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Induction of Somatic Embryogenesis in Carrot by Osmotic Stress

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Segments of cotyledon and apical tip taken from 1-week-old seedlings of *Daucus carota* L. were cultured for 3 weeks on hormone-free Murashige and Skoog's (MS) medium containing high amounts of sucrose (0. 3-0. 7 M) and then transferred to the same medium with lower sucrose concentration (0. 09 M). Somatic embryos were formed from the segments 3 to 6 weeks after the transfer. On the other hand, hypocotyl segments given the same treatment did not produce somatic embryos. When the primary culture medium contained 0. 61 M mannitol and 0. 09 M sucrose, cotyledon segments and apical tip segments also produced somatic embryos when transferred to hormone-free MS medium with 0. 09 M sucrose. In all cases, somatic embryos were formed directly from the explants without visible callus formation.

Introduction

It is well known that somatic embryogenesis in many dicotyledonous plants can be readily induced by a transfer of explants from auxin containing medium to auxin-free medium[1-5]. Effects of various growth regulators on somatic embryogenesis have been examined in detail[4-9]. Only auxin, especially 2, 4-D, is known to play an important role in induction of somatic embryogenesis. A similar situation was reported in several monocotyledonus plants such as *Zea mays, Panicum maximum, Dactylis glomerata* and *Sorghum bicolor*[10-14]. In these cases, however, the source of explants is very important; only young tissues and organs, such as immature embryos, young influorescences and young leaves, are found to produce somatic embryos.

Some biochemical, physiological and molecular biological analyses of somatic embryogenesis have been carried out using carrot cultures[15-19]. However, most investigations have centered primarily on the developmental process from embryogenic cells to mature embryos. The induction of embryogenic cells from somatic tissues have not been dealt with. This is because the diverse effects of auxin make it difficult to detect changes relating directly to somatic embryogenesis. In view of this, there is a need to develop new induction methods other than auxin treatment.

It has been reported that formation of somatic embryos from embryogenic cells cultured in medium containing 2, 4-D was promoted by a short-term treatment with osmotic stress in carrot[20]. Adventitious shoot formation in tobacco callus was also found to require osmotic stress[21]. In *Helianthus annuus* and *Carica papaya*, somatic embryogenesis was stimulated by culturing explants on a medium containing 2, 4-D and high concentrations of sucrose (6-12%)[22,23].

Recently, it has been reported that carrot somatic embryogenesis was induced when apical tip segments with meristems were cultured in a hormone-free medium containing high concentrations of sodium chloride or cadmium ion and then transferred to a hormone-free medium without those substances[24,25]. Furthermore, somatic embryogenesis of carrot was also induced when the seeds were treated for a long time (over 30 min.) with a concentrated sodium hypochlorite solution and then cultured in a hormone-free medium[26,27]. In these induction methods, no exogenous growth regulators were used throughout the experiments.

These results may indicate that some stress treatments induce somatic embryogenesis. In this report, we examined the effects of osmotic stress on the induction of somatic embryogenesis in carrot. We found that somatic embryogenesis in carrot was induced by osmotic stress without any hormonal treatment and that there were some differences in the response of explants taken from different parts of carrot seedlings.

Materials and Methods

One-week-old seedlings of *Daucus carota* L. cv. US-Harumakigosun grown on vermiculite were surface-sterilized with 10% hypochlorite solution for 15 min., then rinsed 3 times with sterilized distilled water. Cotyledons, hypocotyls and apical tip segments (1 mm length) with meristems were excised from the seedlings. Cotyledons and hypocotyls were each cut into 3 equal sized segments. The explants were cultured in petri-dishes (6 cm in diameter) containing 20 m*l* of hormone-free Murashige and Skoog's agar (1%) medium (hereafter referred to as MS medium)[28]. The concentrations of sucrose in the medium varied from 0.1 to 0.7 M. After 3 weeks in culture, one half of the number of explants were transferred onto MS medium in which the concentration of sucrose was decreased to 0.09 M. The remainder of the explants were continuously cultured on the same medium with high sucrose concentration. In experiments with mannitol, MS medium containing both 0.61 M mannitol and 0.09 M sucrose was used as the primary culture medium instead of MS medium with 0.7 M sucrose. Cultures were placed under a 16 h light period (approx. 3,000 lux) at 25°C. Experiments were conducted with at least 10 replicates for each treatment and repeated thrice.

For scanning electron microscopic observations, apical tip segments with meristems producing somatic embryos were prefixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h at 4°C. The specimens were post-fixed for 12 h in similarly bufferred 1% osmium tetraoxide, dehydrated with a graded ethanol series, subjected to critical point drying, and coated with gold. Observations were made with a JEOL Scanning Electron Microscope type JSM-T 20.

