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β-Glucan Synthetase II Activity Upon Callose Formation in the Flower of *Arabidopsis thaliana*

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Callose formation was observed in the pollens during flower development and pollen tube grown in the pistil of *A. thaliana*. The accumulation of callose occurred in the tetrad in the flower bud and pollen tube. Therefore, the activity of β-glucan synthetase II (GS II), which is responsible for synthesizing the callose, was measured in the flowers on the same developmental stages. The enzyme activity was increased by about 10% while the level of callose contents was increased by about 70% in tetrads. Then, callose accumulation was increased during pollen tube growth by about 30% higher than the other stages and enzyme activity was detected, 30% more too. These results suggest that callose plays an important role in the growth of pollen and pollen tube by increasing GS II activity.

Keywords: β-glucan synthetase II (GS II), callose, pollen tube, tetrad

Callose is a polysaccharide containing a high proportion of β-1,3-linked glucose (Eschrich, 1975) and fulfills an important biological role in acting as a temporary wall. The callose wall functions as a molecular filter isolating the developing microspores from the surrounding diploid tissue or sister spores (Heslop-Harrison and Mackenzie, 1967). In addition, callose is formed as a transient wall material at sites which are altered during special developmental states, for example sieve pores formation and pollen maturation (Fincher *et al.*, 1981). Newly synthesized pollen tube walls of *Tradescantia* labelled with ^{14}C-sucrose contained labelled glucose, arabinose, galactose, and minor contained various sugars (Mascarenhas, 1970). Lily pollen tube walls also consist of glucose, arabinose, galactose, xylose and uronic acid (Larbarca and Loewus, 1972). Glucose is the most abundant monosaccharide component of pollen tube walls (Rae *et al.*, 1985).

Plants possess one or more synthetases capable of producing β-glucans (Stone, 1984). β-Glucan synthetases which use UDP-glucose as substrate have been found associated with the Golgi vesicle fraction isolated from pollen tubes of *Petunia hybrida* (Helsper *et al.*, 1977). Golgi-localized β-1,4-glucan synthetase (GSI, EC 2.4.1.12) synthesizes predominantly cellulose, β-1,4-linked glucose (Robinson *et al.*, 1988). On the contrary, callose, β-1,3-linked glucose, is generally considered to be synthesized by β-1,3-glucan synthetase (GS II, EC 2.4.1.34) in the plasma membrane (Luttengger and Mevins, 1985).

The main purpose of the present work is to examine GS II activity in relation to the callose contents in pollen tube of *Arabidopsis* because pollen tube is very important part to carry the sperm nuclei into the embryo sac for seed formation in flowering plants. On the other hand, there are several *Arabidopsis* mutants showing different pollen tube growth (Kim *et al.*, 1996). Experiments also carried on the flavonoid deficient mutant (tt4) and sinapate ester defecient mutant (fah1-7) to find whether callose content and the corresponding enzyme activity is depended on the degree of pollen tube growth.

MATERIALS AND METHODS

Plants and Growth Conditions

Arabidopsis thaliana cv. *Landsberg erecta* (LER)

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and ethylmethane sulfonate-induced mutants (tt4, fahl-7) were used in this study. Mutation resulting in transparent seed coats represent a locus which is named as tt4 mutant (tt4) at the tt4 locus are characterized by lack of flavonoids (Koorneef, 1990). When a mutant that lacks sinapate esters was published, it was named as fah1-7, but fah1-7 is now used in its place (Chapple *et al.*, 1992). Those seeds were sterilized 5 min in 70% ethanol and 5 min in 30% bleach plus 0.1% Triton X-100 and then washed 5 times with sterile water and dispersed onto sterile soil. Seeds were grown in pots in a growth room with a photoperiod of 16 hrs light/8 hrs dark cycle at 22°C for 4 weeks (Ian *et al.*, 1996). In 4-week-old plants, flower buds at various stage of development were harvested for samples and stored at −70°C.

Callose Stain for Tetrads

Flower buds were fixed in 3% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.0) for 24 hrs. The fixed flower buds were dehydrated through 30, 50, 70, 90 and 100% ethanol and embedded in paraffin (Feder and O'Brien, 1968). Samples were sectioned to 10 μm in thickness on a rotary microtome. Serial sections were attached to slides with drops of water. A set of slides was stained in 0.25% lacmoid with 30% ethanol containing 1% $NaHCO_3$ for 24 hrs, rinsed in 1% $NaHCO_3$ and then observed by a light microscope (Olympus, Japan) (Schneider, 1960).

