*-inositol, 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, and 2% sucrose. Plants were grown in a phytotron chamber at 22°C and illumination with LB-80 white luminescent lamps at a 16-h photoperiod. Stem cuttings of single nodes were prepared from these plants. These cuttings were planted on the nutrient medium containing 8% sucrose and devoid of phytohormones or containing 1 mg/l IAA or 1 mg/l kinetin. Cuttings were cultured in continuous darkness at 20°C. Microtubers started to develop from the axillary buds on the sixth day after cutting planting. Cuttings were cultured for six weeks, and the sizes of starch granules and starch content in microtubers were periodically estimated during this period. Figures present the mean values and their standard errors.

Granule sizes were measured in their suspensions obtained from entire tubers; the number of granules was counted on transverse sections of the middle parts of tubers. The suspensions of starch granules were prepared after the homogenization of whole tubers with subsequent removing of cell debris, staining of starch granules with 0.2% iodine solution in 2% KJ, and adding glycerol. In order to obtain transverse sections, tubers were fixed in 8% paraformaldehyde, washed, and embedded in paraffin. Transverse sections 20–25 μm thick were prepared using a rotation microtome (Reichert, Austria), stained with a iodine solution, dehydrated in a series of alcohols and xylol, and embedded in Canadian balsam. In order to determine granule sizes (projection area), we photographed preparations with an Amplival light microscope (Carl Zeiss, Germany). The projection areas of starch granules were measured on negatives using a VOP Videoplan (Reichert).

Starch content in tubers was estimated by routine methods [14]. Tissue samples were fixed and homogenized in 95% ethanol; soluble sugars were removed by washing with 82% ethanol (three times with centrifugation); starch was hydrolyzed by 7 N HCl with added dimethylsulfoxide at 70°C; after hydrolyzate neutralizing, glucose content was assayed spectrophotometrically using a Biosub-GLU reagent (Germany).

RESULTS

Figure 1 shows an age dependence of starch content in tubers of C_{wt}, *rolC*, and *rolB* plants. Tubers of all genotypes contained less starch than those of potato plants grown under field conditions [15]. This fact can be explained by plantlet culturing in continuous darkness and by small tuber sizes (their fresh weight was 30–40 mg on the average). The content of starch in cultured tubers increased gradually with their age. During the first three weeks of culturing, there was no significant difference in starch content between genotypes. After five weeks, tubers of transformed plants, especially *rolB* forms, contained more starch than C_{wt} tubers.

Figure 2 shows the effects of IAA and kinetin treatments on starch content in three-week-old tubers. When plantlet were cultured on hormone-free medium, expression of *rol* genes almost did not affect starch content in tubers of this age, which corresponds well to results presented in Fig. 1. Auxin addition to the medium increased slightly (by 13%) starch content in control tubers, but its effect was more substantial (up to 31%) in *rolB* plants. In *rolC* transformants, starch content in tubers decreased considerably in the presence of auxin, which might be related to a negative response of tuber growth of these plants to auxin observed earlier [5]. Kinetin affected starch content in tubers weaker than IAA.

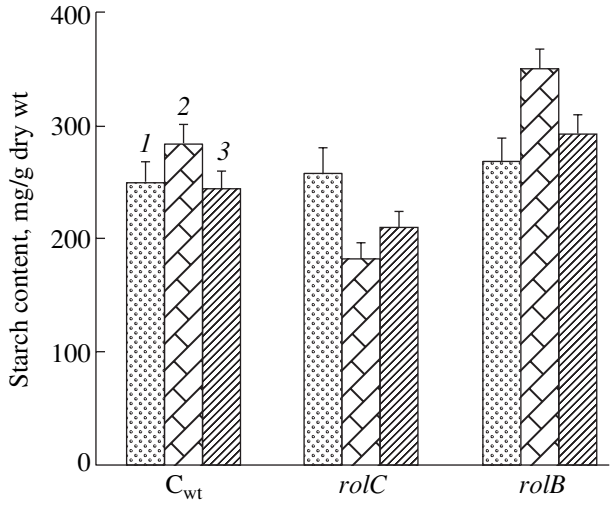

Fig. 2. Starch content in three-week-old potato tubers of transformed and wild-type plants cultured on (*1*) hormone-free, (*2*) IAA-containing, and (*3*) kinetin-containing media.

In *rolC* tubers, kinetin reduced starch content relative to control tubers, whereas in C_{wt} and *rolB* plants, kinetin effects were insignificant.

In general, neither plant transformation nor IAA and kinetin treatments resulted in considerable changes in starch content in potato tubers, especially during the early stage of their development.

