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Continuous Embryogeny and Plant Regeneration System from Seocondary Embryos in *Brassica napus* cv. 'Topas'

By

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Summary : A method for continuous secondary embryo formation and plant regeneration in *Brassica napus* cv. 'Topas' is described. Secondary embryos that emerged from immature zygotic embryos via heat shock were separated and subcultured onto B5 plant growth regulator-free medium. Most secondary embryos (i.e. 70%) produced secondary embryos in a subsequent generation directly on the surface of elongated hypocotyls. Similarly, about 70% of the secondary embryos formed newly produced secondary embryos in a subsequent generation after subculturing, and the embryogenic potential of these secondary embryos has been maintained by repetitive subculture. The application of 10μM abscisic acid (ABA) induced desiccation tolerance in secondary embryos. Consequently, 60% of the desiccated embryos grew, all of which regenerated into normal plants. On the other hand, ABA-treated secondary embryos without desiccation treatment grew abnormally having an elongated hypocotyl, and all secondary embryos not treated with ABA lost their viability after desiccation.

Key words : *Brassica napus* cv. 'Topas', secondary embryos, ABA, desiccation tolerance, plant regeneration

Introduction

Secondary somatic embryogenesis is a phenomenon that new somatic embryos are initiated from zygotic and somatic embryos[1]. Successful secondary embryogenesis has been reported in several species[2,3]. In particular, repetitive secondary embryonic culture reported in soybean[4], peanut[5] and *Arabidopsis*[6] has great value in clonal propagation and genetic engineering. This is because it has certain advantages when compared to primary somatic embryogenesis - such as a high multiplication rate, independent of the explant source and repeatability. Furthermore, embryogenicity can be maintained for prolonged periods of time by repeated cycles of secondary embryogenesis. However, in order to establish a culture system using secondary embryos in which embryogenicity is maintained, control of normal plant regeneration from such embryos is indispensable. Secondary embryos may often regenerate plants at a low frequency, but may also show morphological anomalies on medium supplemented with plant growth regulators[2,3]. In *Brassica,* though the formation of secondary embryos by repetitive culture and the plant regeneration from secondary embryos on medium supplemented with cytokinin have been reported in rapid-cycling *Brassica,* i.e. 'Fast Plant' using seed as the starting material[7], there is also no report which shows the same result in any another cultivar. In addition, regeneration from secondary embryos on plant growth regulator-free medium has not been achieved.

ABA is considered to be involved in maturation to acquire embryonic characteristics such as suppression of precocious germination and induction of desiccation tolerance during the process of embryo development[8,9]. Several previous studies showed that ABA was required in the culture of embryos not only for physiological changes, but also for morphological changes such as stabilization of the cell-membrane system[10,11]. Cultured embryos treated with ABA acquired characterisics similar to natural seeds. Specifically, desiccated embryos treated with ABA before-

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hand possessed capacities such as conversion to seedlings under various soil conditions, long-term storage and easy handling of delivery[12,13].

In this study, we report continuous secondary embryo formation using secondary embryos acquired from immature embryos by application of heat shock used in the microspore culture of *Brassica napus* cv. 'Topas'. Furthermore, we examined the ability of secondary embryos to regenerate into plants on plant growth regulator-free medium by the exogenous application of ABA and/or desiccation.

Materials and Methods

Production and repetitive culture of secondary embryos

Rapeseed (*Brassica napus* cv. 'Topas') plants were grown in an uncontrolled-environment greenhouse. At the commencement of bolting, the plants were transferred to a growth chamber under a 13℃/8℃ (day/night) regime with cool-white illumination at 200μE $m^{-2}s^{-1}$. Flowers were pollinated by bud pollination just before anthesis. At 26–33 days after pollination (DAP), pods removed from the plant were sterilized in sodium hypochlorite (1.0% active chlorine) (Kanto Chemical co., Inc) with 1–2 drops of Tween-20 (Kanto Chemical co., Inc) for 20 min. After rinsing in sterile distilled water three times, the ovules containing immature zygotic embryos were excised from pods and placed into 25×120 mm glass tube including B5[14] agar (0.8%)-solidified medium containing 2% sucrose (B5-2). Three ovules were placed in a tube, and 10 tubes per culture were incubated in the dark at 32℃ for 1 day as heat shock prior to maintenance at 25℃. Culture was repeated 4 times.

