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Involvement of phenylalanine ammonia-lyase in the development of pollen in broccoli (*Brassica oleracea* L.)

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Summary. To determine whether phenylalanine ammonia-lyase (EC 4.3.1.5) is involved in the maturation of microspores to fertile pollen, anthers of a fertile strain of broccoli (*Brassica oleracea* L.) were studied in a comparison with anthers of a cytoplasmic male sterile strain. In the normal fertile strain, immature anthers of about 2 mm in length exhibited higher phenylalanine ammonia-lyase activity than mature anthers or those shorter than 2 mm. The 2-mm-long anthers corresponded to the mononucleate stage, just after release of the microspores during pollen development. Immunohistochemical localization of phenylalanine ammonia-lyase in the anthers indicated that the protein was present predominantly in the tapetal cells. The immature anthers of cytoplasmic male sterile broccoli had a lower phenylalanine ammonia-lyase activity than those of the normal fertile strain. The level of phenylalanine ammonia-lyase activity in the immature anthers was positively correlated with the number of fertile pollen grains at the flowering stage in both strains. It seems possible, therefore, that phenylpropanoid metabolism, which involves phenylalanine ammonia-lyase, may play an important role in the maturation of microspores in flowering plants.

Key words: *Brassica oleracea* L. – Broccoli – Cytoplasmic male sterility – Phenylalanine ammonia-lyase – Pollen development

Introduction

Flavonoids are a principal component of pollen pigments, as are carotenoids. It has been suggested that the transition of phenylpropanoids to flavonoids is essential for the development of viable pollen (Stanley and Linskens 1974; Wiermann 1970). Phenylalanine ammonia-lyase (PAL; L-phenylalanine ammonia-lyase, EC 4.3.1.5) catalyzes the first step in the biosynthesis of the phenylpropanoid skeleton from phenylalanine by deaminating the latter compound to generate cinnamic acid in higher plants (Jones 1984). Rittscher and Wiermann (1983) demonstrated that the specific activity of PAL in isolated tapetum cells of *Tulipa* anthers is very high, as it is in tapetum cells in anthers of several other species (Beerhues et al. 1989). It has also been demonstrated immunohistochemically that PAL is located predominantly in the tapetum of anthers (Kehrel and Wiermann 1985). Thus, evidence suggests that PAL in tapetum cells of anthers might play an essential role in the development of pollen. If this hypothesis is correct, pollen development should result in sterile pollen grains when the PAL activity of anthers is reduced for any reason.

The maturation of pollen can be interrupted not only by environmental factors, such as low or high temperature, and by chemical treatment, but also by the action of genetic factors, such as male sterility, in particular, cytoplasmic male sterility (CMS) (Satake and Hayase 1970; Kaul 1988). Although physiological mechanisms involved in the maturation or the sterility of pollen grains have not yet been clarified, tapetal functions appear to be critical to pollen development. The tapetum plays an important role in pollen development after the completion of meiosis; it is the source of the sporopollenin of the pollen exine, and is responsible for the breakdown of callose around microspore tetrads, as well as for the supply of nutrients to developing pollen (Shivanna and Johri 1985; Chapman 1987).

It is difficult to elucidate the exact developmental stage at which microspore abortion occurs in CMS plants. It seems that developmental aberrations that result in male sterility can occur at any stage of reproductive or floral development, but they occur most commonly during microsporogenesis or microgamatogenesis (Laser and Lersten 1972; Kaul 1988). In the CMS systems known in *Brassica* species, sterility in the "*ogu*" system is the result of degeneration of microspores and

Abbreviations: CHS, chalcone synthase; CMS, cytoplasmic male sterility; DAPI, 4′, 6-diamidino-2-phenylindole dihydrochloride; PAL, L-phenylalanine ammonia-lyase.

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the tapetum after the tetrad stage, while sterility in the "*nap*" system results from delayed pollen development and indehiscent anthers (Bartkowiak-Broda et al. 1979). In the "*ctr*" system, the breakdown in microsporogenesis occurs some time after meiotic prophase 1 (Grant and Beversdorf 1986). In the "*mur*" system (Hinata and Konno 1979), the events causing male sterility occur subsequent to the early microspore stage after tetrad formation, with a resultant reduction in the number of fertile pollen grains and with indehiscent anthers (T. Nii and K. Hinata, personal communication). We have chosen broccoli (*Brassica oleracea*), which provides an example of CMS that involves the "*mur*" system CMS as a system in which to examine the physiological events that are likely to be associated with the maturation of microspores. This system provides opportunities to obtain significant information, with some limitations.

