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RESEARCH ARTICLE

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Active oxygen metabolism in the floral buds and leaves of the new cytoplasm male sterile (CMS) line and its maintainer line of non-heading Chinese cabbage

Abstract The cytoplasm male sterile (CMS) line and its maintainer line of the newly-bred non-heading Chinese cabbage were taken as experimental materials. The production rate of superoxide anion radical (O_2^-), contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), and the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) as well as ascorbate peroxidase (APX) were determined in buds of different developmental stages and leaves during vegetative and florescence stages. As showed by the results, there were higher levels of MDA and H_2O_2 contents, higher O_2^- production rate and higher activities of SOD, POD, and CAT in the buds of the CMS lines than that in the buds of the fertile lines, while the APX activity was different from the other three enzymes above. There were differences in activities of protective enzymes in leaves but no significant differences in MDA contents and O_2^- production rate, which showed that the differences in metabolism of active oxygen between CMS and its maintainer were mainly apparent in reproductive organs.

Keywords non-heading Chinese cabbage, cytoplasm male sterility (CMS), lipid peroxdation, active oxygen metabolism, protective enzymes

1 Introduction

The non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino var. *communis* Tsen et Lee), an allogamy crop with obvious heterosis, originated from China. It is a good material for creating hybrids through male sterility. Ogu cytoplasm male sterile (CMS) line of non-heading Chinese cabbage obtained from the cytoplasm male sterile line of radish was difficult to apply because of its leaf etiolation in the seedling period under low temperature and flower hypogenesis. The newly-bred OguCMS, created through asymmetric electric fusion of protoplast cell technology by Hou et al. (2001), had not only overcome the disadvantages of its original cytoplasm male sterile material such as etiolation under the conditions of low temperature and lack of nectary, but also made its sterile rate reach 100%, and enabled its seeding rate to be not significantly different from that of the maintainer line. Since most of the present work was focused on the application of somatic cells, this research on the mechanism of male sterility was very primary. Starting with the metabolism of active oxygen, we analyzed the physiological and biochemical characteristics of male sterility of non-heading Chinese cabbage and try to find out the possible mechanism.

2 Materials and methods

2.1 Materials

The cytoplasm male sterile line and its maintainer line of the newly-bred non-heading Chinese cabbage were provided by the College of Horticulture in Nanjing Agricultural University. They were seeded in the field in October 2003, and transplanted to the experimental fields in January 2004 with normal field management. The central functional leaves and the floral buds, which were divided into different bud-size groups of big (⩾3 mm), middle (2–3 mm), and small (⩽2 mm), from five plants of each line in the stages of rosette and florescence were mixed evenly and respectively. Related indices were determined with three replicates, and mean values for each index were calculated.

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2.2 Methods

2.2.1 Determination of O_2^- production rate and contents of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2)

Production rate of superoxide anion radical (O_2^-) was determined by using O_2^- oxygenated hydroxylamine method (Wang and Luo, 1990), i.e., the oxidative product NO_2^- was colored in minobenzenesulfonicacid sulfanilic acid and α-aniline solution, then the equal-volume butanol was added, followed by the determination of A_{530} of aqueous phase, and finally a standard curve was made by using $NaNO_2$. The MDA content was determined by TBA color method (Zhao et al., 1991) with a substitution of 10% for 5% of extraction liquid of trichloroacetic. The H_2O_2 content was determined according to the method of Lin et al. (1998).

2.2.2 Determination of enzyme activities

0.3 g fresh leaves or flower buds were homogenized in 3 mL phosphate buffer (pH 7.8, 0.05 mol/L). The homogenate was then centrifuged at 3 000 r/min for 20 min, and then the supernatant volume was measured for the determinations of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) as well as ascorbate peroxidase (APX).

Superoxide dismutase enzyme activity was assayed according to the method of Wang et al. (1983) with some modifications. The 3 mL of reaction mixture contained 6.0 μmol/L L-riboflavin, 13 mmol/L L-methionine, 75 μmol/L NBT and 0.05 mol/L phosphate buffer (pH 7.8). Then it was illuminated under fluorescence at 4 000 lx for 20 min after adding 0.05 mL enzyme solution. 50% inhibition efficiency of photo reduction was regarded as a unit of the enzyme activity.

Peroxidase activity was determined by the guaiacol colorimetric method (Xu and Ye, 1989), i.e., successive determination of A_{470} (Absorptiophotometric value of wavelength 470 nm) at 25°C and an increase of 0.01 in OD value per minute was regarded as a unit of enzyme activity.