The first-generation of secondary embryo (SE_1) that emerged from the immature zygotic embryos were separated at the cotyledonary stage from the parent plant, transferred to fresh B5-2 hormone-free medium at pH 5.7 and cultured at 25℃ under a 16-h photoperiod of cool-white illumination at 30μE $m^{-2}s^{-1}$. The second-generation of SEs (SE_2s) that developed from cultured SE_1s were subcultured in the same way onto fresh medium. SEs of each generation were maintained in a 30-day subculturing cycle.

ABA treatment and desiccation to secondary embryos

The third-generation of SEs (SE_3s) that formed from subcultured SE_2s were treated with ABA to induce desiccation tolerance. SE_3s excised from SE_2s were placed in 1/2 NLN-13[15] liquid medium supplemented with 0, 1, 10 and 100μM ABA in 60×15 mm plastic petri dishes (Falcon) at a density of about 10 SEs per dish. Three dishes for each treatment were incubated in the dark at 25℃ for 2 days. Following incubation, the SEs were placed onto a 200μm nylon mesh and then washed with sterile distilled water. They were transferred to a sterile filter paper (Advantec No. 2), which was moistened with a few drops of sterile distilled water, in 60×15 mm plastic petri dishes.

SE desiccation was performed according to the method of TAKAHATA *et al.* (1993)[16] by drying through a series of desiccators in which the relative humidity (RH) was kept constant using a saturated solution of K_2SO_4 (RH 87%), Na_2CO_3 (80%), NaCl (70%), NH_4NO_3 (61%), $Ca(NO_3)_2 \cdot 4H_2O$ (50%) and $K_2CO_3 \cdot 1.5H_2O$ (40%). SEs were transferred daily from desiccators at a higher RH to those at a lower RH. After the desiccation treatment, the filter paper holding the SEs was transferred to B5-2 solidified medium and incubated at 25℃ under a 16-h photoperiod.

Data were subjected to one-way ANOVA with Scheffe's multiple range test.

Results

After 1 to 2 weeks of ovule culture on B5-2 medium, callus was formed in the region surrounding the hypocotyl of the developed immature zygotic embryos (Fig. 1A), and subsequently, SEs from immature zygotic embryos (SE_1s) formed via callus phase (Fig. 1B). Direct SE_1 formation on the surface of the hypocotyl was also observed (Fig. 1C). The frequency of SE_1s emerged from the immature zygotic embryos in plant growth regulator-free medium was 21.7±5.2% after 2 months' culture. SE_2s formed on the surface of hypocotyls of the developing SE_1 within 1–3 weeks after subculture of SE_1 (Fig. 1D). SE_2s adhering to the parent plant produced roots (Fig. 1D *arrow*). After subculture of SE_2s, the hypocotyls elongated, and were able to form the subsequent generation of SEs, i.e. SE_3s, on their surface within 1–2 weeks (Fig. 1E). Although the direction in which the SEs formed on the hypocotyls was not uniform, the part that adhered to the parent explant was invariably a hypocotyl or a cotyledon (Fig. 1 E, F).

Investigation of secondary embryogenesis using 10 SE_1s indicated that SE formation was continuous. In seven of the 10 subcultured SE_1s, a total of 116 SE_2s formed in 9 weeks. SE_2s that developed into cotyledonary embryos on SE_1 were subcultured, and 37–100% (average of 69.3%) of them formed SE_3s (Table 1). An average of about five SE_3s per SE_2 embryo formed on embryogenic SE_2s and about 13% of the embryogenic SE_2s produced more than 10 SE_3s. As a result of repetitive subculture, 451 SEs were obtained in all

