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Effect of Abscisic Acid and High Osmoticum Concentration on the Induction of Desiccation Tolerance in Microspore-derived Embryos of Chinese Cabbage (*Brassica campestris* L.)

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Summary

Induction of desiccation tolerance by abscisic acid (ABA) and osmotic stress was investigated in microspore-derived embryos of Chinese cabbage. Exogenous application of ABA was effective for germination and plant regeneration from dry embryos whose water content decreased to approximately 10% by transfer to a series of desiccators in which the relative humidity remained constant. Induction of desiccation tolerance depended on the ABA concentration, but not on the genotype. The maximum percentages of germination (87.6%) and plant regeneration (46.3%) were obtained in the embryos treated with 10μM ABA. Though osmotic stress by high concentration of sorbitol also induced desiccation tolerance, its effectiveness was less appreciable than that of ABA. On the other hand, all the embryos lost their viability after desiccation, when they were not treated with ABA or exposed to osmotic stress. The dry embryos treated with ABA were able to regenerate into plants in soil. Protein analysis revealed that ABA and osmotic stress increased the accumulation of seed storage proteins in microspore-derived embryos.

Key Words : *Brassica campestris,* abscisic acid, artificial seed, desiccation tolerance, microspore culture, osmotic strees.

Introduction

Desiccation of uncoated somatic embryos to a level of water content similar to that of true seeds is important for artificial seed production (Fujii *et al.* 1987). It is also useful for germplasm preservation as well as easy handling of materials. Some attempts have been made to desiccate somatic embryos in several plants such as orchardgrass (Gray *et al.* 1987), alfalfa (Senaratna *et al.* 1989; 1990), carrot (Iida *et al.* 1992) and broccoli (Takahata *et al.* 1993). Recently, the desiccation tolerance of somatic embryos has been reported to be enhanced by the exogenous application of abscisic acid (ABA) (Senaratna *et al.* 1989; 1990, Brown *et al.* 1990, Iida *et al.* 1992, Takahata *et al.* 1993). On the other hand, many of the phenomena regulated by ABA are also influenced by osmotic strees (Finkelstein and Crouch 1986, Finkelstein and Somerville 1989). Zeevaart and Creelman (1980) and Ronald *et al.* (1990) reported that osmotic stress stimulated endogenous ABA accumulation. However, the relationship between ABA and osmotic stress in the induction of desiccation tolerance of developing embryos has not been elucidated.

The objective of this study was to determine whether exogenous ABA and osmotic stress pretreatments would induce desiccation tolerance in microspore-derived embryos of Chinese cabbage. In addition, direct plant regeneration ability of dry embryos in soil was examined, and protein analysis of embryos which became tolerant to desiccation was carried out.

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Materials and Methods

Plant materials

Four cultivars of Chinese cabbage (*B. campestris* var. *pekinensis*) were used. Seeds of three cultivars ('Ho Mei', 'Formosa 45 Days' and 'His Fu Early 30 Days') were kindly provided by T. Sato, National Research Institute of Vegetables, Ornamental Plants and Tea and 'Springtime' was obtained commercially. These plants were grown in a greenhouse, and then they were transferred to a growth chamber under a 13/8°C day/night regime with natural photoperiod at the beginning of bolting.

Microspore culture

Flower buds 2.0-3.0mm in length were collected. Sterilization and microspore isolation were carried out as described previously (Takahata and Keller 1991). After washing, the microspores were suspended at a density of 3×10^4/ml in 1/2NLN-10 medium, in which 13% sucrose of the 1/2NLN-13 medium (Takahata and Keller 1991) was replaced by 10% sucrose. Two ml of the microspore suspension was plated in a 60 × 15mm plastic petri dish. The microspores were incubated in the dark at 32.5°C for 1 day before incubation at 25°C.

ABA and sorbitol treatments and embryo desiccation

After two weeks of culture, the medium was replaced by 2ml fresh 1/2NLN-10 medium supplemented with ABA and sorbitol at various concentrations as indicated in Tables 1 and 2, respectively. After one week of incubation in the dark at 25°C, embryos were selected by sieving through a 1mm nylon mesh and were washed with sterile deionized water. All the embryos were at the cotyledonary stage. They were transferred onto a sterile filter paper, which was moistened with a few drops

of sterile deionized water, in a 60×15mm plastic petri dish.