Starch accumulation in tuber amyloplasts occurs via its deposition as starch granules, which have an ordered semi-crystalline structure from amylose and amylopectin chains [4]. Preliminary microscopic observation demonstrated that the tuber tissues, such as cortex, pith, phellodermis, and others, differed in the sizes of starch granules in their cells and the ratio between the tissue volumes also differed in tubers of *rol* transformants and wild-type plants [16]. In this connection, we studied the effect of *rol* genes on the population of granules in the suspensions from whole tubers. Figure 3 presents the appearance of such suspensions. It is evident that starch granule populations were not homogenous: granules differing in their sizes occurred in all genotypes. However, it is clearly seen that *rolC* transformants produced smaller and *rolB* transformants larger granules than C_{wt} plants.

The measurement of starch granule sizes (average projection areas) as dependent on tuber age showed that, in all genotypes, granule enlargement occurred during tuber growth (Fig. 4). The average size of starch granules in *rolC* tubers remained smaller and in *rolB* tubers was larger than in control tubers during the entire period of observation. Thus, expression of *B33rolC* gene created conditions for the formation of smaller starch granules, whereas expression of *B33rolB* gene, for larger starch granules.

Since in genotypes studied, starch content differed much less than the size of starch granules, it seems evident that tubers contain different number of granules. We met some methodological difficulties when tried to count the number of starch granules in various tissues on tuber transverse sections. Therefore, we obtained only rough preliminary results. Our measurements showed that various tissues of *rolC* tubers contained 2–5-fold more starch granules and *rolB* tubers by 80–90% less starch granules than corresponding tissues of wild-type plants. Thus, *rolC* and *rolB* transgenes affected not only the size but also the number of starch granules.

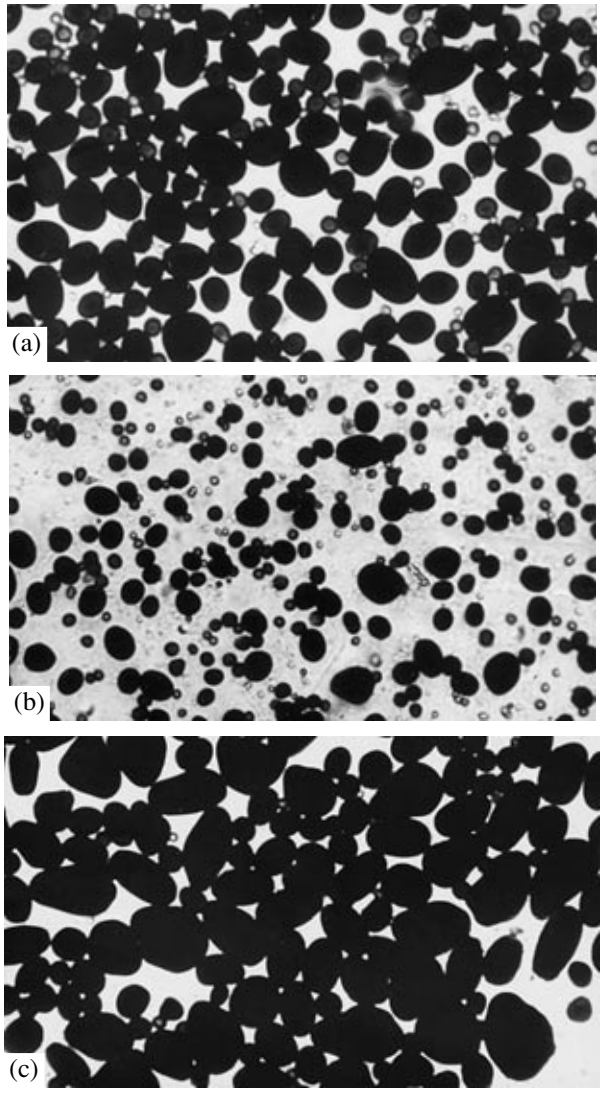

Fig. 3. Suspensions of starch granules from potato tubers. (a) C_{wt}; (b) *rolC*; (c) *rolB*. Magnification of ×800.

Figure 5 compares the effects of *rol* transgenes and exogenous IAA and kinetin on the size of starch granules. On hormone-free medium, the projection areas of starch granules in tubers of *rolC* plants was twofold smaller and in *rolB* plants 1.5-fold larger than in wild-type plants, which corresponds well to the data presented in Fig. 4. On IAA-containing medium, a slight

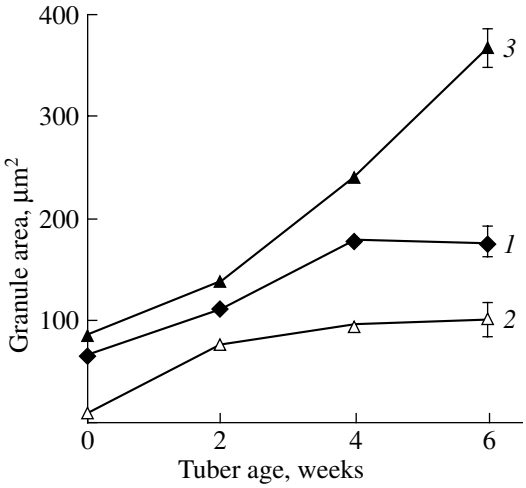

Fig. 4. An average size of a starch granule in tuber suspensions in transformed and wild-type potato plants of various age. (*1*) C_{wt}; (*2*) *rolC*; (*3*) *rolB*.