Callose Stain for Pollen Tube

Callose staining was performed according to Vogts method (1994) with minor modifications. Briefly, the anthers were emasculated by removing them one day prior to pollination and then cross-pollinated by applying fresh pollens from one anther locule to each flower stigma. Pistils were harvested 6 hrs after pollination, fixed in ethanol/acetic acid (3 : 1, v/v) for 16 hrs, rinsed with 1 M phosphate buffer (pH 7.0) and incubated in 8 N NaOH for 3 hrs to clear the tissues. Pistils were stained with decolorized 0.1% aniline blue in 0.1 M K-phosphate buffer (pH 9.0) for 2 hrs, infiltrated with 100% glycerol for 1 hr and mounted on glass slides. Pollen tubes in the pistil were observed with a fluorescence microscope (Olympus, Japan), emitted at 405 nm and photographed with Kodak Tmax 400 ASA.

Enzyme Assay

GSII activity was carried out according to the method described by Cerenius and Soderhae (1984) with minor modifications. The sample was suspended in homogenization buffer, 0.1 M Tris-HCl (pH 8.0), containing 1 M sucrose, 25 μM GTP, 4 mM Na_2EDTA and 1 mM DTT. This sample suspension was further ground in a glass potter homogenizer and remaining fragment were then discarded by centrifugation at 6800×g for 15 min at 4°C. The resulting supernatant was centrifuged at 20,000 rpm for 45 min at 4°C and the pellet was suspended in 0.1 M Tris-HCl (pH 8.0) and used as a source of enzyme. The standard incubation mixture contained 10 mM UDP-[^{14}C]glucose (8.62 GBq mmol ^{1}), 5 mM cellobiose, 0.8% (w/v) bovine serum albumin, 0.1 mM GTP, 0.1 M Tris-HCl (pH 8.0) and enzyme suspension to a total volume of 1 mL, the reaction mixture was incubated at 30°C, and after 2 hrs, the reaction was terminated by adding 0.1 mL of 10% (w/v) trichloroacetic acid, filtered through a Whatman GF/C glass fiber filter followed by washing the filter with 3×3 mL TCA, and 3×3 mL 96% ethanol. After drying, the filter was transferred into 15 mL of scitillation liquid (Aquasol-2, PACKARD) and counted in a PACKARD TRICARB 2300 TR scintillation counter.

Callose Determination

Callose extraction and determimation were performed according to Kohles method (1985) with minor modifications. The samples were suspended for 30 min with shaking in 50 ml of 10 mM Tes/NaOH (pH 7.0) with 2% sucrose and collected by vacuum filtration on glass fiber discs (Whatman type GF/C, 2.5 cm) and washed with about 15 mL of sterile water. To remove autofluorescent soluble material the samples were soaked at least 2 min in 10 mL of ethanol. The suction-dried samples were transferred into a glass potter homogenizer and disintegrated in 3 mL of 1 N NaOH. The resulting suspension was incubated at 80°C for 15 min to solubilize the callose and centrifuged (5 min, 380×g). Aliquots (200 μL) of the supernatant were used for the callose assay. For determination of the callose, supernatant (200 μL) were mixed with 400 μL of 0.1% (w/v) aniline blue, resulting in a violeted color. After addition of 210 μL of 1 N HCl the color changes to deep blue, indicating neutral to acidic pH values. The final pH value was adjusted by addition of 590 μL 1 M glycine/NaOH buffer (pH 9.5) and the tubes were mixed vigorously. During the following

incubation for 20 min at 50°C and further 30 min at room temperature, the aniline blue becomes almost completely decolorized. Fluorescence of the assay was read in a SHIMADZU RF-5000 spectrofluorometer (excitation 400 nm, emission 510 nm). The standard of fluorescence used were a laminarin.

Protein Assay

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Callose Accumulation

To examine the callose accumulation in the flower of *Arabidopsis*, flower developmental stages were divided into five groups. The stages of flower are as follows; stage I(tightly closed bud before meiosis of PMC), stage II (slightly closed bud carrying tetrads), stage III (closed bud carrying pollens), stage IV (pollinated open bud) and stage V (wilted bud). Fig. 1 represents the cross section of flower buds in four different stages, I, II, III and VI, respectively. Cross