Embryo desiccation was performed according to the method of Senaratna *et al.* (1989). Embryos were transferred through a series of desiccators, in which the relative humidity (RH) was kept constant by using a saturated solution of K_2SO_4 (RH 87%), Na_2CO_3 (80%), NaCl (70%), NH_4NO_3 (61%), $Ca(NO_3)\cdot 4H_2O$ (50%) and $K_2CO_3\cdot 1.5H_2O$ (40%). They were transferred daily from a desiccator at a higher RH to one at a lower RH.

Determination of germination and plant regeneration ability of dry embryos

After the desiccation treatment, the dry embryos were transferred to B5 agar (0.8%)-solidified medium (Gamborg *et al.* 1968) together with a filter paper and incubated at 25°C under 16 hour photoperiod. As a control, non-desiccated embryos were also subcultured onto a filter paper placed on top of B5 agar medium after washing the embryos with sterile deionized water. Germination and plant regeneration of the dry embryos were investigated after one and four weeks of culture, respectively.

A part of the dry embryos was directly sown to vermiculite moistened with B5 mineral nutrient lacking vitamins and carbohydrates.

Protein analysis

Some of the embryos, which were prepared for desiccation, were weighed, submerged in liquid nitrogen, and stored at −80°C until protein analysis. Embryos (approximately 60mg) were homogenized with a plastic pestle in a microcentrifuge tube containing 300μl extraction buffer (50mM Tris-HCl pH6.8, 60mM β-mercaptoethanol, 10mM EDTA, 2% SDS, 1mM PMSF). After boiling for one min, the homogenate was centrifuged at 10,000rpm for 5min. The supernatant was then mixed with 5 volumes of cold acetone and placed at −20°C for 2 hours. The proteins were collected by centrifugation at 10,000rpm for 10min, dried and resuspended in 100μl solubilization buffer (60mM Tris-HCl pH6.8, 60mM DTT, 8mM EDTA, 2% SDS, 12% sucrose). Concentration of the proteins was determined using the Bio-Rad protein assay kit. Seed proteins were also extracted in 50μl extraction buffer per mg dry seed weight. For each sample, 15μg of the proteins were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970).

After SDS-PAGE, the proteins were electroblotted onto a nitrocellulose membrane for Western blotting. Storage proteins were detected using antibodies against rapeseed 12S (cruciferin) seed storage proteins, which were kindly provided by T. Sakai, Plantech Research Institute.

Results

Effect of ABA on induction of desiccation tolerance

Water content of the microspore-derived embryos was approximately 10% after 6 days of desiccation treatment. When the dry embryos were rehydrated, those which became tolerant to desiccation turned green and developed a root within five days. The embryos of all genotypes became tolerant to desiccation by the applica-

Table 1. Effect of ABA treatment for induction of desiccation tolerance of microspore-derived embryos of Chinese cabbage (*B. campestris*)

Concentration of ABA (μM)	Cultivar	No. of embryos tested	Normal germination frequency[1] (%)	Plant regeneration frequency[2] (%)
0	Springtime	30	0.0	0.0
	Formosa[3]	27	0.0	0.0
	Ho Mei	9	66.7	0.0
	His[4]	44	9.0	0.0
	Total	**110**	**9.1**	**0.0**
1	Springtime	26	34.6	15.4
	His	22	100.0	59.1
	Total	**48**	**64.6**	**35.8**
10	Springtime	35	100.0	74.3
	Formosa	23	73.9	43.5
	Ho Mei	12	100.0	58.3
	His	51	84.0	25.5
	Total	**121**	**87.6**	**46.3**
100	Springtime	32	87.5	59.3
	Formosa	20	45.0	25.0
	Ho Mei	9	0.0	0.0
	His	59	25.4	22.0
	Total	**120**	**43.3**	**30.8**

[1] Examined 1 week after rehydration
[2] Examined 4 week after rehydration
[3] Formosa 45 Days, [4]His Fu Early 30 Days

Fig. 1. Embryo germination from desiccated embryos of Chinese cabbage 'Springtime' 7 days after rehydration. Embryos were treated with 0 (top left), 1 (top right), 10 (bottom left) and 100μM ABA (bottom right).

