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Research article

Does the expression of the maize gene Zm-p60.1 encoding the enzyme β-glucosidase inactivate enzymes of starch metabolism in transgenic tobacco pollen?

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Abstract

The dynamics of starch metabolism in tobacco pollen was studied in the developing pollen of positive plants (i.e. plants expressing Zm-p60.1 in the leaf) in comparison to that of the negative plants (i.e. plants not expressing Zm-p60.1 in the leaf), in order to elucidate the possible role of Zm-p60.1 in starch metabolism. Fifty percent of the pollen population from the plants expressing the β-glucosidase was found to be sterile. The transgenic tobacco pollen development was divided into six developmental stages/phases which correlated with distinct cytological characteristics. On the basis of microscopic and histochemical studies, using X-glc (5-Bromo-4-chloro-3-indolyl-β-D-glycopyranoside) as substrate, β-glucosidase activity was found in half the pollen of the transgenic tobacco plants. Detection of the β-glucosidase (Zm-p60.1) was achieved using β-glucosidase histochemical and biochemical assays. The activities of selected enzymes of sucrose metabolism (UDPG-pyrophosphorylase) and starch metabolism (ADPG-pyrophosphorylase, starch-phosphorylase, and amylase) were biochemically analyzed. Histochemical studies with potassium iodide revealed that 50% of the pollen of the positive plants (i.e. tobacco plants expressing β-glucosidase activity) exhibited normal starch accumulation. Biochemical determination of starch revealed that the pollen expressing Zm-p60.1 contained significantly lower amount of starch in comparison to the pollen from negative plants (i.e. tobacco plants not expressing β-glucosidase activity). About 50% of the pollen population expressing Zmp-60.1 had decreased starch content. The same starchless pollen exhibited β-glucosidase activity.

Keywords: β-glucosidase; Zm-p60.1; enzymes of starch metabolism; transgenic tobacco; transcription inhibitors; pollen stages, biochemical and histochemical assays; transformants; streptomycine resistant; cytokinins; etc.

Introduction

This research work was devoted to studies on the influence of the expression of maize β-glucosidase in tobacco plants on pollen development and starch metabolism. The work was initiated by the finding that half of the pollen of one transgenic tobacco cv. Petit Havana SR1 was sterile and had very low starch content. As this specific β-glucosidase is able to cleave cytokinin-O and N3-glucosides, the eventual correlation of the studied processes with cytokinins was also of interest. The experimental system for this study was as follows: pollen from positive plants (i.e. plants expressing β-glucosidase) and from negative plants (i.e. plants not expressing β-glucosidase). Histochemical staining of pollen grains showed the gene expression. Pollen was studied in 6 phases/stages of development during which starch in control plants accumulates and disappears again. Centrifugation in Percoll gradient separated pollen into 3 fractions according to their starch content. Aside from the key characteristics of the pollen from this tobacco plant, i.e. starch, reducing sugars and protein contents, the activities of enzymes of sugar and starch metabolism were studied.

Materials and Methods

Description of the Experimental Material

The experimental material used for this study was the pollen isolated from amphidiploids tobacco plants *Nicotiana tabacum* L. cv Petit Havana SR1 (streptomycine resistant). The maize gene Zm-p60.1 encoding the enzyme β-glucosidase whose expression takes place in maize root tips was cloned and used for the transformation of tobacco (Brzobohaty et al. 1993). This gene under the constitutive promoter CaMV 35S was transferred into *in vitro* cultured tobacco leaf discs using *Agrobacterium tumefaciens*. In the population of the transformants regenerated, a plant which was later designated #7 was found. These #7 plants expressed the transferred gene for β-glucosidase both in the vegetative plant (i.e. the sporophyte) and in 50% of the pollen grains. Following self-pollination of #7 plants, the offspring obtained segregated at a ratio of 1:1. Offspring of self-pollination showing β-glucosidase activity were designated +#7 (genotype G/-), while plants that did not express β-glucosidase activity were designated -#7 (genetotype -/-).

Offspring of the tobacco plants #7 maintained as the heterozygous line were screened for β-glucosidase activity and used for this study. Plants were raised from seeds *in vitro* between late February and March in agar medium after Murashige and Skoog, 1962). The seedlings were transplanted *ex vitro* into soil in April after they had attained a height of about 7cm. A month later they were transferred into large plastic containers in the greenhouse where they grew until the end of September. To maintain the plants at the flowering phase from late June until September, old shoots were continuously cut-off, and old (unused) flowers at post-anthesis stage were picked and discarded regularly.