We now report evidence for a possible involvement of PAL in the development of fertile pollen grains, obtained from a comparison of a CMS strain to a normal fertile strain of broccoli. Although genes that regulate the development of pollen grains have not yet been identified, regulation via the PAL pathway may be a component of the regulatory mechanism, as suggested by an earlier report by Elkind et al. (1990). Recently, the importance of phenylpropanoid metabolism in pollen development was demonstrated using an antisense chalcone synthase gene in transgenic petunia plants (Van der Meer et al. 1992). Other recent evidence also indicates the critical importance of flavonols for pollen functions (Coe et al. 1981; Taylor and Jorgensen 1992; Ylstra et al. 1992).

Materials and methods

Plant materials

Fertile broccoli (*Brassica oleracea* L. cv. De Cicco) plants and CMS plants were used. The broccoli CMS strain, with the nucleus of broccoli and with the cytoplasm of *Diplotaxis muralis*, was developed by N. Konno and K. Hinata (unpublished). Plants were propagated by growth of cuttings from individual normal fertile and CMS plants to obtain uniform plants. The plants were grown in an unheated greenhouse.

To determine whether tetrads or uninucleate or binucleate microspores were present on developing anthers, the relationship between the length of the anther and the stage of pollen development was clarified by fluorescence microscopy after staining with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI; Wako Pure Chemical Industries, Osaka, Japan), which is a fluorescent DNA-binding agent for the detection of DNA that is very simple to use (Williamson and Fennell 1975). Anthers were selected according to their length, placed immediately in liquid nitrogen and stored at −80° C until use. The number of fertile pollen grains per flower was determined by the method of Hinata and Konno (1975).

PAL activity

PAL activity was determined by the method of Tanaka et al. (1974). The activity was expressed in terms of the amount (micromoles) of cinnamic acid produced. Protein was quantitated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Immunohistochemical detection of PAL in anthers

Immature anthers were embedded in 1% gelatin with cooling under ice-water, frozen and stored at −80° C. Cross sections (10–15 μm thickness) were made at −20° C using a Cold tome CM-41 (Sakura Seiki, Tokyo, Japan), mounted on cover glasses coated with 0.1% poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.), and then fixed in cold acetone for 5 min after air-drying. The procedures for indirect immunoalkaline-phosphatase staining of PAL were those described by Satoh and Fujii (1988), except that alkaline phosphatase-conjugated antibody, instead of peroxidase-conjugated antibody, was used for the secondary antibody. Nonspecific binding of proteins was saturated by a 30-min incubation with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4 pH 7.3) that contained 1% skim milk. The primary antibody was raised against sweet potato PAL (Tanaka and Uritani 1977), and samples were incubated for 1 h in a humid chamber at 37° C. The sections were rinsed for 1 h with three changes of PBS, after which they were overlaid with alkaline phosphatase-conjugated antibody, raised in goat against rabbit IgG (Promega, Madison, Wi), which had been diluted 100-fold in PBS that contained 1% BSA. Samples were then incubated for 2 h in a humid chamber at 37° C. Preimmune serum and only secondary antibody were used as negative controls. The sections were rinsed first as described above and then with AP buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.02% NaN_3). They were then stained with the color reagent, 0.033% nitro blue tetrazolium (Sigma) and 0.0165% 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Sigma) in AP for up to 5–10 min. After a final rinse in distilled water, the sections were mounted with 50% glycerol and viewed under a light microscope.

Results

PAL activity in the floral organs

In mature buds, 1 day before anthesis, petals and anthers had predominantly higher PAL activity than that found in sepals and pistils (Fig. 1A). However, in immature buds of the same inflorescences, immature anthers, in particular those about 2 mm in length, had the highest level of activity. Immature anthers less than 2 mm long had similar activity to that of the mature anthers (Fig. 1B). The activity of petals could not be determined, since the petals were small and undeveloped. This stage in the development of pollen, when anthers are 2 mm long, corresponded in broccoli to the mononucleate stage just after release of the microspores, as determined by staining with DAPI.