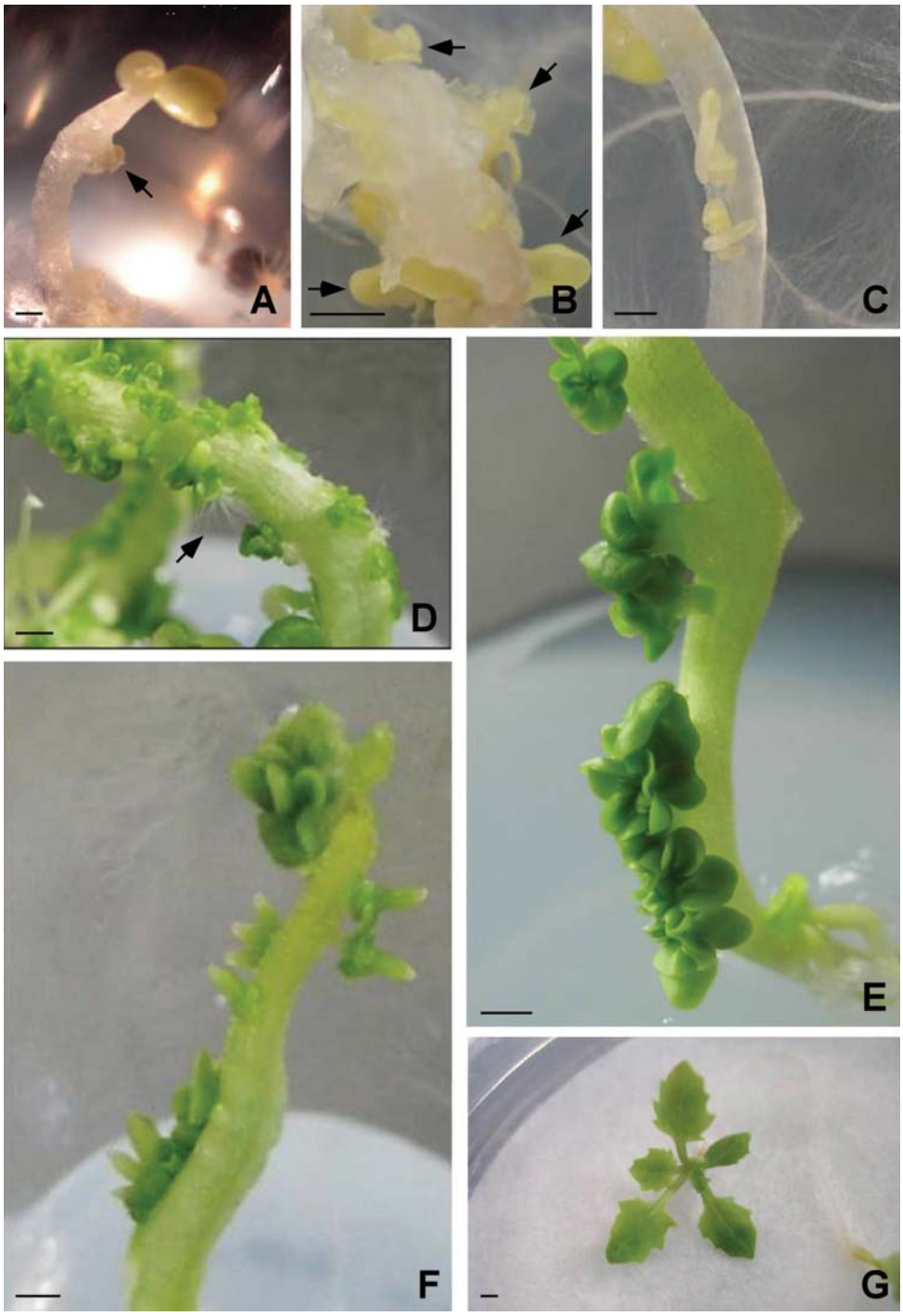

Fig. 1 Secondary somatic embryogeny of *B. napus* cv. 'Topas'. (A) Callus formation (*arrow*) on the hypocotyl of a developed immature zygotic embryo. (B) First-generation of SE_1s (*arrows*) formed via callus on the hypocotyl of developed immature zygotic embryo. (C) SE_1s formed direcly on the hypocotyl of a developed immature zygotic embryo. (D) Second-generation of SE_2s formed directly on the hypocotyl of subcultured SE_1. (E) and (F) Third-generation of SE_3s formed directly on the hypocotyl of subcultured SE_2. (G) Plant regenerated from desiccated SE_3 in which desiccation tolerance was induced by the application of 10μM ABA. Bar=1 mm.