Results

Cotyledon explants cultured on MS medium with 0.5 M or 0.7 M sucrose produced somatic embryos 3 to 6 weeks after transferring to MS medium with 0.09 M sucrose (**Table 1**). Somatic embryos appeared directly at the cut-end of explants without visible callus formation (**Fig. 1-A**). When apical tip segments were used as explants, the first and second leaves protruded and elongated when cultured on MS medium containing sucrose at low concentrations (0.1 and 0.3 M), but did not at high concentrations (0.5 and 0.7 M). When explants cultured on 0.3 M sucrose medium were transferred to medium with 0.09 M sucrose for 3 to 6 weeks, somatic embryos were formed on the tip and surface of the elongated leaves without visible callus formation (**Fig. 1-B**). When apical tip segments cultured on 0.5 M or 0.7 M sucrose medium were transferred to medium with 0.09 M sucrose, the first leaves became slightly elongated and somatic embryos were formed

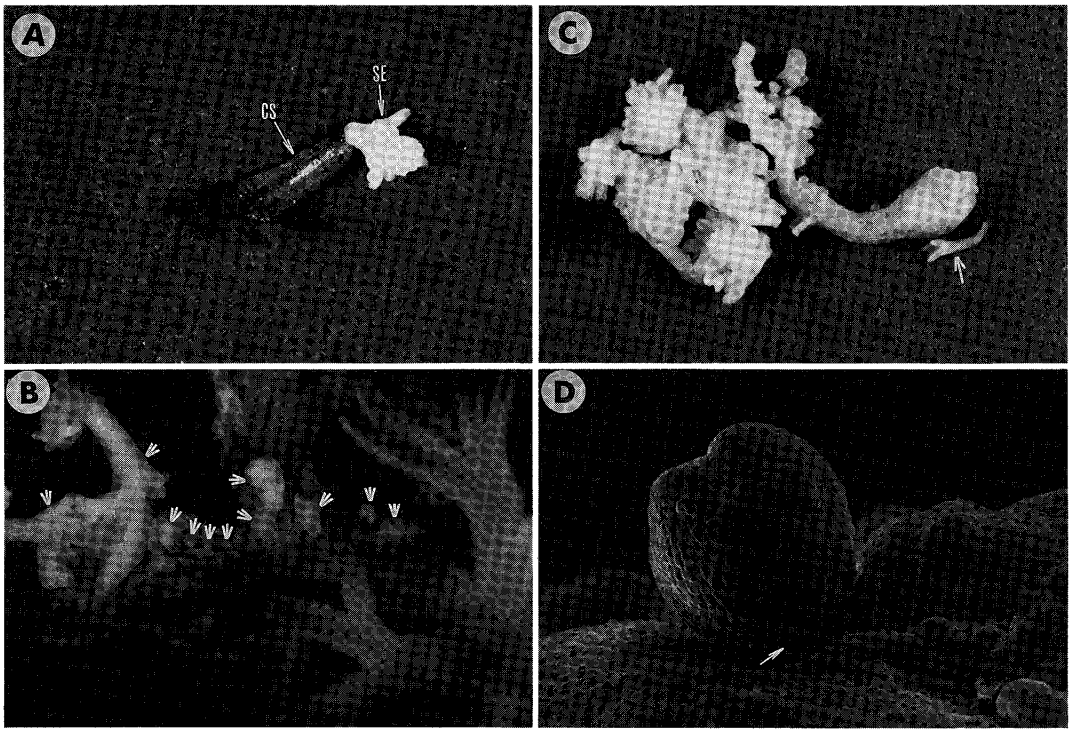

Fig. 1 Somatic embryogenesis induced by osmotic stress in carrot.
A : Mass of somatic embryos (SE) formed at a cut end of a cotyledon segment (CS) without visible callus formation. The cotyledon segment was cultured for 3 weeks on hormone-free MS medium with 0. 7 M sucrose, then transferred to hormone-free MS medium with 0. 09 M sucrose. The photograph was taken 4 weeks after the transfer (×7). B : Somatic embryos (arrows) formed directly on a tip-end surface of the first leaf grown from an apical meristem. The apical tip segment was cultured on hormone-free MS medium with 0. 3 M sucrose for 3 weeks, then transferred to hormone-free MS medium with 0. 09 M sucrose. The photograph was taken 4 weeks after the transfer (×10). C : Mass of somatic embryos formed directly on the surface of the first leaf slightly grown from an apical meristem. Some of them developed to mature embryos with cotyledons (arrow). The apical tip segment was cultured for 3 weeks on hormone-free MS medium with 0. 7 M sucrose, then transferred to hormone-free MS medium with 0. 09 M sucrose. The photograph was taken 6 weeks after the transfer (×20). D : Scanning electron micrograph of a somatic embryo arising directly from the first leaf. The embryo is attached directly to the leaf by a suspensor-like structure (arrow) without callus formation. The apical tip segment was treated in a same manner as in (C). The photograph was taken 4 weeks after the transfer (×100).