Immunohistochemical detection of PAL in the immature anthers

Figure 2 shows the immunohistochemical localization of PAL with antibody against sweet potato PAL (Tanaka and Uritani 1977). The result of western blot analysis using this antibody showed that the antibody reacted specifically with PAL protein (80 kDa) of broccoli anthers (data not shown). In this experiment, an internal phosphatase in the anther tissues was not detected. The immunochemically visualized PAL protein was localized predominantly in tapetal cells, which form a heavily

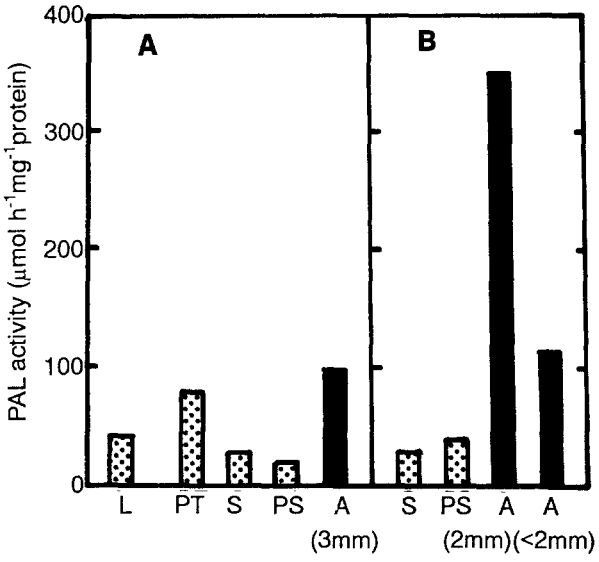

Fig. 1A, B. PAL activity in floral organs and leaves of broccoli. **A** Mature buds 1 day before anthesis; **B** immature buds on the same inflorescence. *L*, leaves; *PT*, petals; *S*, sepals; *PS*, pistils; *A*, anthers, with lengths indicated in *parentheses*. Each value is the mean of two determinations

stained layer around the loculi that contain the microspores, and in the vascular-bundle cells of connective tissue. In fertile anthers, staining in the tapetal cells was heavier than in CMS anthers.

Comparison of PAL activity in the immature anthers

We measured the PAL activity in immature anthers about 2 mm length from the CMS strain of broccoli and from the normal fertile strain. CMS immature anthers had significantly lower activity than that of the normal fertile strain in both years (Table 1). The PAL activity shown in Table 1 was somewhat variable, depending on the sampling years, probably as a result of fluctuating temperature in this season. Even though the activity was variable, a significant difference in PAL activity between the normal fertile strain and the CMS strain was apparent in each case (Table 1). Next, we examined the relationship between PAL activity in the immature anthers and the production of fertile pollen grains at the flowering stage. A scatter diagram of PAL activity versus the number of fertile pollen grains per