Table 1 Second- and third-generation of secondary embryo (SE_2 and SE_3) formation from an independent line of 10 first-generation of secondary embryos (SE_1s) for 9 weeks in *B. napus* cv. 'Topas'.

SE_1 line	No. of SE_2 formed	Embryogenic SE_2 frequency (%)	No. of an average (max.) of SE_3 formed per embryogenic SE_2
1	0	-	-
2	6	-	-
3	9	60.0	1.0 (1)
4	40	76.2	3.9±1.0 (16)
5	21	37.5	11.8±7.2 (40)
6	35	80.0	4.8±0.7 (13)
7	4	100	3.7±0.3 (4)
8	1	-	-
9	0	-	-
10	0	-	-
Average		69.3	4.9±0.8

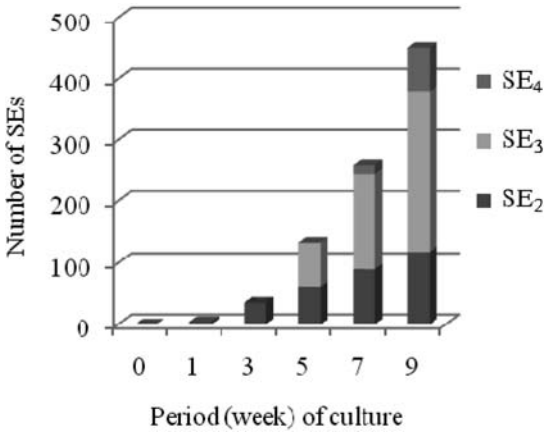

Fig. 2 Continued secondary embryogeny from an independent line of the 10 first-generation of secondary embryos (SE_1s) until fourth-generation of secondary embryos (SE_4s) for 9 weeks' culture in *B. napus* cv. 'Topas'.

Table 2 Effect of ABA treatment and desiccation for third-generation of secondary embryos (SE_3s) of *B. napus* cv. 'Topas'.

Concentration of ABA (μM)	Desiccation[a]	No. of embryos tested	Growth[b] frequency (%)	Morphology of plantlet grown	SE formation[c] of subsequent generation
0	-	31	100	Hypocotyls elongated	+
	+	30	0	n.d.[d]	n.d.[d]
1	-	29	100	Hypocotyls elongated	-
	+	34	8.8±8.3	Normal	-
10	-	33	97.0±2.6	Hypocotyls elongated	-
	+	30	60.0±8.2**	Normal	-
100	-	30	93.3±6.7	Hypocotyls elongated	-
	+	31	3.2±3.0	Normal	-

Asterisks (**) indicate significant differences between growth frequencies of the desiccated embryos after ABA treatment of each concentration at the 1% level according to Scheffe's multiple range test.
[a] +: Desiccated, -: Not desiccated
[b] Examined 4 weeks of rehydration
[c] +: With SE formation, -: Without SE formation
[d] Not detected

(from SE_2 to SE_4) in 9 weeks' culture from 10 SE_1s (Fig. 2).

In order to investigate whether this was a switch into plantlet regeneration from secondary embryogenesis, we investigated the effect of exogenous application of ABA and/or desiccation treatment to SEs. As a result of the application of ABA, SEs acquired desiccation tolerance and the desiccated SEs grew on B5-2 solidified medium (Table 2, Fig. 3). Plant regeneration frequency of the desiccated SEs depended on the concentration of ABA. Highest growth frequency (60%) of the desiccated SEs was achieved when they were treated with 10 μM ABA. Both 1 and 100 μM ABA slightly induced desiccation tolerance. From desiccated SEs