Methods

Determination of β-glucosidase activity in leaf extracts

This method is based on the cleavage of the β-D-glucopyranoside part of PNPG (4-nitrophenyl-β-D-glucopyranoside) by β-glucosidase, enabling the determination of the resulting product of the reaction. After stopping the reaction by adding 2M Na_2CO_3 to the incubation mixture, the product of the reaction immediately developed a yellow colouration. Young tobacco leaves (200-400mg), initially stored at -70^{o}C were homogenized in liquid nitrogen. Samples were finely crushed in a mortar using a pestle, the extraction buffer was added (1µl/mg sample) and the mixture was thoroughly stirred. Centrifuge tubes

with homogenates were kept in an ice bath before and after centrifugation. The mixture was transferred into centrifuge tubes and centrifuged for 15 minutes at 4°C, at 3000rpm. 10µl of the supernatant was added to the reaction mixture consisting of 290µl citrate buffer and 100µl PNPG. The reaction was thoroughly stirred using a vortex stirrer and incubated at 30°C in a thermostat with shaking for 30 to 35 minutes, depending on how quickly the activity of β-glucosidase was registered. The reaction was stopped by adding 600µl 2M Na_2CO_3. A yellow colouration quickly developed in the sample, confirming a positive reaction to β-glucosidase activity. The β-glucosidase activity was measured on a microplate reader as change in absorbance at 405nm.

Determination of β-glucosidase activity in pollen samples

This method is based on the cleavage of the β-D-glucopyranoside part of X-glc (5-Bromo-4-chloro-3-indolyl-β-D-glycopyranoside) by β-glucosidase, releasing a blue stain as a product of the reaction. 100µl of the staining solution was pipetted onto pollen sediments in a centrifuge tube. This mixture was left to stand with shaking on a vortex shaker for 8 hours at 25°C. The product of the reaction developed deep colouration after 8 hours and stained the pollen population blue, which indicated a positive reaction to the β-glucosidase.

Description of tobacco flower buds developmental stages

Tobacco flower buds were collected in small labelled beaker with moist filter paper. The lengths of the tobacco flower buds coincided with their developmental stage which correlated with distinct cytological characteristics. For instance, stage 1: 13-17mm long (from the base of the flower bud to the petal apex) – petals are hidden in the sepal; Stage 2: 18-24mm long, petals slightly protruding from the sepal; Stage 3: 25 -30mm long, petals very visible, pollen filled with starch; Stage 4; 32-38mm long, petal tube usually bent (a lot of starch still present); Stage 5: 39-43mm long, the flower bud apex starts turning yellow or purple. The amount of starch in the pollen decreases; and Stage 6: 44-50mm long, the flower bud is almost open - anthers are still closed.

Isolation of tobacco pollen grains from anthers

A pollen isolation medium which constituted of 5% manitol, 0.01% H_3BO_3, 1mM Ca $(NO_3)_2$, and 5mM KNO_3 was prepared for the isolation of tobacco pollen from the anthers. In the laboratory, the flower buds which had been grouped according to their developmental stages were opened using forceps. The flower anthers were isolated from the stamens into a clean cooled mortar. Immediately, 0.5ml of the pollen isolation medium per flower bud was pipetted onto the anthers, so that the anthers did not float on the medium. Pollen grains were isolated from the anthers by gently pressing them with a pestle. The resulting pollen suspension was transferred into clean labelled centrifuge tubes, using a pipette. Stages 1 and 2 pollen were centrifuged at 200g for 90 seconds. The resulting supernatant was discarded and the sediment was suspended once again in the same medium. The pollen was collected on a filter paper by filtration and applying vacuum using the water pump, and then transferred onto a massed and labelled aluminium foil. The aluminium foil together with the enveloped pollen was re-massed, and the new mass registered. The pollen from the remaining pollen developmental stages (i.e. 3 through 6) was filtered without prior centrifugation. Pollen obtained as described above was then stored at -70°C for further use.

Tobacco pollen *in vitro* culture experiments

The mediums used for this study were Mediums B, BSach, BS1 or C. These mediums were used separately as an environment for the cultivation experiments, with an inhibitor or growth regulator (W)

or without a growth regulator (Wo). Medium B after Harada and Kyo (1986) was the basic medium for the rest. Medium BSach contained the same medium compounds as medium B, except for the substitution of 300mM manitol with 10% sucrose (292mM). In medium BS1, 300mM manitol was substituted with 1% sucrose. Medium C had all medium B compounds in addition to 3mM glutamine and 1mM sucrose.