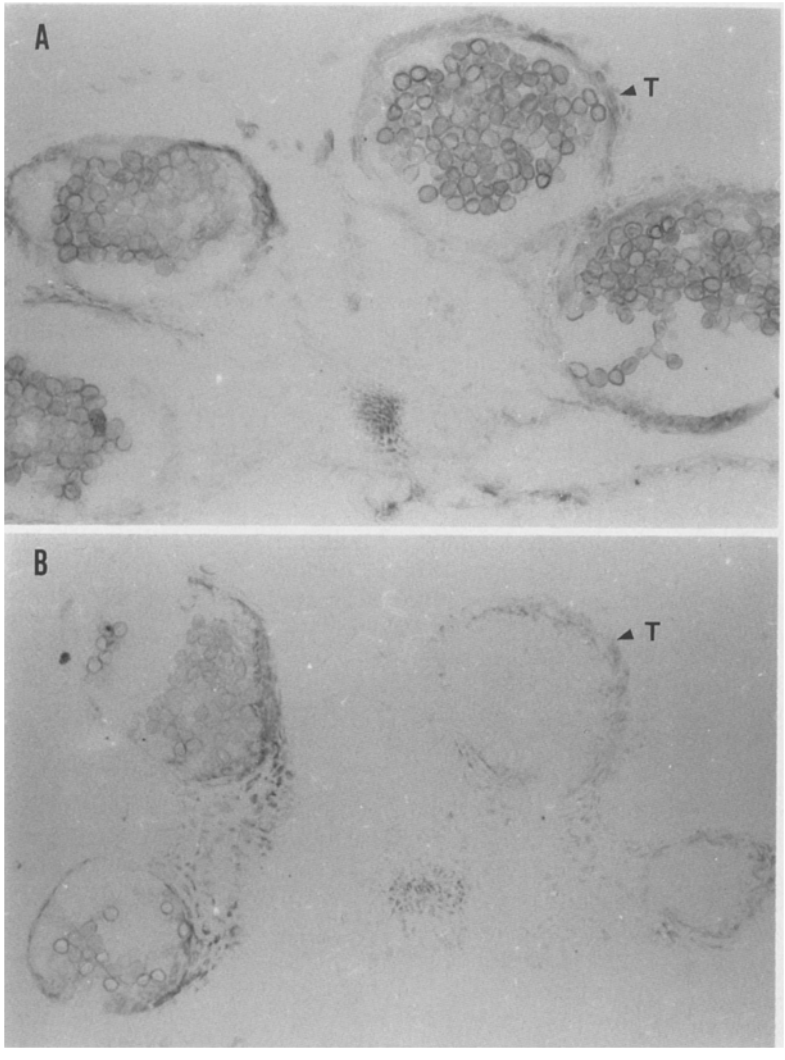

Fig. 2A, B. Immunohistochemical detection of PAL in fertile (**A**) and CMS (**B**) immature anthers in broccoli. Cross sections were incubated with primary antibody raised against sweet potato PAL and then with alkaline phosphatase-conjugated secondary antibody. The antigen was visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt. *Arrowheads* indicate tapetal cells (T). Sections were photographed in a light microscope. × 50

Table 1. Comparison of PAL activity in immature anthers between CMS and normal fertile strains of broccoli. Each determination was conducted with 40–50 mg fresh weight of anthers, and pairs of samples were handled together. Four determinations were made on each occasion.
* $P<0.05$; ** $P<0.01$ (difference from the fertile strain)

Measurement	PAL activity (μmol h^{-1} (mg protein)$^{-1}$)	
	Fertile strain	CMS strain
M-1	305±20	207±33*
M-2	229±22	89±19**

M-1, sampling at 10 April 1990; M-2, sampling at 12 April 1991

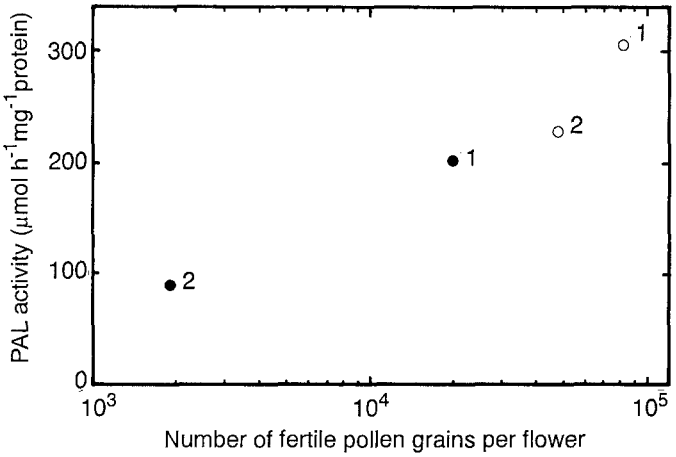

Fig. 3. The relationship between the number of fertile pollen grains per flower at anthesis and PAL activity in immature anthers about 2 mm in length, for fertile (*open circles*) and CMS (*solid circles*) strains of broccoli. PAL activity corresponds to that in Table 1, which was determined on two different occasions. Fertile pollen grains were deeply stained with 0.025% cotton blue

flower (shown on a logarithmic scale) indicated that the correlation between the parameters was significant at the 5% level ($r=0.976$) (Fig. 3). This correlation suggests that high PAL activity in immature anthers might be necessary for pollen maturation.

