Inhibitors of Cyanide-insensitive Respiratory Pathway Induce Male Sterility in Broccoli (*Brassica oleracea*)

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We have found that inhibitors of the cyanide-insensitive respiratory pathway induce male sterility in Broccoli (*Brassica oleracea* L. cv. De Cicco). All three major specific inhibitors; salicylhydroxamic acid (SHAM), propyl gallate and disulfiram (tetraethylthiuram disulfide), were effective in inducing male sterility whenever they were applied to the flower buds with anthers less than about 2mm in length, which those pollen developing stages corresponded to be earlier than early microspore stage. These chemically induced male sterile flowers which were characterized by shorter filaments and a reduction in the number of total and fertile pollen grains per flower, were similar to the cytoplasmic male sterile flowers. However, when pollinated with normal pollen, the percentage of seed-setting in the chemically induced male sterile flowers decreased considerably in comparison with that in the cytoplasmic male sterile flowers. All the progeny plants derived from the male sterility (female) × normal pollen grains showed male fertility. Morphological characters of the progeny plants were very similar to those of normal Broccoli. Although the physiological significance of the cyanide-insensitive respiratory pathway remains unknown, some physiological pathways, such as the alternative pathway may be associated with male sterility in *Brassica* species.

KEY WORDS: *Brassica oleracea*, Broccoli, chemical male sterility, cyanide-insensitive respiration, cytoplasmic male sterility.

Introduction

Cytoplasmic male sterility (CMS) in higher plants which is a phenomenon distributed widely among various plant genera, is agronomically beneficial for the production of hybrid seeds (HANSON and CONDE 1985, KAUL 1988). Recently it has been suggested that mitochondrial dysfunction during microsporogenesis is in some way related to CMS and that nuclear genes modulated this dysfunction (LEVINGS 1983, PRING and LONSDALE 1985). Little information is available about the functions of mitochondria that are responsible for the CMS phenomenon.

MUSGARAVE *et al.* (1986) compared cyanide-insensitive respiration of leaf and root tissues in seven male sterile lines from four species, including CMS maize-C, -S, and -T, with that of corresponding fertile line tissues. They reported that all the male sterile lines with the exception of the CMS-T line lacked cyanide-insensitive respiration, unlike fertile lines. Although these results *in vivo* were not obtained in the anthers themselves, it suggested that the cyanide-insensitive respiratory pathway might be involved in the male sterile phenomenon in plants. If it is certain, it is interesting whether the plants have the male sterile flowers when the cyanide-insensitive respiratory pathway of normal plants is inhibited reversely.

The cyanide-insensitive respiratory pathway is widespread among plant species. This cyanide-insensitive O₂ uptake takes place in the inner mitochondrial membrane and appears to constitute a pathway "alternative" to the main cyanide-sensitive cytochrome pathway. Since this pathway is associated with the less production of ATP, it is considered to be useless and wasteful (LANCE *et al.* 1985, SIEDOW and BERTHOLD 1986). Three major specific inhibitors of

the cyanide-insensitive respiratory pathway; namely salicylhydroxamic acid (SHAM), propyl gallate and disulfiram (tetraethylthiuram disulfide), have been studied extensively (LANCE *et al.* 1985). These compounds are also strong inhibitors of lipoxygenase. Recently the terminal oxidase of the alternative pathway has been identified by ELTHON and MCINTOSH (1987), but its physiological significance and its role in plant metabolism still remain unknown.

The purpose of this report is to present an evidence that the inhibitors of the cyanide-insensitive respiratory pathway induced the male sterility in Broccoli plants, and to discuss a possible mechanism concerning in the induction of the male sterility.

Materials and Methods

Broccoli (*Brassica oleracea* L. cv. De Cicco) plants were used for the application of the chemicals. A cytoplasmic male sterile (CMS) strain was also used to compare the following observations. The CMS Broccoli strain, with a Broccoli nucleus and *Diplotaxis muralis* cytoplasm, has been recently developed by KONNO and HINATA (unpublished). Plants were sown in autumn and grown in a glasshouse. In the next spring at the bud stage with anthers less than 2-2.5mm in length, the treatment was initiated at the stem just below a bud ball in the way that the chemical solution could be absorbed through cotton strings from a 2ml container during 7-10 days. The compounds used were Salicylhydroxamic acid (Sigma Chemical Co.), n-Propyl gallate and Tetraethylthiuram disulfide (Wako Pure Chemical Industries, Ltd.). The concentration of the solutions was 1-10mM for SHAM and 0.1-1mM for propyl gallate and disulfiram. Each compound was dissolved in a small amount of 2-methoxyethanol, then diluted about one thousand times with distilled water. This concentration of 2-methoxyethanol had no effect on the plants and flowers. Application to seeds was also attempted during the germination in the 1-5mM SHAM solution.