tion of ABA (Table 1, Fig. 1). The germination and plant regeneration frequency of the dry embryos depended on the concentration of ABA, but not on the genotypes. Highest percentages of germination (87.6%) and plant regeneration (46.3%) were achieved when the embryos were treated with 10μM ABA. Both 1μM and 100μM ABA also induced desiccation tolerance, especially, in the case of His Fu Early 30 Days, in which 1 μM ABA induced a higher desiccation tolerance than 10 μM. In contrast, desiccation tolerance could not be induced in embryos in the absence of treatment with ABA. Though 9.1% of the embryos could germinate after one week of rehydration, they lost their viability after 4 weeks of rehydration.

Germination frequency of the non-desiccated embryos was higher than that of the desiccated ones (Fig. 2a). In the absence of desiccation treatment, almost all the embryos germinated normally, but the embryos treated with 100μM ABA showed a reduced germination rate (70%). When the embryos were treated with 10μM ABA, the germination ability of the desiccated embryos was not different from that of the non-desiccated ones. Plant regeneration frequency of the non-desiccated embryos varied from 37.9% on 1μM ABA to 60.0% on 10μM ABA (Fig. 2b). Desiccated embryos which were treated with ABA showed a similar ability of plant regeneration to that of the non-desiccated embryos.

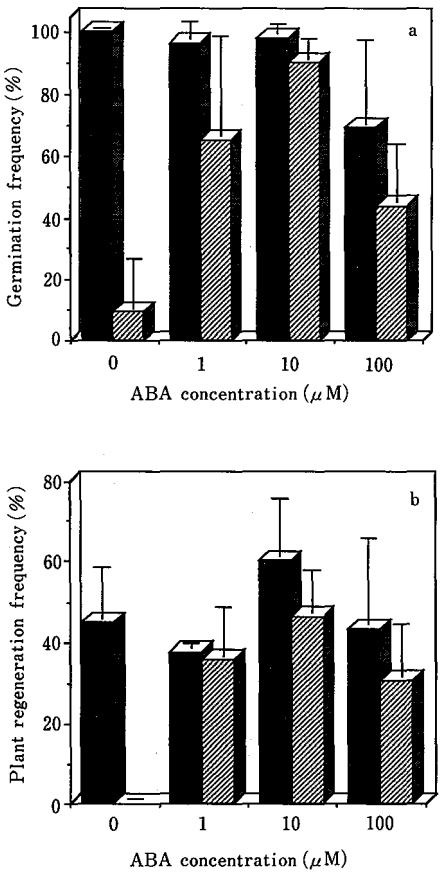

Fig. 2. Effect of desiccation of microspore-derived embryos of Chinese cabbage on (a) germination and (b) plant regeneration frequency on B5 agar medium. Embryo germination and plant regeneration of desiccated (▨) and non-desiccated embryos (■) were examined after one and four weeks of culture, respectively.

The regenerants from desiccated and non-desiccated embryos grew normally in soil. No morphological and ploidy differences were observed between them. The percentages of diploidy were 75.0% and 81.8% for the plants from desiccated and non-desiccated embryos, respectively.

Effect of sorbitol on induction of desiccation tolerance

The embryos also became tolerant to desiccation when they were treated with sorbitol at various concentrations (Table 2). However, the frequencies of germination and plant regeneration in these embryos were lower than those in the ABA-treated embryos. Though genotypic differences were observed, the highest percentages of germination and plant regeneration in the embryos treated with 10.5% sorbitol were 17.1% and 7.1%, respectively. 12.5% sorbitol was also effective in Springtime and Ho Mei. At a higher concentration of sorbitol, the frequency of survival rate of embryos decreased and embryo growth was strongly inhibited.