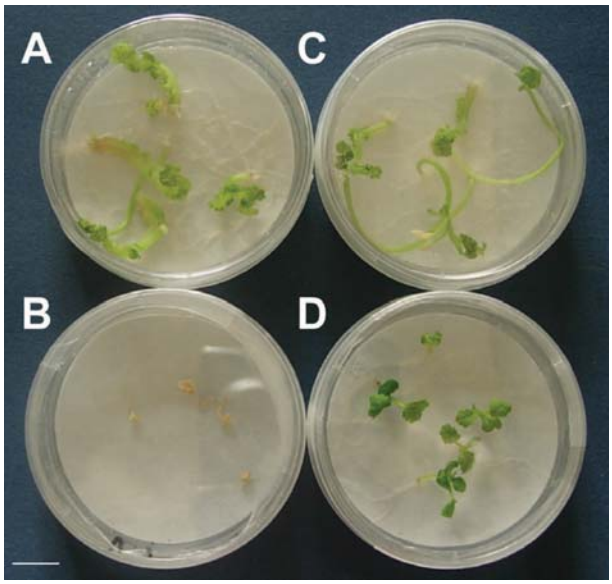

Fig. 3 Growth of non-desiccated (A, C) and desiccated (B, D) third-generation secondary embryos (SE_3s) in *B. napus* cv. 'Topas' 4 weeks after culture on B5-2 solidified medium. SE_3s were treated with ABA at 0 (A, B) and 10μM (C, D), prior to desiccation treatment. Bar=1 mm.

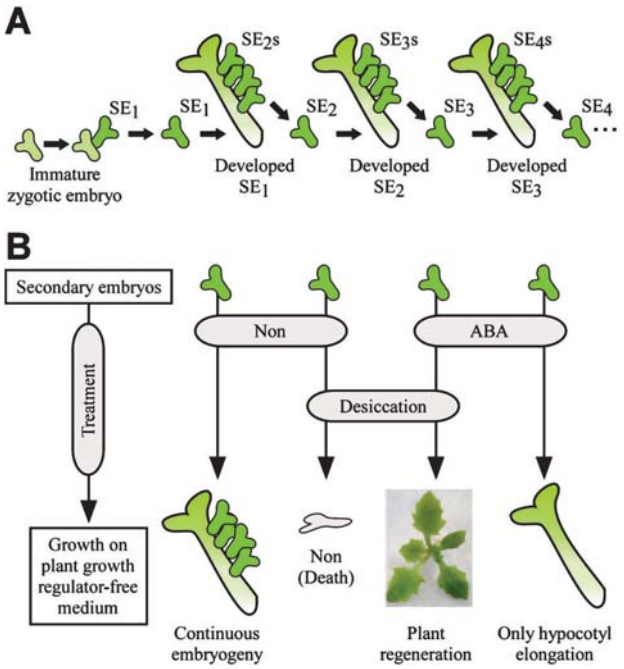

Fig. 4 A flow diagram showing the link between each SE series, i.e. SE_1, SE_2, SE_3 and SE_4 (A) and growth of SE after ABA treatment and/or desiccation.

after ABA treatment, all germinated SEs regenerated into normal plants (also Fig. 1G). On the other hand, desiccated SEs not treated with ABA did not germinate after rehydration and lost their viability by desiccation. Non-desiccated SEs treated with 1–100μM ABA showed a growth frequency of 93–100%, but these SEs grew abnormally, i.e. without forming a subsequent generation of SEs on their surface of elongated hypocotyls. Non-desiccated SEs without ABA treatment formed the subsequent generation of SEs on the surface of the elongated hypocotyls.

Discussion

In this study, we investigated the potential in *B. napus* for continuous SE formation and the effect of exogenous application of ABA and/or desiccation of SEs for plant regeneration. One of the results was that SEs could be obtained continuously by formation of a subsequent generation of SEs that formed on developing SEs after subculture (Fig. 4A). The growth of these SEs and their ability to form new SEs changed when combined with ABA and desiccation treatments (Fig. 4 B).

In this study, to examine continuous SE formation SE_1s were obtained from immature zygotic embryos by heat shock. In *B. napus,* microspore embryogenesis is induced by heat shock of at least 8 hours at 32℃ after a culture start[17–19]. Heat shock used in microspore culture was applied in our study, and immature zygotic embryos were incubated at 32℃ for 1 day prior to maintenance at 25℃. SE_1s were formed at 21.7±5.2% frequency, which suggests the possibility of increasing the efficiency of SE formation. This is because, when immature zygotic embryos were cultured without heat shock, SE_1s were formed at 8.3±3.3% frequency, a significantly lower frequency than that with heat shock (data not shown).

