

Dynamics of β -Glucosidase Zm-P60.1 Ectopic Expression during Transgenic Pollen Development: A Histochemical Approach

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Abstract. *Zm-p60 1* is maize cDNA coding cytokinin-glucoside specific β -glucosidase. Indigogenic method was used for histochemical localization of Zm-p60 1 β -glucosidase activity in various developmental stages of transgenic tobacco anthers. Expression of *Zm-p60 1* cDNA in T7 tobacco plants is controlled by the CaMV 35S promoter. Another type of tobacco transformant expresses *Zm-p60 1* under the control of LAT 52 promoter. Histochemical detection has proved different patterns of β -glucosidase activity during tobacco pollen development in these two types of transformants. Zm-p60 1 β -glucosidase activity had no direct influence on pollen germinability.

Key words: Indigogenic method — β -glucosidase — Tobacco — Pollen

Abbreviations: CaMV – cauliflower mosaic virus, RT-PCR – reverse transcription-polymerase chain reaction, DHFR – dihydrofolate reductase, dNTPs – deoxynucleoside triphosphates

Plant hormones regulate many features of plant development. They often occur in plant tissues covalently bound to low molecular compounds, as sugars or amino acids, forming so-called conjugates. Phytohormone conjugates are abundant in plant tissues and are often considered as a reserve or transport forms (Schliemann 1991). It was shown that in maize seedlings cytokinins are transported as glucosides from the endosperm to the embryo, where they can be activated by hydrolysis. Brzobohatý *et al.* (1993) has cloned the maize complementary DNA, designated *Zm-p60 1*, coding cytokinin-glucoside specific β -glucosidase. This β -glucosidase has been shown to liberate free active cytokinins from their glucosides both, *in vitro* and *in vivo*. Transgenic tobacco plants expressing *Zm-p60 1* cDNA constitutively under the control of the CaMV 35S gene promoter were constructed. Mouse dihydrofolate reductase non-sensitive to methotrexate acts as a marker gene. It is

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present on T-DNA and cosegregates with the β -glucosidase activity. Studying effects of *Zm-p60 1* ectopic expression in transgenic tobacco plants, one transformed plant (T7) with distorted Mendelian segregation of transgene in reciprocal crosses was identified. From the segregation ratio, it was apparent that the transgene is transmitted only maternally (Žárský *et al* 1997). These facts led us to more detailed study of the pollen development and viability in the T7 transgenic plant. Recently, we have constructed also transgenic tobacco plants expressing *Zm-p60 1* under the pollen specific promoter LAT 52, expression of which is specific for late pollen developmental stages (Twell *et al* 1990).

Sporogenous cells (pollen mother cells) undergo meiosis, giving rise to a tetrad of haploid cells. The individual cells of the tetrad are released as free microspores. These microspores undergo an asymmetric mitotic division, resulting in young pollen grain with two cells (McCormick 1993). Next steps including growth and dehydration of pollen grain are called pollen maturation. Development from the microspore to the mature pollen is divided into six developmental stages (Říhová *et al* 1996). The aim of this study was to compare *Zm-p60 1* expression and pollen development in two transformants and to decide if the special phenomenon displayed in crossing T7 transgenic tobacco is caused by β -glucosidase *Zm-p60 1* activity.

Plant material used in experiments Control plants (wild type) *Nicotiana tabacum* L. cv. Petite Havana SR1, transgenic plants T7 plants *Nicotiana tabacum* L. cv. Petite Havana SR1 with *Zm-p60 1*, promoter CaMV 35S, marker gene coding for a dihydrofolate reductase non-sensitive to methotrexate, LAT plants *Nicotiana tabacum* L. cv. Petite Havana SR1 with *Zm-p60 1*, promoter LAT 52, gene coding for a phosphinothricin acetyl transferase was used as a selectable marker. Plants were planted both in field and greenhouse conditions.