The effect of the chemical applications was evaluated on the flower with abnormalities such as indehiscent or stunted anthers. In the sterile flower, the number of pollen grains per flower, percentage of fertile pollen grains, anther size and relative length of stamens to pistil were examined in comparison with normal and CMS flowers, according to the method of HINATA and KONNO (1975).

Induced male sterile flowers were pollinated with normal pollen to analyse the pistil function and the seeds obtained were planted to test the progeny.

Results

Of the three major specific inhibitors used, SHAM and propyl gallate were able to induce effectively male sterility in Broccoli (Figs. 1 and 2). The male sterile flowers induced by disulfiram exhibited a side-effect such as petals that failed to open at the flowering stage, therefore SHAM and propyl gallate were mainly used in this experiments. Compared with a normal flowers, the male sterile flowers thus obtained showed smaller petals and shorter filaments with indehiscent and stunted anthers. Such morphological characteristics were similar to those of the CMS flowers (Figs. 1 and 2). In Table 1, the number of pollen grains per flower and characteristics of the anther observed on the flower buds one day before anthesis are presented as mean values. The number of pollen grains per flower and percentage of

Fig. 1 SHAM-induced male sterile (B), fertile (A) and CMS(C) flowers.

Fig. 2 Male sterile (B) induced by propyl gallate, fertile (A) and CMS (C) flowers.

Table 1. Comparison of number of pollen grains per flower and characteristics of anther among SHAM- and propyl gallate-treatments, normal and CMS strains. Each value is a mean of five to six different flowers

Strains	Number of pollen grains per flower		Characteristics of anther		
	Total number of pollen grains	% of fertile pollen ¹⁾	Length	Width	Relative length of stamens to pistil
	$\times 10^3$	%	mm	mm	
Normal	83.8	57.1	2.9	1.0	1.10
CMS	9.5	20.4	2.5	0.9	0.78
SHAM treatment	19.0	15.3	2.7	0.9	0.84
Propyl gallate treatment	18.0	9.7	2.3	0.8	0.94

1) Fertile pollen grains were stained by 0.025% cotton blue.

fertile pollen grains decreased in the sterile anthers induced by the chemicals to a similar degree to those of the CMS anthers. The anther size was also a little smaller in the chemically induced sterile anthers than in normal anthers. The shorter relative length of the stamens to pistil in the anthers chemically induced and CMS anthers reflected mainly the presence of shorter filaments, as shown in Figs. 1 and 2.

The percentage of seed-setting in the chemically induced male sterile flowers decreased to 0-30%, when compared with that in the normal and CMS plants. It varied depending on combined factors including the concentration of the chemical solution applied, the physiological status of the plants, environmental conditions, and so on. When 1-5mM SHAM was applied to the seeds during germination, the rate of occurrence of the male sterile plants was approximately 18%. These male sterile flowers exhibited a percentage of seed-setting of about 50%, which was slightly higher than that of the flowers in which chemicals were applied to the inflorescences. Apparently toxic side-effects of the chemicals on the pistil function should be considered.

All the progeny plants derived from the chemically induced male sterile flowers that were

crossed with normal pollen grains showed male fertility. Morphological characters of the progeny plants were quite similar to those of normal Broccoli. When the concentration of the chemical was high enough to cause bud blight (10mM for SHAM or 1mM for propyl gallate), the adjacent shoots exhibited male sterile flowers. Furthermore, the male sterile plants induced by the application of the chemical to the seeds continued to produce male sterile flowers even when new shoots sprouted after the cutting of the dead shoots. The persistence of the effectiveness which is interesting, was presumably not associated with some mutation induced by the chemicals, because it was not inherited.

Discussion

The morphological observations in the male sterile flowers chemically induced suggest that the mechanism responsible for the male sterility induced by the inhibitors of the alternative pathway may be analogous to that of CMS in Broccoli. The mechanism may be related to the sequence of developmental stages from microspore to pollen maturation, because the anthers exhibited the sterility at the flowering stages whenever the inhibitors were applied to the inflorescences with anthers less than about 2mm in length, which those pollen developing stages corresponded to be earlier than early microspore stage.

The inhibitors of the alternative pathway may have been first described as one of the gametocides (KAUL 1988). Apparently toxic side-effects of those chemicals on the pistil function is considered, but a suitable concentration of the chemicals to minimize the side-effects may be able to be decided for each case of the applications. The reduction in the female fertility is unavoidable as shown in cases of male sterility induced by several other chemical gametocides (KAUL 1988).