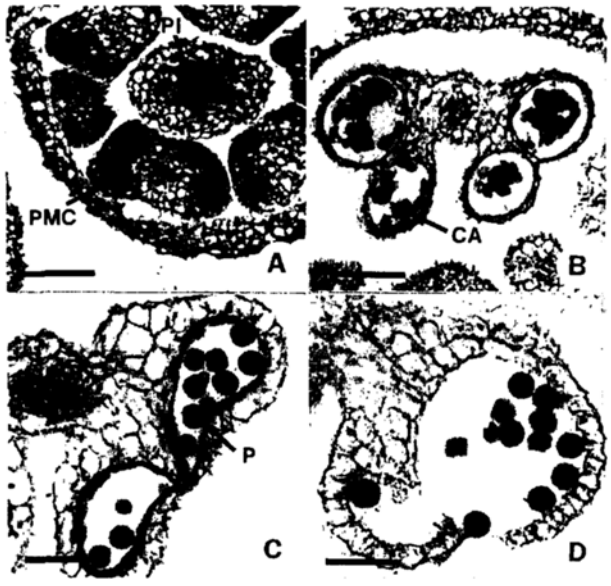

Fig. 1. The development of pollens in flower buds of *A. thaliana* (LER) and callose was stained with lacmoid (200×). (Bars=50 μm). A, transverse section of flower bud in stage I; B, transverse section of flower bud in stage II; C, transverse section of flower bud in stage III; D, transverse section of flower bud in stage IV (PI, pistil; PMC, pollen mother cell; P, pollen; CA, callose).

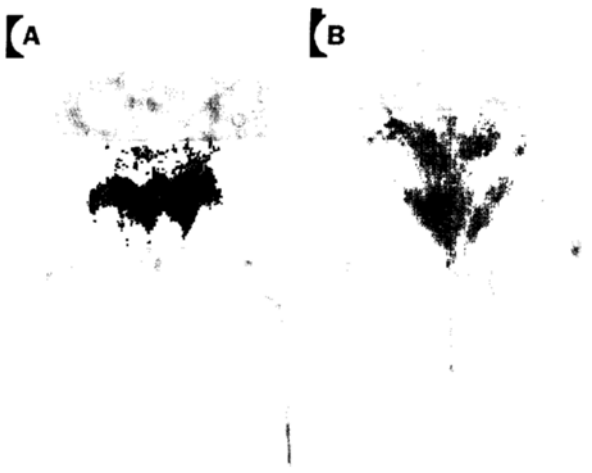

Fig. 2. The photograph of pollen tube growth in a pistil of *A. thaliana* (LER) at 6 hrs after pollination. Pollen tubes fluoresced intensely after staining with aniline blue (100×). A, pistil of *A. thaliana* 2 hours after pollinaton; B. pistil of *A. thaliana* 6 hours after pollinaton (PT, pollen tube).

sections of flower were stained specifically with lacmoid to distinguish callose deposition in the tissues (Schneider, 1960). Each flower (A, B, C and D) showed developmental charateristics, especially in male gametophytic tissues. Only tetrad cells in stage II were positively stained in blue. The callose act as a temporary wall to isolates the products of meiosis so that the cells were prevented from cohesion and then released upon callose dissolution (Waterkeyn, 1962). Since callose was not satisfactorily identified in pollen tubes at stage V, the pollen tubes was stained differently. The component of pollen tube wall have not yet been satisfactorily characterized, but callose is usually detected as fluorescence material after staining with aniline blue which is primarily specific for β-1,3-glucans (Stone *et al.*, 1984). Fig. 2 shows pollen tube growth *in vivo* from the stigma using aniline blue staining and fluorescence microscopy. Pollen tubes fluoresced intensely after staining with aniline blue at 6 hours after pollination.

Determination of Callose Content

Since callose deposition was observed in tetrad and pollen tube walls, callose contents were spectrofluorometrically determined in five developmental stage of flower. Flowers in stage II and IV accumulate high amount of callose comparatively about 170% and 127% of other stages, respectively

(Fig. 3). During pollen development, the microsporocytes undergo meiosis to form tetrads of haploid microspores. Just before meiosis, microsporocytes synthesize a special cell wall consisting of callose between the cellulose cell wall and plasma membrane. After the completion of meiosis, the callose wall is broken down by callase, a tapetally secreted β-1,3-glucanase (Steiglitz, 1977). In addition, the growth of pollen tubes is closely linked to deposition of their cell wall, which is rich in a β-1,3-glucan, callose (Herth *et al.*, 1974).

GSII Activity

In flower stage II, GSII activity was increased by about 10% while the callose contents increased by about 70%. Again, GSII activity was elevated by about 30% during pollen tube growth at stage IV but, callose accumulation was less severe than in stage II (Fig. 3). These results show a relationship between callose content and GS II activity. To confirm whether GS II is reposible for the synthesis of callose in *Arabidopsis* flower, same experiment was carried out using biochemical mutants.

It was previously reported that the growth of pollen tubes was significantly different in flavonoid mutants. The tubes in the pistil of fah1-7 elongated to a greater length relative to those of the wild type or tt4 (Kim *et al.*, 1996). In the mutants of *A. thaliana*, both callose content and GS II activity was generally increased in stage II and VI (Fig. 4). That is, enrichment of callose coincides with the increase in GS II activity, especially during the pollen tube growth. Concurrently, less activity of GS II results in lower amount of callose in the comparison of three genotypes (Fig. 4).