The pollen suspensions in centrifuge tubes were purified by centrifugation at 100 rpm for 2 minutes after which anther remains were discarded. Centrifugation of re-suspended pollen sediments in medium B was repeated twice for each centrifuge tube, and the supernatant was removed by sucking with adjustable pipette. Specific volume of the pollen suspension samples at a density of 50,000 pollen grains per ml was transferred into labelled sterile petri dishes containing medium B and cultured for the required time.

Separation of Pollen Samples on Percoll discontinuous gradients

1ml 65% Percoll was obtained by mixing 6.5ml absolute Percoll (supplied by SIGMA) with 3.5ml medium B. This 65% percoll was pipetted into a clean dry 10ml centrifuge tube with a screw cap. 1ml 55% Percoll was carefully layered onto the 65% Percoll, such that it formed a clear band with it. Now, the pollen suspension was layered onto the top of this discontinuous gradient. Following centrifugation at 1000 rpm for 5 min, each pollen population formed bands or pellet, in this order: top = T (water/55% Percoll interface); middle = M (55%/65% Percoll interface), and bottom = B (sediment). Each pollen population was collected with a pipette and diluted with 1ml of medium B. The re-suspended pollen fractions in medium B were centrifuged at 7000 rpm for 1 minute. The sediment in each centrifuge tube was used for further experiments such as β-glucosidase assay, starch estimation, etc.

Determination of protein content in tobacco pollen

The BIORAD method, which is a modification of the Lowry method, was used for measurement of the amount of protein in the transgenic pollen. This method is based on two reactions, and uses two reagents: **A** and **B**. First, the formation of a coordination complex between Cu^{2+} and four hydrogen atoms, and from each of the two adjacent peptide chains, and second - the reduction of the phosphomolybdate-phosphotungstate reagent (Folin-Ciocalteau reagent) by tyrosine and tryptophan residues.

20 µl of each pollen sample (supernatant) was pipetted into each clean and dry test tube. Then, 100µl of reagent A (see BIORAD method) was added to each test tube and the mixture was stirred using a Vortex mixer. Next, 800µl of reagent B was added after mixing reagent A with the pollen sample. The reaction mixture was immediately stirred and incubated for 15 minutes at room temperature. The absorbance was read at the end of the 15 minutes incubation, in a UV-Spectrophotometer, at 750nm against a blank containing 20µl extraction buffer instead of pollen extract. The absorbance was stable for at least 1 hour.

Determination of starch in tobacco pollen

This method was based on a simplified extraction of starch with 32% perchloric acid, a selective retention of the starch-iodine complex on a glass fibre disk (Whatman GF/A). The starch on the disk is dissolved in 0.75M sulphuric acid, and estimated with phenol (Lustinec et al. 1983). This method is able to detect as little as 5µg of starch in different amounts of plant material.

Determination of Amylase activity in tobacco pollen samples

The determination of amylase activity was based on the reaction of the released reducing sugars at 40^{o}C with 3,5-dinitrosalicylic acid (Bernfeld, 1966) during which a brownish-yellow compound is formed. Then, the absorbance of the product of the reaction was then read at 570nm against a blank in a spectrophotometer.

The solution of amylopectin (0.2ml) was pipetted onto 0.2ml of the enzymatic extract (i.e. pollen supernatant) in a 15 ml test tube and the reaction mixture was incubated for 20 minutes at 30^{o}C. After the incubation time has elapsed, the reaction was stopped by adding 0.4ml of 3,5-dinitrosalicylic acid (DNSA). Then, these stoppered test tubes (i.e. test tubes having with screw caps) placed on a test tube rack were transferred onto a boiling water bath for 5 minutes. During this time, the product of the reaction developed a brownish-yellow colouration of varying intensities depending on the strength of the amylase activity.

Determination of selected enzymes activities in the tobacco pollen

This method was based on the reduction of NADP (Nicotinamide adeninine dinucleotide phosphate). Each of the enzymes determined produces glucose-1-phosphate *in vivo*. The enzymes which activities were measured are as follows: UGPase, AGPase, and starch phosphorylase. Determination of enzymatic activity was by the help of two enzymes namely phosphoglucomutase and glucose-6-phosphate dehydrogenase (i.e. PGM and GDH), and consisted in the conversion of glucose-1-phosphate to glucose-6-phosphate, and its subsequent oxidation to phosphogluconolactone (PGL), while the NADP is reduced to NADPH. The amount of NADPH formed during the reaction was then monitored using spectrophotometer. The components of the reaction (i.e. incubation mixture including the chilled pollen extract) were added consecutively into centrifuge tubes and the reaction was commenced by adding 2.5mM Sodium pyrophosphorylase.