on the surface, also without visible callus formation (**Fig. 1-C** and **D**). On the other hand, when hypocotyl segments were used as explants, they elongated slightly but failed to produce somatic embryos in any of the media used (**Table 1**).

Frequency of explants producing somatic embryos in apical tip segments was higher than that in cotyledons (**Table 1**). The treatment with high concentrations of sucrose was also effective for somatic embryo formation (**Table 1**). When cotyledon segments or apical tip segments were cultured continuously on high sucrose medium (0. 3 to 0. 7 M), somatic embryos were also formed during the 9-week culture period with generally lower frequency than that in the transfer experiment (**Table 1**). These somatic embryos did not develop further on the high sucrose medium, but upon transferring them to medium with 0. 09 M sucrose, they developed into plantlets.

To clarify whether or not the induction of somatic embryogenesis by the treatment with a high

Table 1. Effects of sucrose concentration on somatic embryo formation in carrot.

treatment		explant source		
concentration	duration	cotyledon	hypocotyl	apical tip
0.1 M	3 weeks	0.0(%)	0.0(%)	0.0(%)
0.3 M	3 weeks	0.0	0.0	39.6
0.5 M	3 weeks	20.5	0.0	35.3
0.7 M	3 weeks	24.7	0.0	62.8
0.1 M	continuous	0.0	0.0	0.0
0.3 M	continuous	0.0	0.0	22.2
0.5 M	continuous	15.8	0.0	23.6
0.7 M	continuous	20.8	0.0	39.6

Cotyledons, hypocotyls and apical tip segments with meristems were cultured for 3 weeks on hormone-free MS medium with sucrose at the concentrations indicated. Half of the explants were then transferred to hormone-free MS medium with 0.09 M sucrose (3-week treatment). The rest of the explants were continuously cultured on the same medium (continuous treatment). Each value indicates a percentage of explants producing somatic embryos 9 weeks after the start of culture. Experiments were conducted with at least 10 replicates for each treatment and all experiments were repeated at least thrice.

concentration of sucrose was caused by osmotic stress, mannitol, which could not be utilized by carrot cells as a carbon source (data not shown here), was used in combination with sucrose. Cotyledon segments and apical tip segments were transferred to hormone-free MS medium with 0.09 M sucrose after culturing for 3 weeks on MS medium containing both 0.61 M mannitol and 0.09 M sucrose. Morphogenetic response of the explants was similar to that observed in the case of the treatment with 0.7 M sucrose, except that the frequency of explants producing somatic embryos was lower than that with 0.7 M sucrose (**Table 2**). Continuous treatment of explants with 0.61 M

Table 2. Effects of combined treatment with 0.61 M mannitol and 0.09 M sucrose on somatic embryo formation in carrot.

duration of treatment	explant source	
	cotyledon	apical tip
3 weeks	12.5(%)	38.4(%)
continuous	0.0	0.0

Explants of cotyledons and apical tip segments with meristems were cultured for 3 weeks on hormone-free MS medium with 0.61 M mannitol and 0.09 M sucrose. Half of the explants were then transferred to hormone-free MS medium with 0.09 M sucrose (3 week treatment). The remaining half of the explants were continuously cultured on the same medium (continuous treatment). Each value indicates a percentage of explants producing somatic embryos 9 weeks after the start of culture. Experiments were conducted with at least 10 replicates for each treatment and all experiments were repeated at least thrice.

mannitol and 0.09 M sucrose did not induce somatic embryogenesis in cotyledons and apical tip segment explants (**Table 2**).

Discussion

The results presented in this report show that somatic embryogenesis in carrot could be induced by osmotic stress, although the development of embryos to plantlets was suppressed under high osmotic conditions. It has been reported that osmotic stress caused an increase of the endogenous level of abscisic acid in several plant species[29-31]. On the other hand, it has also been reported that treatment with abscisic acid inhibited development of somatic embryos from embryogenic cells in carrot[4-6,9].