Callose deposition appeared to be varied according to the developmental condition of flower tissue. Therefore, regulation of callose metabolism is apparently well regulated in the sporogenetic tissue. Although there are numerous reports in the literature

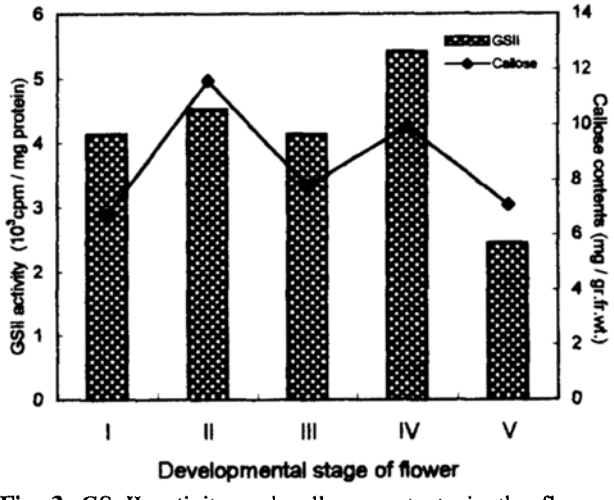

Fig. 3. GS II activity and callose contents in the flower of *A. thaliana* (LER). [^{14}C]-UDP glucose was used as a substrate and [^{14}C] glucan formed was measured. The callose contents was determined by spectrofluorometer (excitation 400 nm, emission 510 nm). Each value was the average of three separate examinations (I, tightly closed bud before meiosis of PMC; II, slightly closed bud carrying tetrads; III, closed bud carrying pollens; IV, pollinated open bud; V, wilted bud).

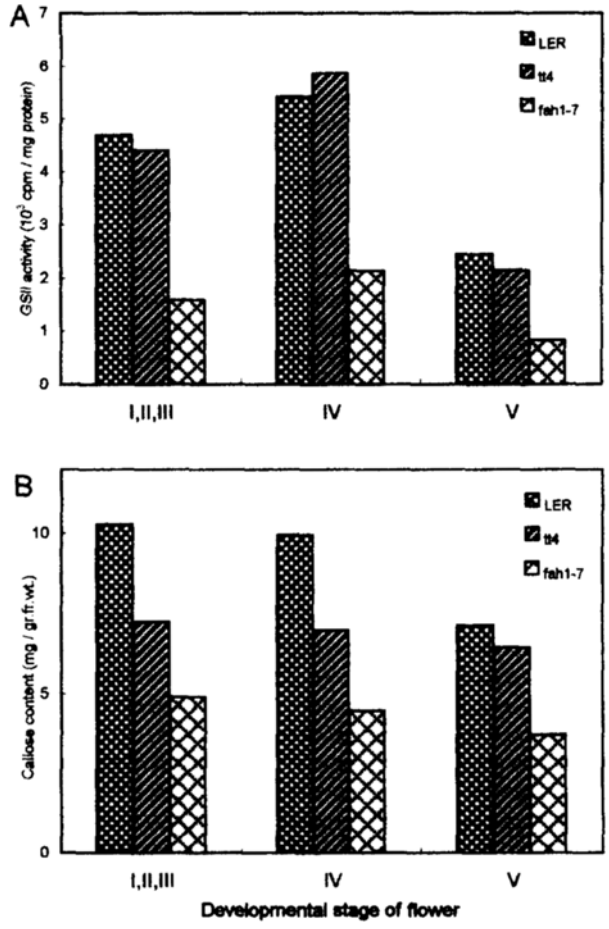

Fig. 4. GSII activity and callose contents the flower of *A. thaliana* mutants (tt4 and fah1-7). [^{14}C]-UDP Glucose was used as a substrate and [^{14}C] glucan formed was measured. The assay of callose contents was determined by spectrofluorometer (excitation 400 nm, emission 510 nm). Each value was the average of three separate examinations. A, GS II activity; B, callose contents (I, II, III, before flowering; IV, pollination; V, after pollination).

on GS II, no report has been concerned with sporogenetic tissue. In this experiment, the results show that GS II activity was clearly related with callose formation in the flower tissues. It suggest that callose plays an important role in the growth of pollen tube by increasing GS II activity responsible for synthesizing the callose. Recently, GS II has been shown to be activated by Ca^{++} (Delmer *et al.*, 1984; Kauss, 1985; Morrow and Lucas, 1986). In pollen tubes, the existence of a tip-focused gradient of cytosolic free calcium has been shown to be essential for growth (Obermeyer and Weisenseel, 1991; Rathore *et al.*, 1991; Miller *et al.*, 1992; Pierson *et al.*, 1994) and it is maintained by an asymmetric activity of Ca^{++} channels (Malho *et al.*, 1994, 1995). In our next study, we will examine the effects of Ca^{++} on pollen tube growth in relation to the GS II activity.

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