Reaction mixture (incubation mixture) for UDPG-pyrophosphorylase (UGPase)

The total volume of the incubation mixture was 700µl, which included: 200µl buffer (750mM HEPES pH 7.5); 50µl 9mM NADP; 50µl PGM (2.5U); 50µl water solution of GDH (1U); 50µl 10mM UDPG; 50µl 1000mM $MgCl_2$; 50µl 100mM NAF; 50µl water; and 50µl pollen extract. The reaction was commenced by adding 100µl 2.5mM Sodium pyrophosphorylase. The measurement of the enzymatic activity lasted between 5 to 10 minutes, contingent on the speed of the reaction. HEPES stands for: N(2-hydroxyethyl) piperazine-N'-2-ethanesulphunic acid.

Reaction mixture (incubation mixture) for ADPG-pyrophosphorylase (AGPase)

The incubation mixture for AGPase was the same as in the case of UGPase, but with the only difference that the substrate was ADP-glucose (20mM), and the 50µl of 10mM 3-PGA (3-phosphoglyceric acid) replaced 50µl of water. Measurement of enzymatic activity lasted between 10 to 20 minutes, contingent on the speed of the activity.

Reaction mixture (incubation mixture) for starch-phosphorylase

The incubation mixture of total volume 800µl contained: 200µl of buffer (750mM HEPES pH 7.5); 50µl 8mM NADP; 50µl 100mM $MgCl_2$; 50µl 200mM glucose-1,6-biphosphate; 50µl PGM (2.5U); 50µl GDH (1U); 50µl 100 mM NAF; 100µl of pollen extract, and 200µl of soluble starch solution in

40mM phosphate buffer pH 7.0. The organic components of the incubation mixture were stored in water solution at – 20^{o}C.

The colloidal solution of amylopectin was prepared fresh. Amylopectin was dissolved in water at increased temperature. After it was cooled the phosphate buffer was added to the water solution of amylopectin, so that a final concentration of 40 mM amylopectin was achieved. The mixture containing the pollen extract was pre-incubated for 5 to 10 minutes before the addition of the soluble amylopectin solution, and the reaction was run for 40 minutes. The reason for the pre-incubation was to adjust the temperature of the incubation mixture at which the enzymatic activity was read.

Key Results

After pollen mitosis, starch accumulation gradually begins in the pollen and attains its maximum point at pollen developmental stage 4, just three days prior to pollen maturity and anthesis. Expression of β-glucosidase in half the population of the positive plants correlated with the defect in starch synthesis, while the second half of the pollen population showed normal starch accumulation.

The course of activity of UDPG-pyrophosphorylase in the pollen of positive plants was notably different in comparison to pollen of negative plants (i.e. plants not expressing β-glucosidase activity). UDPG-pyrophosphorylase activity increased with progress in pollen development in the positive plants, while the opposite was the case in negative plants. At stage 6, UDPG-pyrophosphorylase activity in the positive plants had tripled in comparison to the negative plants. Very distinct differences were observed with regard to the activity of ADPG-pyrophosphorylase in the developing pollen between positive and negative plants. At stages 3 and 4, ADPG-pyrophosphorylase activity had almost tripled in the pollen of negative plants in comparison with positive plants. Increase in starch accumulation correlated with increase in ADPG-pyrophosphorylase activity. Starch phosphorylase activity increased with progress in pollen development in both types of pollen studied, with meagre differences between pollen at the same stage of development. However, its activity was 1.4-fold higher in positive plants as compared to the negative plants at pollen developmental stage 4. The dynamics of amylase activity during pollen development was similar in both types of pollen. However, there were significant differences in the level of amylase activity in pollen at the same stage of development. For instance, at stage 6, amylase activity was 1.7-fold higher in the positive plants against the negative plants.

The binucleate pollen of the positive plants cultured in a medium supplemented with enough sucrose is capable of accumulating starch just as the pollen not expressing ZM-60.1. This means that the starch synthesizing machinery in the pollen expressing ZM-p60.1 remains intact. Gluconic acid lactone or kinetin stimulated starch accumulation in both the pollen expressing β-glucosidase activity and that not expressing the enzyme. The content of starch in pollen cultured in the medium with actinomycin-D and cordycepin was about three-fold higher in comparison with the control. There was no significant difference in starch accumulation between the treatments with the transcription inhibitors namely actinomycin or cordycepin. The activities of amylase and starch-pyrophosphorylase were down-regulated using actinomycin-D or cordycepin pointing to the transcriptional regulation of both enzymes activities.