Discussion

The results obtained here suggest that a high level of PAL activity in the immature anther, in particular at the stage of pollen development that corresponds to the early microspores, may be essential for the normal development of microspores into fertile pollen grains. The major components of pollen pigments are flavonoids, such as quercetin, kaempferol and naringenin in *B. oleracea*. The main carbon skeleton of these compounds is derived from shikimic acid via phenylalanine and *p*-coumaric acid (Stanley and Linskens 1974; Wiermann 1970). Rittscher and Wiermann (1988) observed a high level of incorporation of the radiolabel from (U-^{14}C)-phenylalanine into sporopollenin. Their finding suggests that phenylpropanoid metabolism and, therefore, PAL are involved in the biosynthesis of sporopollenin. Sporopollenin, the chemical nature of which is still unknown, is transported in a highly polymerized form from the tapetum to the exine, the outer wall of the pollen grain (Stanley and Linskens 1974; Shivanna and Johri 1985). If the phenylpropanoid metabolism that is involved in pigmentation and/or the biosynthesis of sporopollenin is essential for the development of pollen, insufficient synthesis via this pathway for any reason might result in the failure of the formation of normal pollen. Our results with the CMS strain support this hypothesis.

The positive relationship between PAL activity in immature anthers and the number of fertile pollen grains at the flowering stage indicates that PAL activity in immature anthers is a significant factor in the development of pollen (Fig. 3). The results suggest that the production of less fertile pollen by CMS broccoli, even when flowers appear normal, is partly due to a lower PAL activity in the tapetum of young anthers at the early microspore stage of pollen development. Moreover, the regulation via PAL might be under common regulatory control in the fertile and CMS strains of broccoli, acting in addition to the genetic factors responsible for the CMS phenomenon.

In *Tulipa* and several other species, the key enzymes in the biosynthesis of flavonoids, namely PAL and chalcone synthase (CHS), have been located in the tapetum fraction from a pollen-tapetum-fractionation (Rittscher and Wiermann 1983; Beerhues et al. 1989) and in the tapetum by immunohistochemical staining (Kehrel and Wiermann 1985). In our study, we confirmed the location of PAL using an immunohistochemical method and found that PAL was located predominantly in the tapetal cells of immature anthers about 2 mm in length (Fig. 2). In the CMS anthers, the staining of tapetal cells seemed to be rather weak, suggesting that less PAL protein might be synthesized in the tapetal cells of the CMS strain. This observation corresponds to a lower PAL activity at M-2 in Table 1.

When inflorescences of the normal fertile strain were treated with aminooxyacetate (AOA), which is a competitive inhibitor of PAL (Amrhein et al. 1976; Hoagland and Duke 1982), the number of sterile pollen grains in mature anthes clearly increased and approached levels in CMS flowers in terms of percentages of sterile pollen grains (data not shown). This treatment with AOA in vivo may affect other biochemical pathways, but the result suggests that AOA may inhibit the development of microspores into fertile pollen grains. This suggestion is supported by the light microscope observation that anthers treated with AOA developed normally until the early microspore stage, after which they became abnormal, with a partly enlarged tapetum and an increase in the number of sterile pollen grains at the mature stage. These observations are similar to those made with a CMS strain of broccoli (T. Nii and K. Hinata, personal communication).

Why do immature anthers of the CMS strain have lower levels of PAL activity than the fertile strain? It

is difficult to demonstrate unequivocally that reduced PAL activity causes sterility or that reduced PAL activity is a result of the failure of pollen development. In future studies, observations are required of the floral phenotypes of transformants generated by introduction of PAL genes into CMS plants and of the results of introduction of antisense PAL genes into normal plants, under control of a promoter that is active in the tapetum. Such transformants should provide further information about the involvement of PAL in the production of fertile or sterile pollen. We are attempting such analyses at the present time. A novel way to introduce the antisense genes of CHS, another key enzyme in the biosynthesis of flavonoids, has already been accomplished in *Petunia* plants (Napoli et al. 1990; Van der Meer et al. 1992).

Recently Elkind et al. (1990) obtained 23 transgenic tobacco plants that carried the PAL2 gene from bean; 6 of them had altered floral development with shorter filaments, fewer pollen grains and reduced numbers of viable pollen grains. The levels of PAL activity were markedly reduced in leaves, petals and anthers of the transformants. Thus, it appears that a perturbation of phenylpropanoid metabolism caused by the altered expression of PAL genes in transgenic plants can lead to morphological alterations in floral organs, in particular in anthers. In our CMS strain of broccoli, a similar perturbation of the function of PAL genes in anthers may occur.

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