The effect of disulfiram application differed from that of SHAM and propyl gallate in the lack of persistence of the effectiveness and a side-effect such as petals that failed to open at the flowering stage. SHAM and propyl gallate may thus affect male sterility in the same ways or at the same site. LANCE *et al.* (1985) reported that SHAM and propyl gallate acted on the same site, while SHAM and disulfiram showed different sites of action on the specific inhibitors of the cyanide-insensitive respiratory pathway. Since the high performance liquid chromatography analysis did not enable to detect propyl gallate inside the plant, it is assumed that the compound may have been incorporated into the metabolic pathway or decomposed in the plant.

It remains to be determined whether the inhibitors *in vivo* act on some site of the cyanide-insensitive respiratory pathway itself or on an other site. It is possible to consider that two mechanisms control the involvement of the inhibitors in the development of abnormal pollen. First, the heat generation in this alternative pathway may play a significant role in pollen development, although this mechanism has not been identified in *Brassica* species. Thermogenicity of the alternative pathway is well known in the arum lily family, which releases heat to serve as a "volatilizer" for the floral odors that attract the insect pollinators (MEEUSE and RASKIN 1988). Another possibility is that these compounds are also inhibitors of lipoxxygenase (E.C.1.13.11.12) which catalyzes the incorporation of molecular oxygen into polyunsaturated fatty acids such as linolenic and linolenic acids in plants (VICK and ZIMMERMAN

1987). Evidence for the role of lipoxygenase in ethylene formation was also presented (VICK and ZIMMERMAN 1987). AHOKAS (1982) first reported the possible role of the lipoxygenase activity in the phenomenon of CMS in barley. He emphasized that lipoxygenase is important in anthers and may be associated with the production of sporopollenin in the tapetum. Sporopollenin, a chemical component which is present in the outer wall of pollen grains, is an oxidative polymer of carotenoids (BROOKS and SHAW 1978). The physiological functions of the alternative pathway and metabolites of the lipoxygenase pathway are poorly understood. Further studies should be carried out to understand the physiological and biochemical processes of the male sterile phenomenon.

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シアン耐性呼吸経路阻害剤によるブロッコリーの雄性不稔誘発

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ミトコンドリアの内膜で起こっているシアン耐性呼吸の阻害剤により雄性不稔が誘発された。ブロッコリー (*Brassica oleracea* L. cv. De Cicco) の花芽分化後の蕾に、主要な阻害剤; SHAM (salicylhydroxamic acid), Propyl gallate, Disulfiram, をそれぞれ, 1-10 mM, 0.1-1 mM, 0.1-1 mM の濃度で7-10日間処理した。処理方法は蕾の下部の茎に糸を通して、吸収させる方法を採用した。いずれの阻害剤も適当な濃度で雄性不稔を誘発したが、Disulfiram は花卉が開きにくい等の影響がみられたので、主に SHAM と Propyl gallate を用いた。花器を観察すると、正常型と比べて、誘発された不稔花は花卉の大きさが小さくなり、葯の柱頭に対する相対位置も低くなるなど、細胞質雄性不稔ブロッコリーとよく似た形態を示した (Figs. 1, 2)。1花当りの総花粉数と稔性花粉の割合 (%) はともに大きく低下しており、葯の大きさもやや小さくなり、開花時に葯が裂開しない点でも、対照の細胞質雄性不稔系統と類似していた (Table 1)。

阻害剤の処理方法として、種子に処理した場合には、約18%の割合で雄性不稔個体が出現した。

阻害剤により誘発された雄性不稔花に正常な花粉を交配したところ、細胞質雄性不稔系統と比較して、種子稔性が0-30%に低下したことから、これら阻害剤は同時に雌ずいにも異常を引き起こしているようである。しかしながら、濃度等の処理条件をさらに検討することにより、雄性不稔性は維持しつつ、雌ずいへの影響を最低限にとどめる方法が見いだせるかも知れない。

雄性不稔花に、正常な花粉を交配して得られた種子の次代の花は花粉稔性・形態等すべて正常であった。

シアン耐性呼吸阻害剤が雄性不稔を誘発する機構については、今後の検討を要するが、花粉の発育過程になんらかの影響を与えているようである。またこれら阻害剤は、同時に Lipxygenase の強い阻害剤としても知られているので、雄性不稔の生理・生化学的発現機構を解明する上で、興味ある知見が得られたものと考えられる。