Discussion

Histochemical studies using potassium iodide confirmed that only 50% of the pollen population of positive plants exhibited normal starch accumulation. Starch formation and degradation in the pollen at respective pollen developmental stages were first observed microscopically. It was possible to observe

starch granules in the developing pollen prior to and after pollen histochemical staining. Further histochemical studies using X-glc (5-Bromo-4-chloro-3-indolyl-β-D-glycopyranoside), a β-glucosidase substrate, showed that the substrate was hydrolyzed in the active pollen thereby releasing a blue product. This revealed that the majority of the pollen grains that showed the activity of Zm-p60.1 contained very low amount of starch.

The connection between starch accumulation and the activity of ADPG-pyrophosphorylase had been proved in many starchless mutants, for instance, during studies with maize mutants, *shrunken-2 and brittle-2*, which are both deficient in this enzyme and starch formation (Tsai and Nelson, 1966). In experiments with peas, Smith et al. (1989) showed that a pea line having recessive *rb genes* (the gene controlling the level of ADPG-pyrophosphorylase (AGPase) in developing pea embryos) exhibiting 3-5% of AGPase activity had only between 38-72% of starch as compared to the amount of starch found in normal pea line. In *Arabidopsis thaliana*, Lin et al. (1988a) isolated a mutant containing less than 2% of starch in comparison to the normal strain, and less than 2% AGPase activity. In potato tuber, Muller-Rober et al. (1992) expressed a chimeric gene encoding the antisense RNA for the AGPase small unit which caused a reduction in enzymatic activity to 2-5% of the normal levels and this led to a reduction in starch content.

Caspar et al. (1985) obtained a null mutant of Arabidopsis thaliana for the chloroplatic phosphoglucomutase (PGM) that was defective in starch synthesis. This starchless phenotype of null plastidic PGM mutant supports the prevailing view that carbon flow into starch occur via the gluconeogenesis pathway within the plastid compartment. Okita (1992) reported that the absence or depression in the levels of starch synthesis can be attributed to a direct causal relationship between defects in the expression of the structural genes for AGpase and the concomitant lower amounts of this enzyme's activity. It is apparent from the results of this study that there were notable differences in the activities of starch-degrading enzymes (starch-phosphorylase and amylase) and AGPase (a starch synthesizing enzyme) between the positive pollen (i.e. pollen expressing Zm-p60.1) and negative pollen (i.e. pollen not expressing Zm-p60.1) or the control.

The results of this study substantiate the general view that AGPase plays a key role in starch biosynthesis. When the second phase of starch synthesis attained its maximum point at pollen developmental stages 3 and 4, the activity of AGPase rose considerably in both types of pollen with the most significant difference that the pollen expressing Zm-p60.1 had 2.5-fold lower amount of starch in contrast to the control. The above data are in agreement with the general view that AGPase activity is positively correlated with starch biosynthesis. These and other results support the notion that in the event of enough level of the enzyme, its activity and subsequently starch synthesis are principally contingent on the presence of inhibitors and activators, source of needed precursors and ATP (Sun et al. 1999).

The impact of an allosteric activator called 3-phosphoglycerate (3-PGA) on AGPase and UGPase activities was also investigated. Generally, it was noted that this allosteric activator elevated the activities of these enzymes progressively with increase in its concentration (from 1mM to 20mM) in the reaction mixture. The highest increase in the enzymatic activity per additional mM 3-PGA in the case of UGPase was observed between the concentration of 5 and 10mM, where the increase was more than two-folds. In contrast, for AGPase, the highest increase in the enzymatic activity per additional mM 3-PGA was three-folds between the concentration of 1 and 5 mM. Comparing the influence of 3-PGA on the enzymatic activities of UGPase and AGPase, this allosteric activator increased UGPase activity by three-folds against AGPase at a concentration of 10mM.

Several experiments have been cited to support a direct correlation between the concentration of 3-phosphoglycerate and starch accumulation, and an inverse one between inorganic phosphate (Pi) and starch content. This is true for photosynthetic tissues where Pi and 3-PGA levels within the chloroplast are good indicators of energy and carbon status, and in this way the regulation of AGPase provides a good mechanism for modulating the flux of photosynthate into starch (Okita, 1992). Also, in non-photosynthetic tissues of maize endosperm, potato tubers, cassava root and rice endosperm, Zhang et al. (1996) observed that the AGPase activity was highly contingent on the presence of phosphoglycerate. This is a situation relevant to pollen development.