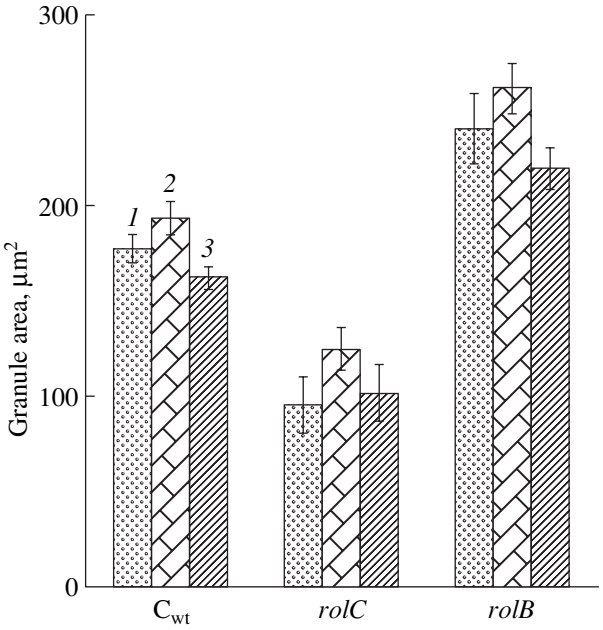

Fig. 5. An average size of the starch granule in suspensions from three-week-old tubers of transformed and wild-type potato plants cultured on (*1*) hormone-free, (*2*) IAA-containing, and (*3*) kinetin-containing media.

increase in the starch granule size was observed. On kinetin-containing medium, a tendency to granule reduction was manifested. However, these hormone-induced slight changes were statistically insignificant. Phytohormones did not prevent stronger and clear effects of *rol* genes on the size of starch granules. Under all hormonal treatments, the average projection area of the starch granule in tubers of *rolC* transformants was 2–2.5-fold smaller than in the similarly treated *rolB* transformants.

DISCUSSION

A comparison between effects of *rolC* and *rolB* transgenes and those of exogenous kinetin and IAA showed that there were common and specific features in their action.

Both expression of the *rolB* gene and IAA addition to the culture medium resulted in a small increase in starch content in tubers. However, the time course of such increase differed in these treatments: a tendency to starch accumulation in *rolB* transformants appeared only in five-week-old mature tubers, whereas IAA-induced increase in starch content was noticed earlier, in three-week-old tubers. Kinetin effects on starch content were weaker than IAA effects and depended of the genotype. Similarly, *B33rolC* gene expression was less efficient than *rolB* expression.

Phytohormones and *rol* genes affected differently also the size of starch granules. Both IAA and *rolB* increased the granule size, a transgene being much more efficient than IAA. The insertion of *B33rolC* resulted in a sharp decrease in the average area of the starch granule (twofold and more), whereas exogenous kinetin exerted only weak and ambiguous action. Thus, *rol* transgene effects on the size of starch granules was substantially more pronounced than the effects of corresponding phytohormones.

At present, many research teams study actively the mechanisms controlling the formation of starch granules and their sizes [3, 4]. By genetic-engineering approaches, it was shown that selective suppression of one of two forms of starch synthase resulted in a considerable (two- or threefold) reduction of starch granule sizes in potato tubers [17]. Similar result was observed when the two forms of the enzyme responsible for glucan-chain branching were suppressed [18]. In both studies, a sharp decrease (two- to fivefold and more) in the starch content in tubers was observed along with the formation of small granules. The insertion into the potato genome of a gene from *Bacillus subtilis* encoding levansaccharase and stimulating fructan accumulation in amyloplasts also resulted in a decrease in the starch granule sizes and a sharp reduction of starch content in tubers [19]. In our experiments, *rol* transgenes inducing changes in the sizes of starch granules did not result in such a considerable suppression of starch accumulation and evidently acted otherwise than in aforementioned studies.

Processes controlling starch granule initiation and the termination of their growth remained obscure. Granule formation was shown to start from the lipid structures on the derivatives of the amyloplast inner membrane with subsequent clustering of glucan, chemically resembling starch, at these sites [20]. Thereafter, granules grow by deposition of new starch portions on this starch nucleus. The data obtained in this work per-

mit a supposition that one of the *rolC* and *rolB* gene effects in potato tubers is their involvement in the control of starch granule initiation (changing the number of granules) and their growth completion (determination of granule sizes).

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