SE formation was continued by repeated subculture, revealing that the embryogenic potential was maintained over generations during an embryo's development (Fig. 4A). Thus, clonal SEs are continually available from this culture. In addition, the genetic stability of SE formation is ensured by avoiding a callus phase. Embryogenic potential was maintained by repetitive subculture for more than one year (data not shown). This continuous SE formation is similar to the results obtained in previous studies of rapid-cycling *Brassica*[7]. However, the proliferation efficiency of those SEs was higher than cv. 'Topas', which might reflect genotypic differences. Rapid-cycling *Brassica* was the first plant developed for the purpose of growing small, rapid-flowering, highly fertile forms under optimum and standardized laboratory growth conditions[20].

The exogenous application of ABA induced desicca-

tion tolerance in SEs, which is in agreement with the results obtained in previous studies of microspore-derived embryos of *Brassica* spp.[12,16,21]. However, the optimum concentration of ABA was different from the result reported by Brown *et al.* (1993)[21], who demonstrated that 100μM ABA was most effective for induction of desiccation tolerance in microspore-derived embryos of *B. napus*. Finkelstein *et al.* (1985)[8] reported that endogenous ABA increased to a level needed for maturing embryos by application of 10μM ABA in cultured zygotic embryos, but 1μM ABA was insufficient. The difference in the optimal ABA concentration for induction of desiccation tolerance might be based on the difference in ploidy since microspore-derived embryos were haploid. On the other hand, ABA treatment did not affect subsequent germination since SEs grew with the hypocotyl elongated on B5 solid medium as well as freshly subcultured SEs. However, the subsequent-generation of SEs did not form on such elongated hypocotyls at all, indicating that ABA controlled negatively the subsequent-generation of SEs in this culture. The previous study showed morphological changes such as the stabilization of the cell-membrane system and hardening of the tissue structure in response to the application of exogenous ABA to SEs[11]. In addition to induction of desiccation tolerance, stabilization of the cell-membrane system by application of ABA may also have contributed to the inhibition of the subsequent generation of SEs or SE formation.

In desiccated SEs after ABA treatment, when placed on plant growth regulator-free medium, all germinated SEs regenerated into normal plants (i.e. without an elongated hypocotyl). Finkelstein *et al.* (1985)[8] reported that desiccation, the final phase of normal embryonic development in most angiosperms, appears to be important in the transition from embryogeny to the ability to germinate and form normal seedlings. The effect by desiccation after ABA treatment on SEs was drastic in this culture. These results suggest that desiccation acts as a factor or switch providing regenerative ability to the SE for forming new SEs continuously.

In conclusion, we have established a method of continuous secondary embryo formation and plant regeneration in *B. napus* cv. 'Topas'. This method will be available as a useful tool for clonal propagation of specific *Brassica* genotypes and for transformation studies, and support further studies for elucidation of a trigger or a switch in somatic embryogenesis.

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Brassica napus 品種 'Topas' における連続的胚形成及び二次胚からの植物体再生系

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要約：*Brassica napus* 品種 'Topas' における連続的な二次胚形成及び植物体再生系の確立を試みた。熱ショックによって未熟種子胚より誘導した二次胚を，植物成長調節物質無添加の B5 培地へ継代した。培養後 70%の二次胚において，徒長した胚軸の表面に次代の二次胚が直接形成された。これら二次胚を同様に継代培養した結果，約 70% の胚より新たな二次胚が形成され，次胚形成能が継代培養を通じて維持されていたことが示された。二次胚に 10μM アブシジン酸（ABA）処理をした結果，胚は乾燥耐性を獲得し，60% の二次胚が乾燥後においても生育しその全てが正常に再生した。一方，ABA 処理後，乾燥処理を行わなかった胚は胚軸が徒長する異常な生育を示し，ABA 無処理胚は乾燥処理によって全て枯死した。

キーワード：*Brassica napus* 品種 'Topas'，二次胚，ABA, 乾燥耐性，植物体再生

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