However, it has been reported that pea embryo (Hylton and Smith, 1992) and barley AGPases were not highly activated by 3- phosphoglycerate (Kleczkowski et al. 1993). Activation of the bean cotyledon AGPase by 3-PGA was only 1.5 fold when the activity was measured in the pyrophosphorolysis direction (Weber et al. 1995). It has been observed that both plant and bacterial AGPases were usually much less activated by allosteric activators in the pyrophosphorolysis direction than in the synthesis direction (Ghosh and Preiss, 1996). Thus, the higher activation of for the bean cotyledon or the tobacco extract enzyme being discussed herein may be observed in the synthesis direction, which is the physiological direction.

The temperature and pH optimums were studied for AGPase, UGPase, starch-phosphorylase and amylase. The optimal pH range (pH 6.5 to 7.5) obtained in this study was in agreement with the optimal pH range reported in papers where different biological materials were used (Ghosh and Preiss, 1966; Preiss and Levi, 1980). During this research, studies on the characterization of starch enzymes, it was discovered that the enzymes had maximum activity at temperatures between 40 and 50^{o}C in the tobacco pollen extract. The maximum activities of UGPase and starch-phosphorylase in the transgenic tobacco were observed at 50^{o}C, while the optimum temperature for AGPase was 40^{o}C. The optimum pH and temperature for starch-phosphorylase were pH7 and 50^{o}C. It may have been interesting to measure starch-phosphorylase activity at pH values higher than 8.5, but the HEPES buffer did not effectively buffer at higher pH. Similar temperature optimum was reported by Khanna et al. (1971) for the enzyme in the leaves of *Dendrophyte falcata*. These temperatures had no remarkable denaturing impact on the enzymes, phosphoglucomutase (PGM) and glucose-6-dehydrogenase (GDH), used for the estimation of the produced glucose-1-phosphate. The optimum temperature of 40^{o}C obtained for tobacco pollen AGPase during this study is in conformity with the results reported for AGPase in wheat and maize endosperm (Keeling et al. 1994). At the temperature of 10^{o}C AGPase activity was almost impossible to be measured. However, at increasing temperatures AGPase activity rose steadily until it attained its maximum at 40^{o}C. The most significant effect of increase in temperature on the AGPase activity in the tobacco pollen was between 35 and 40^{o}C. The optimum temperature for starch synthesis in wheat and maize grains, however, lies in the interval of 25 to 30^{o}C. Denyer et al. (1994) acclaimed that low starch content in storage organs caused by low or high temperatures was a result of high temperature sensitivity of the soluble starch synthase (SSS).

The activity of UGPase increased by 2 and 3-folds in the pollen expressing Zm-p60.1 against the control at pollen developmental stages 5 and 6 respectively. Stages 5 and 6 in pollen development correspond to the stages where starch degradation occurs. While the UGpase activity increased with progress in the development of the pollen expressing Zm-p60.1, it was the opposite in the control. When the binucleate positive pollen was supplemented with enough sucrose in *in vitro* culture, it was capable of accumulating as much starch as the pollen of control (i.e. negative pollen). This finding attests that one of the causative factors for low starch accumulation in the pollen expressing Zm-p60.1 could be insufficient endogenous supply of sucrose which could be converted to starch.

Clement and Audran (1994) in their findings reported that neither microspores nor pollen grains are probably able to regulate starch metabolism, but that the regulation comes from the sporophytic tissues of the anthers. The content of starch in anthers of *Lily* from the meiocyte stage to pollen maturity has been described by Clement et al. (1996). Their report showed two distinct phases of amylogenesis. Two phases of starch accumulation in the pollen were also reported in *Lycopersicum peruvianum*, and these phases are accompanied by some structural changes, from meiosis to pollen maturity (Pacini and Juniper, 1984). The authors affirmed that the first phase commences before the microspores are released from the tetrad and ends before first pollen mitosis. The second phase begins (after first mitosis) at the two-celled pollen stage and terminates with the maturation of the pollen structures accompanied by dehydration, during which starch is degraded to sucrose. The above findings show that the level of sucrose can indirectly affect the amount of starch synthesized by AGPase. There are evidences that strongly indicate that the activity of UGPase *in vivo* is regulated by substrate availability (Vella and Copeland, 1990).

Starch biochemical studies in conjunction with in vitro cultures which revealed that the pollen expressing ZM-p60.1 contains considerably lower amount of starch in comparison to the control negative, but in rich sucrose medium accumulated starch, suggests that the starch-synthesizing machinery in the transgenic plant expressing Zm-p60.1 probably remains intact. This is because if it were the opposite the enzymes of starch biosynthesis would have been defective. This along with other biochemical data of this study point to the fact that the starch-synthesizing enzymes such as AGPase are present in the pollen expressing the β-glucosidase, but are not in full function. This could be caused by several factors such as insufficient level of endogenous ADP-glucose that could be converted to starch or by low level of glucose-1-phosphate, the substrate for AGPase, which could result to a reduced pool of ADP-glucose needed for starch biosynthesis or by low level of allosteric activator (3-PGA) of ADPG-pyrophosphorylase.