Plant regeneration ability of dry embryos in soil

The desiccated embryos treated with ABA were able to germinate and regenerate into plants in soil (Table 3, Fig. 3). Fifty % of His Fu Early 30 Days embryos could

Table 2. Effect of sorbitol treatment for induction of desiccation tolerance of microspore-derived embryos of Chinese cabbage (*B. campestris*)

Concentration of sorbitol (%)	Cultivar	No. of embryos tested	Normal germination frequency[1] (%)	Plant regeneration frequency[2] (%)
0	Springtime	43	11.6	0.0
	Formosa[3]	104	4.8	0.0
	Ho Mei	204	0.0	0.0
	Total	**351**	**2.8**	**0.0**
8.5	Springtime	42	16.7	0.0
	Formosa	166	15.1	5.4
	Ho Mei	213	11.7	5.2
	Total	**421**	**13.5**	**4.8**
10.5	Springtime	40	17.5	2.5
	Formosa	157	31.2	14.6
	Ho Mei	237	7.6	3.0
	Total	**434**	**17.1**	**7.1**
12.5	Springtime	75	21.3	4.0
	Formosa	168	3.6	1.2
	Ho Mei	248	9.3	3.2
	Total	**454**	**9.3**	**2.4**
14.5	Formosa	166	4.8	1.2
	Ho Mei	224	8.0	1.3
	Total	**390**	**6.7**	**1.3**

[1] Examined 1 week after rehydration
[2] Examined 4 week after rehydration
[3] Formosa 45 Days

Table 3. Frequencies of germination and plant regereration in soil from Chinese cabbage ' His Fu Early 30 Days' dry microspore-derived embryos treated with ABA

Concentration of ABA (μM)	No. of embryos tested	Germination frequency[1] (%)	Plant regeneration frequency[2] (%)
0	14	0.0	0.0
10	28	50.0	39.3

[1] Examined 1 week after rehydration
[2] Examined 4 week after rehydration

Fig. 3. Embryo germination from desiccated embryos of Chinese cabbage 'His Fu Early 30 Day' in soil after 10 days from sowing.

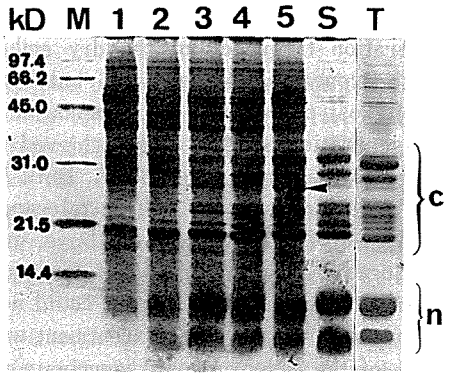

Fig. 4. SDS-PAGE pattern of proteins of Chinese cabbage 'Springtime' microspore-derived embryos treated with various concentration of ABA and sorbitol. M : Molecular weight marker, 1: Control, 2: 1μM ABA, 3: 10μM ABA, 4: 100μM ABA, 5: 12.5% sorbitol, S: Seed of Chinese cabbage 'Springtime', T: Seed of rapeseed 'Topas', c: Cruciferin, n: Napin. The arrowhead marks 27kD protein which was accumulated by treatment with sorbitol.

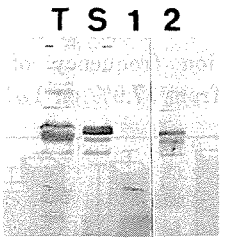

Fig. 5. Western blot analysis of protein extracts from microspore-derived embryos of Chinese cabbage 'Springtime' using antibodies against cruciferin. 1: Control, 2: 10μM ABA, T: Seed of rapeseed 'Topas', S: Seed of Chinese cabbage 'Springtime'

germinate normally and 39.3% of these regenerated into plantlets. On the other hand, no embryos germinated without ABA treatment.

Effect of ABA and sorbitol on protein accumulation of embryos

Proteins of the embryos treated with ABA or sorbitol were analyzed by SDS-PAGE (Fig. 4). The embryos treated with ABA or sorbitol accumulated proteins with molecular weights ranging from 20 to 32kD as well as less than 7kD, both of which almost corresponded to the major seed storage proteins of Chinese cabbage and were also similar in size to cruciferin (12S) and napin (1.7S) of rapeseed. The accumulation of these proteins depended on the concentration of ABA and increased with the increase of ABA concentration. Western blotting showed that antibodies of cruciferin cross-reacted with the proteins whose size was similar to that of cruciferin (Fig. 5). Accumulation of 27kD protein was detected in the embryos treated with sorbitol, but not in those treated with ABA.