Catalase activity was assayed according to Jiang and Wang (1982) with some modifications, i.e., addition of 100 μL of 2% H_2O_2, 50–100 μL of enzyme solution into 1.7 mL phosphate buffer (pH 7.0, 225 mmol/L), consecutively recorded A_{240} at 25°C, and each decrease of 0.01 in OD value per minute was regarded as a unit of enzyme activity. Ascorbate peroxidase activity was determined according to the methods of Asada (1984) and Xu and Ye (1989), i.e., successively recorded A_{290} at room temperature, and each decrease of 0.01 in OD value per minute was taken as a unit of enzyme activity.

2.3 Data analysis

Data achieved were given to *LSD* analysis of variance by using Excel. Different small or capital letters indicated significant or extremely significant differences, at $P < 0.05$ and $P < 0.01$ levels, respectively.

3 Results and analysis

3.1 Changes of O_2^- production rate and contents of MDA and H_2O_2 in the floral buds of the male sterile line and its maintainer line in different stages of development

The O_2^- production rate (Fig. 1A) and H_2O_2 contents (Fig. 1B) in the floral buds of the maintainer line did not change significantly in different stages of development, while the O_2^- production rate and H_2O_2 contents were significantly higher in the male sterile line than that in the maintainer line with the development of floral buds.

In the development process of floral buds, the MDA content of the maintainer line quickly decreased (Fig. 1C), while the MDA content of the male sterile line with a gradual increase was higher than that of the maintainer line in the same stages, i.e., 1.03, 1.56, and 4.66 times as much as that of the maintainer line, respectively.

3.2 Changes of antioxidase activities in floral buds of male sterile line and maintainer line in different stages of development

The POD activity of the male sterile line was all significantly higher than that of the maintainer line in every developmental

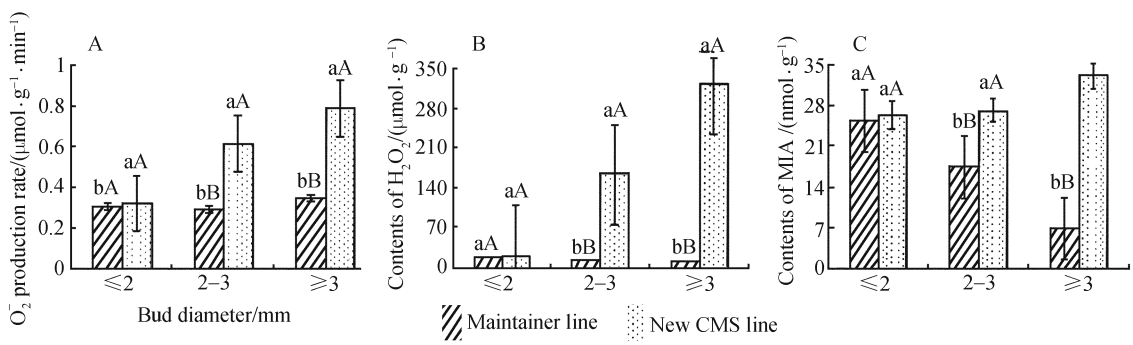

Fig. 1 Comparison of O_2^- generating rate, H_2O_2 and MDA contents in floral buds of new CMS line with its maintainer line during different developmental stages in non-heading Chinese cabbage