The reason for the weaker effects of mannitol (this study) and sorbitol[20] on induction of somatic embryogenesis, compared to that of sucrose, remains unclear. It was reported that short duration (45 min.) high osmotic stress (0.5 to 1.0 M) given to carrot embryogenic cells promoted somatic embryo formation[20]. In Wethrell's report[20], the effects of osmotic stress on the induction of embryogenic cells remains unclear because embryogenic cells induced by 2, 4-D were used as experimental material.

Recently, it has been reported that somatic embryogenesis in carrot was induced when apical tip segments with meristems were cultured for 2 to 4 weeks on a hormone-free medium containing high amounts of NaCl (0.2-0.4 M) or heavy metal ions (0.25-1.0 mM) such as cadmium or nickel, and prior to culturing in hormone-free medium without NaCl or heavy metal ions[24,25]. Somatic embryogenesis could also be induced by treating the seeds of carrot, *Lavendula, Mentha* and *Rosmarinus* with sodium hypochlorite solution and subsequently cultured them in a hormone-free medium[26,27,32,33]. Furthermore, pollen embryogenesis in tobacco and *Brassica napus* could be induced in hormone-free medium by treating the microspores with high temperature (32°C over 8 h) or osmotic stress with mannitol solution (0.4 M for 3 days)[34-36].

The above data lead to a possible conclusion that some stresses, in the form of osmotic, temperature shock or high concentrations of salt or heavy metals, induce somatic embryogenesis in certain plant cells. The hypothesis that some stresses induce somatic embryogenesis could also explain the possible action of 2, 4-D and 2, 4, 5-T on somatic embryogenesis. Both chemicals are synthetic auxins and strong herbicides as well as effective inducers of somatic embryogenesis. It is possible that the herbicidal effects of 2, 4-D and 2, 4, 5-T cause stress on plant cells and as a result such plant cells deviate from their normal developmental pathway and become embryogenic. Natural auxins such as IAA which are non-herbicidal are weaker inducers of somatic embryogenesis[5], although they caused strong physiological responses such as stem elongation in plants. More recently, we applied a temperature stress of 37°C for 3 weeks on cultured apical tip segments of carrot and then transferred them to 25°C for 4 weeks, where somatic embryos developed from the segments (our unpublished data). All these results indicate that the application of stress treatments possibly triggers the induction process leading to the development of embryos from somatic cells and microspores of certain plant species.

It is difficult to clarify whether or not a common mechanism is involved in some successful stress-treatments for induction of somatic embryogenesis. We found that gene expression of a protein (ECP 31) directly related to embryogenic competence was commonly observed in some stress treatments which could induce somatic embryogenesis in carrot[37]. The gene expression of ECP 31 was also controlled by abscisic acid[38]. It was also reported that carrot embryogenic cells induced by 2, 4-D treatment had higher levels of endogenous abscisic acid than those of nonem-

bryogenic cells[39]. Because endogenous levels of abscisic acid generally increase by stress treatments, abscisic acid is thought to play an important role in stress induction of somatic embryogenesis.

The reason for differential morphogenetic responses between cotyledons, hypocotyls and apical tip segments to osmotic stress is not known and needs further investigation. However, examples in which explant source can influence morphogenetic responses are numerous. For example, when hypocotyls of *Capsicum* seedlings were cut into smaller segments, shoots were only produced from upper sections, central sections produced mostly roots, while callus were observed from lower segments[40]. It is very likely that in carrot, different tissues such as cotyledons, hypocotyls or shoot apices have different degrees of sensitivity towards osmotic stress. Varying tissue sensitivity towards plant growth substances was previously suggested by Trewavas[41].

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《和文要約》

浸透圧ストレスによるニンジン不定胚形成の誘導

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発芽1週間目のニンジン(*Daucus carota* L.)の実生から子葉切片および頂芽を含む切片を切り出し，高濃度（0.3-0.7 M）ショ糖を含む植物ホルモン無添加の Murashige-Skoog (MS) 培地で3週間培養した後，0.09 M ショ糖を含む植物ホルモン無添加の MS 培地に移植した．移植後3-6週目に外植片上に不定胚が形成された．一方，胚軸切片では同じ高濃度ショ糖処理によって不定胚は形成されなかった．0.61 M マニトールと 0.09 M ショ糖を同時に含む MS 培地を最初の培地として用い，0.09 M ショ糖を含む植物ホルモン無添加 MS 培地に移植した場合，子葉切片および頂芽を含む切片では不定胚が形成された．どの場合においても，肉眼で見えるようなカルスの形成を経ることなく外植片上に直接不定胚が形成された．