The results of this study suggest that UGpase does not play a direct role in starch synthesis in the pollen in the second phase of starch synthesis. Its higher activity in the uninucleate pollen stage may be caused by its connection to the syntheses of the components needed for the formation of the wall of the pollen grain. Pacini and Juniper (1984) reported that exine and intine start to form before the release of the microspores from the tetrad and that their formation is completed after the first pollen mitosis. UDPG-pyrophosphorylase activity in the tobacco pollen was 10-folds higher than that of ADPG-pyrophosphorylase. This finding is in agreement with that obtained in potato tubers (Sowokinos, 1976), where the ratio of activity of both enzymes (i.e. AGPase and UGPase) was in favour of UGPase as its activity was up to 100-folds elevated against that of AGPase.

The results of this study on the course of activity of starch-phosporylase, which gave a gradual and progressive increase in the activity of this enzyme from stage 1 through 6, support the suggestion that this enzyme partakes in starch degradation. To confirm that starch-phosphorylase is involved in starch hydrolysis in the pollen, its activity was influenced by using transcription inhibitors actinomycin-D or Cordycepin. The amount of starch in the pollen samples cultured with inhibitors was 3-folds higher than the amount found in the control (i.e. without the inhibitor). The higher content of starch in the treatments with the inhibitors correlates to the fact that the number of starch-phosphorylase transcripts were reduced using the inhibitors, thereby leading to the decreased starch hydrolytic activity of this enzyme. In recent years, works have been published which support starch-phosphorylase's regulation in starch metabolism at the transcription level. For example, in developing cotyledons of peas, mRNA of form II (plastidic starch-phosphorylase) was dominant, while its level was less during germination (Van Berkel et al. 1991).

The data measured for amylase activity in this study show that amylase is involved in starch degradation in both types of pollen studied, as its activity was highest at the stages corresponding to those where starch degradation occurs. The high activity of amylase at these stages might have be prompted by the increased expression of the genes for isoezymes of amylase in the pollen or indirectly by the increase in gibberellic acid (GA) synthesis, which induces the expression of the genes for isoenzymes of amylase. In this study the hydrolytic role of amylase in starch degradation and its possible transcriptional regulation was investigated; amylase activity was measured in *in vitro* maturated pollen in the presence of cordycepin or actinomycin-D. The content of starch in the pollen samples where the inhibitors were used was 3-fold higher in contrast to control, and amylase activity in the control was about 2-folds higher in comparison with treatments with the transcription inhibitors. There was no significant difference in the level of activities between amylase and starch-phosphorylase in the pollen maturated in *in vitro* culture in the presence of actinomycin-D or cordycepin. The above results give evidence that amylase partake in starch degradation in the pollen.

The method for starch determination described by Lustinec et al. (1983) could be used without modification for determination of starch content in pollen. This method is faster and more starch-specific than the enzymatic ones used by Pucher et al. (1948). It can be used for different types of starch, including those of unusual waxy starch. Also, it can differentiate between starch and glycogen which could be useful in analyzing plants infected by some pathogenic fungi in which both polysaccharides namely amylose and amylopectin, occur (Holligan et al. 1974).

Protein content in the pollen progressively increased with the pollen developmental stage. The course of changes in protein content in the transgenic pollen was closely in agreement with the results reported by Zarsky et al. (1985) on the content of soluble and insoluble protein fractions in tobacco pollen of cultivar Samsun and White Burley, where the content of protein was expressed per pollen grain. However, they reported a slight decline in the amount of protein at stages 5 and 6 of the tobacco varieties cv. Samsun and White Burley. An increasing content of protein following first pollen mitosis and its slight decrease preceding pollen maturity was also reported in *Lily* pollen (Linskens, 1966) and in tobacco pollen (Villanueva et al. 1985). Gluconic acid lactone (GAL), a β-glucosidase inhibitor, and kinetin were used separately in pollen culture experiments. Each separately stimulated starch accumulation in both types of pollen (positive pollen = pollen expressing the β-glucosidase activity, and negative pollen = pollen not-expressing the β-glucosidase activity), confirming the earlier proposition that the decreased starch formation in the pollen expressing Zm-p60.1 may not be a result of defects in starch-synthesizing mechanism but due to some physiological factors deregulating the metabolic pathway. The increase in starch accumulation in the presence of exogenously supplemented gluconic acid lactone could be due to the use of the lactose part of this substance as a metabolite in the pollen.