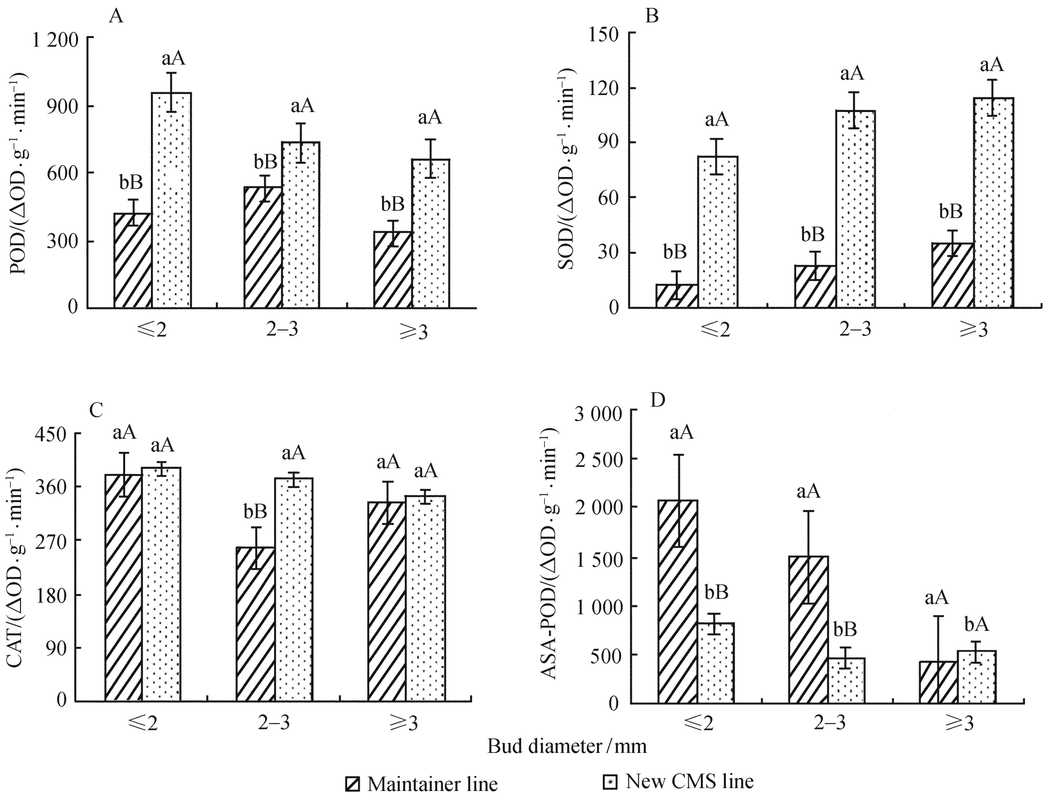

Fig. 2 Comparison of activities of POD(A), SOD(B), CAT(C) and APX(D) in floral buds of new CMS line and its maintainer line during different developmental stages in non-heading Chinese cabbage

stage of the floral buds (Fig. 2A). The POD activity of the maintainer line increased firstly and then decreased. It reached the highest in the middle-bud-sized stage. The POD activity of the male sterile line was the highest in the small-bud-sized stage and then gradually decreased with a significantly higher level than that of the maintainer line all the time.

The SOD activity of the male sterile line and the maintainer line slowly increased in the floral-bud developmental process. The SOD activity of the male sterile line was significantly higher than that of the maintainer line all the time, i.e., 6.67, 4.60 and 3.15 times as much as that of the maintainer line, respectively (Fig. 2B). The variation of SOD activity was a gradual increase which showed the same changing trend as O_2^- production rate. Thus, it can be seen that a high rate of O_2^- production may induce the increase of SOD activity.

Catalase activity in medium-sized buds was higher in the male sterile line than in the maintainer line, while the CAT activity in the other kinds of buds was not significantly different between the male sterile line and the maintainer line (Fig. 2C), indicating that there was a tiny difference of CAT ability in eliminating H_2O_2 between the male sterile line and the maintainer line.

According to Fig. 2D, during the whole development process of the floral bud, the APX activity of the maintainer line decreased significantly, while that of the male sterile line decreased appreciably and the difference was not significant. The possible reason was the very low content of H_2O_2 in floral buds of the maintainer line, resulting in the decrease of the APX activity. But in the male sterile line, the rapid increase of H_2O_2 content and the decrease of APX activity led to a serious peroxidation of lipid membrane, making it sterile. Michaelis-Menten Kinetics (Km) between APX and H_2O_2 was smaller than that between CAT and H_2O_2, which indicated that the former affinity was relatively bigger, so APX played a more important role in eliminating H_2O_2 (Asada, 1992).

3.3 Changes of O_2^- production rate, contents of MDA and H_2O_2 as well as the antioxidase activity in leaves

As shown in Table 1, the O_2^- production rate of the maintainer line was slightly higher than that of the male sterile line in the periods of vegetative growth and florescence. Contents of H_2O_2 and MDA in leaves of the maintainer line were higher than those of the male sterile line in vegetative growth, but they were reverse in the florescence period. The MDA content in the leaves of the male sterile line was significantly higher than that of the maintainer line leaf in the florescence period, while the contents of active oxygen and MDA in leaves were not significantly different during different stages of development.

Table 1 O_2 production rate, H_2O_2 contents, MDA contents and activities of POD, SOD, CAT and APX in leaves during different developmental stages in non-heading Chinese cabbage

Stage	Line	O_2^- prodution rate /μmol·g^{-1}·min^{-1}	H_2O_2 contents /nmol·g^{-1}	MDA contents /nmol·g^{-1}	Activity of enzymes /ΔOD·g^{-1}·min^{-1}			
					POD	SOD	CAT	APX
Vegetative stage	MF	0.51aA	6.40aA	2.52aA	4 540.51aA	131.91bB	3 578.18bA	5 864.14aA
	MS	0.40aA	5.30aA	2.46aA	3 458.34bB	181.42aA	3 718.27aA	5 717.79aA
Flowering stage	MF	0.29aA	40.57aA	43.9bA	2 534.21bA	108.81bA	2 464.30bA	1 876.51aA
	MS	0.250aA	41.56aA	49.6aA	3 281.41aA	132.21aA	2 950.10aA	1 978.41aA

During the whole process of development, O_2^- production rate and the MDA content decreased significantly, while the H_2O_2 content increased significantly.