Modified indigogenic method according to Gossrau *et al* (1987) was used to demonstrate the activity of *Zm-p60 1* β -glucosidase in successive developmental stages of the pollen. Cryostat sections of anthers were prepared following formaldehyde fixation and washing in 15% sucrose. Incubation medium was composed of 0.5 mmol/l 5-bromo-4-chloro-3-indolyl β -D-glucopyranoside in 0.1 mol/l citric acid-phosphate buffer pH 5.6 with addition of 2 mmol/l potassium ferricyanide and 2 mmol/l potassium ferrocyanide. Sections were incubated for 30 min at the laboratory temperature, nonfixed pollen and pollen tubes were incubated in the same medium for 3 hours. After the incubation, sections were mounted and washed in glycerine-jelly. A part of pollen grains was washed out from the sections during the incubation procedure. Therefore, we used the technique of semipermeable membrane to overcome problems with the loss of pollen grains, and indolyl diffusion. Pollen germinability was tested in medium according to Brewbaker and Kwack (1963).

In order to correlate activity staining results with *Zm-p60 1* mRNA level RT-PCR technique was used to detect β -glucosidase *Zm-p60 1* expression pattern. RNA was extracted either from leaves or from pollen according to Chomczynski and Sacchi (1987). Oligo-(dT) primed cDNA was synthesised from total RNA (3.0 μ g of RNA per reaction) using "First strand cDNA synthesis kit" (Boehringer, Mannheim). The subsequent amplification by PCR was performed using a pair of primers specific for *Zm-p60 1* (upstream 5'-GGCGACAAGGTGAAGAAT-3' and downstream 5'-TGGGTTTCCGTATTTGTT-3'). A typical PCR reaction mixture contained 1x PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 0.01% gelatine, (Sigma)), dNTPs (0.25 mmol/l of each), 1.0 μ mol/l of each primer, 2.5 U of *Taq* polymerase (Sigma), an aliquot of reverse transcription reaction mixture corresponding to 0.5 μ g of total RNA, and water added to a final volume of 50 μ l. An amplification step was performed as follows: denaturation at 94°C for 20s, primer annealing at 55°C for 20s, extension at 72°C for 45s. The PCR prod-

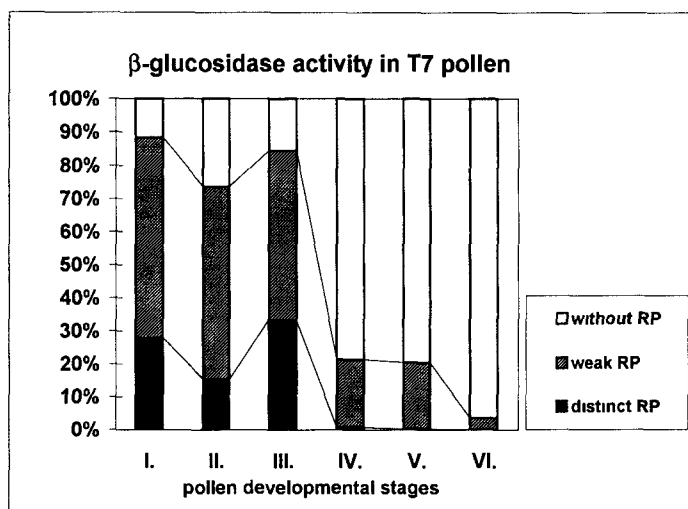


Figure 1. Frequency of pollen grains with reaction product (RP) in successive developmental stages in T7 tobacco plants

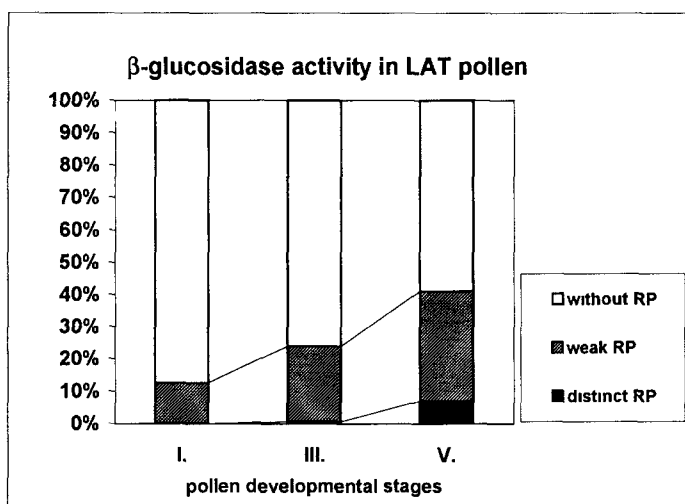


Figure 2. Frequency of pollen grains with reaction product (RP) in three developmental stages in LAT tobacco plants

uct of expected size (663 bp) was visualized on agarose gel by ethidium bromide staining (Fig 3) After incubation of anther sections in medium for histochemical detection of β -