The findings of this work during culture experiments with the binucleate pollen of the positive and negative tobacco plants show that exogenously supplemented kinetin (1mM) in the culture medium highly supported starch accumulation irrespective pollen type (i.e. in both positive and negative pollen). This is consistent with the findings of Miyazawa et al (1999) and Lustinec et al. (1976). The cytokinin (kinetin) might have triggered starch accumulation in the pollen by stimulating increased amyloplast formation and gene expression which enhanced the accumulation of starch and *Agps* (ADPG-pyrophosphorylase small subunit gene), GBSS (granule-bound starch synthase) and SBE (starch branching enzyme) transcripts (Miyazawa et al. 1999). A correlation between the accumulation of ADPG-pyrophosphorylase transcripts and starch was also observed in developing potatoes (Pratt et al. 1990). Hormonal regulation of starch accumulation and cell expansion in tobacco stem pith was studied (Lustinec, et al. 1976) using different concentrations of naphthalene acetic acid (NAA) and a

constant concentration of kinetin (10^{-6}M). These authors reported that the optimum concentration of NAA (10^{-5}M) for cell expansion in tobacco stem pith explants, cultivated on agar media containing 4% glucose and kinetin, was at least ten thousand times higher than for starch synthesis (10^{-9}M NAA) and, that the optimal concentration for cell expansion inhibited starch accumulation in those tissues.

This study showed that only 50% of the pollen population from the plants expressing the β-glucosidase (Zm-p60.1) had normal starch accumulation. The other 50% does not accumulate starch. Since Zm-p60.1 hydrolyzes cytokinin-O-glucosides or kinetin-N3-glucoside, it would be expected that it can release free cytokinins from inactive conjugates and that cytokinin conjugates could be exploited to trigger starch biosynthesis. As cytokinins at optimal concentration range support starch accumulation in plant tissues, it would also be expected that the released cytokinins in the pollen expressing the β-glucosidase will trigger starch accumulation. Instead of this, only 50% not expressing the β-glucosidase was capable of starch accumulation. However, the positive pollen expressing the β-glucosidase was capable of accumulating more starch in a culture medium supplemented with kinetin in comparison with the medium without exogenous supply of cytokinin. The aforementioned results suggest that Zm-p60.1 could also act as a glucosyltraferase, thereby increasing the pool of cytokinin conjugates. Thus, the levels of free cytokinins in the pollen of positive plants were insufficient to trigger starch accumulation in this type of pollen in the absence of exogenous cytokinin source. Results of cytokinin composition analyses of the developing tobacco pollen showed for the first time to my knowledge that higher plant pollen comprised of different highly dynamic cytokinin types. The pollen expressing the β-glucosidase showed significant differences in cytokinin contents and dynamics in comparison with the pollen not expressing Zm-p60.1. However, the differences could not be explained simply by Zm-p60.1 cytokinin-glucoside deglycosylating or glucosyltransferase activities. The starchless phenotype of Zm-p60.1 correlated well with the changes in starch metabolizing enzymes.

Conclusions

The above findings suggest that the decreased starch formation in the pollen expressing the β-glucosidase Zm-p60.1 may not be attributed to defects in the starch-synthesizing mechanism, but probably to metabolic deregulation caused by several physiological factors such as: the slight decrease in the activity of ADPG-pyrophosphorylase (AGPase) which has a pivotal role in starch biosynthesis; increase in the activity of starch-phosphorylase and amylase which on the contrary degrade starch; considerable increase in the activity of UDPG-pyrophosphorylase (UGPase) which influence may indirectly decrease starch biosynthesis, probably by setting increased requirement for glucose-1-phosphate, thereby reducing the pool of glucose-1-phosphate, the substrate for AGPase. As a result, enough amount of ADPG needed for starch synthesis cannot be synthesized. Furthermore, probably to metabolic deregulation caused by physiological factors such as: insufficient endogenous supply of substrates, in the pollen expressing Zm-p60.1, needed to trigger the full function of the enzymes that partake in starch biosynthesis; insufficient levels of free endogenous cytokinins which could stimulate starch accumulation; insufficient levels of endogenous allosteric activator (3-PGA) of AGPase to stimulate full activity of AGPase in the pollen expressing Zm-p60.1. Finally, the aforementioned results suggest that the basic starch-synthesizing machinery in the pollen expressing Zm-p60.1 remains intact.

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