Compared with that of the male sterile line, activities of SOD and CAT in leaves of the maintainer line were lower during the period of vegetative growth, while the POD activity in leaves was significantly higher, and the APX activity in leaves was appreciably higher. After the florescence period, SOD and CAT activities in leaves were still significantly higher in the maintainer line than in the male sterile line; the APX activity in leaves was also appreciably higher than that of the male sterile line. The change of the POD activity was opposite to that of the florescence period, i.e., it was significantly higher in the male sterile line than in the maintainer line.

From the vegetative period to the reproductive period, SOD, POD, CAT, and APX activities were all sharply decreased.

4 Conclusions and discussions

4.1 Relationship between male sterility and superoxide lipid membrane of non-heading Chinese cabbage

In the process of oxygen assimilation, plants can produce some poisonous active oxygen, such as O_2^-, ·OH, and H_2O_2. This active oxygen would harm the plants, i.e., MDA accumulation and damage of the membrane led to a physiological and biochemical disorder in related tissues. In the process of floral development, the microspore metabolized very actively and needed much energy, but the damage to the membrane structure would hamper the formation and transportation of energy, which hence hindered the floral development.

As believed through the study on such sterile materials as rice, corn, and rape, the higher content of active oxygen and MAD in the plant was one of the important causes for male sterility, which was supported by our study. O_2^- production rate and the H_2O_2 content in floral buds of non-heading Chinese cabbage were significantly higher in the male sterile line than that in the maintainer line, so was MDA as a product of membrane peroxidation. These conclusions proved that the male sterility of non-heading Chinese cabbage is closely related to higher level of active oxygen and subsequent peroxidation of membrane.

4.2 Relationship between male sterility and protective enzymes of non-heading Chinese cabbage

There were different viewpoints about the relationship between antioxidase and male sterility because of different crops and materials. As discovered by previous researchers, SOD, POD, CAT, and APX activities in different crops were all lower in the male sterile line than in the maintainer line. Therefore, according to most researchers, the peroxidation of lipid membrane resulted from the restraint of SOD, POD, CAT, and APX activities making the plant unable to clear out the free radicals and finally leading to the accumulation of active oxygen. But the activities of the enzymes to eliminate active oxygen in preserved tuber mustard (Hu et al., 2000) and cauliflower (Zhao et al., 2002) were higher than those of their maintainer lines, indicating that it was a kind of protective response of the male sterile line to the increase of active oxygen in plant.

In this research, SOD, POD, CAT, and APX activities in floral buds were also higher in the Ogu cytoplasm male sterile line of non-heading Chinese cabbage than in the maintainer line, which supported the research result of preserved tuber mustard (Hu et al., 2000). O_2^- can be eliminated by SOD. Although POD and SOD can eliminate some H_2O_2, APX is the key enzyme for H_2O_2 elimination. As discovered in our study, the APX activity of male sterile line was significantly lower, which was unable to respond to the significant increase of H_2O_2 content and finally led to abundant accumulation of H_2O_2. It may be one reason for the outcome that MDA content in the anther of the male sterile line was far higher than that of the fertile line.

Therefore, it is believed that male sterility of non-heading Chinese cabbage was mostly caused by high level of active oxygen and subsequent peroxidation of lipid membrane. There was no direct relationship between activities of protective enzymes and male sterility. The increase of SOD, POD, and CAT activities was induced by high O_2^- level in the sterile material. Further researches on the causes for high O_2^- level and high peroxidation degree of lipid membrane need to be carried out possibly in the aspects of metabolism of matter and energy such as respiration metabolism and hormone difference.

During the period of vegetative growth, compared with that of the maintainer line, the APX activity was not significantly different, SOD and CAT activities were higher, and O_2^- production rate and contents of H_2O_2 and MDA were lower. These results indicated that SOD in the male sterile line can rapidly catalyze O_2^- to H_2O_2 which can then be eliminated rapidly, resulting in no peroxidation of lipid membrane. Furthermore, the results also indicated that the abilities of the male sterile line and the maintainer line to eliminate active oxygen were not significantly different because abnormal metabolism did not take place in the male sterile line. In the florescence period, the contents of H_2O_2 and MDA in leaves were higher in the male sterile line than in the maintainer line, which showed that the sterile feature of the sterile line of non-heading Chinese cabbage could be expressed in leaves to some extent, but the abnormal metabolism of active oxygen was mostly found in reproductive organs.

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