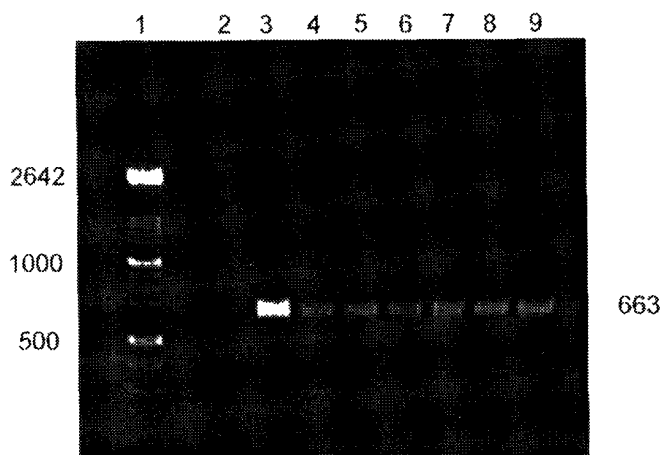


Figure 3. Detection of β -glucosidase *Zm-p60 1* mRNA in course of pollen development with *Zm-p60 1* cDNA specific primers. Lane 1 DNA size marker, lane 2 control plant (tobacco SR1) - leaves, lane 3 T7 plant - leaves (PCR product detected at 23 cycles), lanes 4-9 T7 plant - pollen I-VI stage (PCR products detected at 28 cycles)

glucosidase, the staining of microspores or pollen grains could be distinguished by means of their deep blue or light blue colour. Control incubations of sections from both transgenic plants in substrate-free medium and wild type plants in full incubation medium were without reaction product. Distinct *Zm-p60 1* β -glucosidase activity was present in microspores and pollen of T7 plants during the first three developmental stages. The enzyme activity was decreasing gradually in course of pollen maturation in T7 transformants (see Fig. 1), whereas *Zm-p60 1* mRNA levels did not change significantly based on the RT-PCR assay (Fig. 3). The difference between histochemically detectable enzyme activity and mRNA levels may be explained in several ways. *Zm-p60 1* is a plastid enzyme. Thus, *Zm-p60 1* can become inaccessible for reaction with the substrate by becoming trapped in starch grains accumulating in plastids in later stages of pollen development. Alternatively, inhibitors can accumulate during pollen development that interfere with enzyme activity detection. For example, content of flavonoids increases during pollen maturation. Recently, we have found, that several flavonoids are strong inhibitors of *Zm-p60 1* (Kopčíková et al. 1996).

Approximately 3 000 of pollen grains were evaluated in each pollen developmental stage in T7 plants. Ten-times lower amount of pollen grains was evaluated in second type of transgenic plants (LAT). The mature pollen grains from T7 transformed plants formed pollen tubes in lower ratio than in wild type plants. However, only about 20 % of T7 pollen grains, which did not form pollen tube, displayed a distinct blue staining for β -glucosidase. Detection of β -glucosidase activity in pollen of the T7 transformant is surprising as the CaMV 35S promoter has been reported to be inactive during the pollen development (van der Leede-Plegt et al. 1992). Thus, we assume that the transgene is activated during pollen development by a nearby promoter or enhancer specific for early stages of pollen development.

Microspores and pollen grains from transgenic tobacco plants expressing *Zm-p60 1*

under the LAT 52 promoter revealed the pattern of β -glucosidase activity which increased during pollen maturation (see Fig 2). To the present time, RT-PCR was not done in the pollen of LAT transformants due to the small amount of available pollen in different developmental stages. From the morphological point of view, no significant differences were identified between the pollen of LAT transformants and wild type of tobacco.

Results presented in this paper indicate that accumulation of Zm-p60 1 β -glucosidase in late developmental stages does not interfere with normal morphological pattern of pollen development. In the T7 transformant, the maximal Zm-p60 1 activity was found in the earliest developmental stages, although *Zm-p60 1* mRNA levels do not differ significantly in individual stages of pollen development. Thus, to test a possible causal link between the biological defects in T7 pollen and Zm-p60 1 β -glucosidase activity, a promoter highly active during the earliest stages of pollen development will have to be employed to drive *Zm-p60 1* expression.

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