Discussion

In this study, we investigated the effect of ABA and osmotic strees on the induction of desiccation tolerance in microspore-derived embryos of Chinese cabbage. Our results revealed that the ABA treatment induced desiccation tolerance in the embryos, which is in agreement with the results obtained in other *Brassica* species such as *B. napus* (Brown *et al.* 1990) and *B. oleracea* (Takahata *et al.* 1993). However, the optimum concentration of ABA was different among the species. Previous studies demonstrated that 100μM ABA was most effective for rapeseed (*B. napus*) and broccoli (*B. oleracea*) (Brown *et al.* 1990, Takahata *et al.* 1993). On the other hand, in Chinese cabbage, the application of 10μM ABA was sufficient to induce a complete desiccation tolerance and treatment with higher concentrations of ABA exerted a deleterious effect. These results suggest that Chinese cabbage is more sensitive to ABA than the other two species. When embryos were not treated with ABA, desiccation tolerance could not be induced in embryos. Though high value (66.7%) of germination percentage were observed in Ho Mei, this may be due to insufficient desiccation. In this study, embryos at the cotyledonary stage were used for the desiccation experiments. Embryos at late stages, from the torpedo to cotyledonary stages, were reported to be most responsive to ABA in some plants (Senaratna *et al.* 1990, Iida *et al.* 1992, Takahata *et al.* 1993). Though we did not examine the effect of the stage of development, our

results are compatible with previous works.

Though osmotic stress induced a desiccation tolerance in somatic embryos to some extent, the tolerance was not comparable to that induced by ABA. Ronald *et al.* (1990) reported that a high concentration of osmoticum increased the endogenous level of ABA six times compared with that of the control in microspore-derived embryos of rapeseed. However, the increase of the level by the osmoticum treatment was lower than that obtained by the exogenous supply of ABA. These results indicate that the concentration of ABA synthesized due to osmotic stress was not sufficient to induce a complete desiccation tolerance in the microspore-derived embryos. It is also recognized that ABA is supplied from maternal tissues in the developing zygotic embryo (Karssen *et al.* 1983, Hendrix *et al.* 1987, Le Pase-Degivry *et al.* 1989). Therefore, it can be speculated that the incomplete desiccation tolerance of the microspore-derived embryos may be due to the lack of exogenous supply of ABA from the maternal tissues. Accumulation of enough amount of ABA for inducing desiccation tolerance may not be achieved by a single application of the osmotic treatment.

Recovery of plantlets from desiccated embryos under soil conditions is required for practical use as artificial seeds. The present results which demonstrated that the dry embryos treated with ABA were able to germinate and regenerate into plants in soil are consistent with previous studies on rapeseed (Takahata *et al.* 1992). Accumulation of storage proteins in the microspore-derived embryos which was promoted by ABA may induce plant regeneration ability of embryos in soil. This assumption may be supported by the results obtained by Kawana and Ohkawa (1992) who observed that non-desiccated embryos of rapeseed could acquire a plant regeneration ability in soil when treated with ABA and exposed to a low temperature, though their proteins were not analyzed.

Results of protein analysis in the present study confirmed the importance of the role of ABA in promoting the accumulation of mRNA and its products of seed storage proteins as reported in zygotic embryos of rapeseed (Crouch and Sussex 1981) and soybean (Bray and Beachy 1985) and somatic embryos of rapeseed (Taylor *et al.* 1990, Ronald *et al.* 1990). In addition to the effect on storage proteins, it was reported that the ABA treatment resulted in the increase of fatty acid accumulation in zygotic and somatic embryos of some species (Finkelstein and Somerville 1989, Taylor *et al.* 1990, Kim and Janick 1991). Though these seed reservoirs became appropriate markers for somatic embryo maturation, they were not considered to be candidate materials in relation to the desiccation tolerance of embryos, because the embryos exposed to osmotic stress did not become sufficiently tolerant to desiccation, in spite of the increase of the content of seed storage proteins. In addition, we observed that microspore-derived embryos of rapeseed treated with ABA for 1 day became tolerant to desiccation in spite of the absence of accumulation of seed storage proteins (Takahata *et al.* unpublished data). On the other hand, the accumulation of LEA (late embryogenesis abundant) mRNA's and proteins, which starts at the beginning of zygotic embryo desiccation and is promoted by ABA, is considered to be related to the desiccation tolerance of embryos (Dure *et al.* 1989, Harada *et al.* 1989). Studies of LEA gene expression are currently being carried out.

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