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Direct somatic embryogenesis, plant regeneration and genetic transformation of *Panax ginseng*

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Abstract

Somatic embryogenesis is one of good examples of the basic research for plant embryo development as well as an important technique for plant biotechnology. This paper describes the direct somatic embryogenesis from zygotic embryos of *Panax ginseng* on hormone-free medium. Direct somatic embryogenesis from zygotic embryos of *Panax ginseng* is reversely related to normal axis growth of zygotic embryos by the experiment of various chemical treatments. Under the normal growth condition, the apical tips of embryo axis produced an agar-diffusible substance, which suppressed somatic embryo development from cotyledons. Although the cells of zygotic embryos were released from the restraint of embryo axis, various factors were still involved for somatic embryo development. Electron microscopic observation revealed that the ultrastructure of cells of cotyledon epidermis markedly changed before initiation of embryonic cell division, probably indicating reprogramming events into the cells embryogenically determined state. Polar accumulation of endogenous auxin or cell-cell isolation by plasmolysis pre-treatment is the strong inducer for the somatic embryo development. The cells for the process of somatic embryogenesis might be determined by the physiological conditions of explants and medium compositions. Direct somatic embryos from cotyledons of ginseng were originated either from single or multiple cells. The different cellular origin of somatic embryos was depended on various developmental stages of cotyledons. Immature meristematic cotyledons produced multiple cell-derived somatic embryos, which developed into multiple embryos. While fully mature cotyledons produced single cell-derived single embryos with independent state. Plasmolysis pretreatment of cotyledons strongly enhanced single cell-derived somatic embryogenesis. Single embryos were converted into normal plantlets with shoot and roots, while multiple embryos were converted into only multiple shoots. GA_3 or a chilling treatment was prerequisite for germination and plant conversion. Low concentration of ammonium ion in medium was necessary for balanced growth of root and shoot of plantlets. Therefore, using above procedures, successful plant regeneration of ginseng was accomplished through direct single embryogenesis, which makes it possible to produce genetically transformed ginseng efficently.

In the last two decades, somatic embryogenesis has been studied extensively for plant regeneration in various plant species. The studies particularly emphasized on its effects of genotype of starting materials, on types of explants, and on media and hormone compositions. However, little progress has been achieved toward understanding the mechanism leading to the process of somatic embryogenesis. This paper deals with the influences of maternal plant tissues on direct somatic embryo development, the structural events in early stage of direct somatic embryogenesis, and the high frequency of plant production and the efficient genetic transformation of *Panax ginseng*.

Early event of direct somatic embryogenesis in Panax ginseng

1. Regulation of somatic embryo formation from axis of zygotic embryos

It is generally accepted that direct embryogenesis is closely related with physiological disorder of normal growth of plants [23, 28]. Smith and Krikorian [28] suggest that the cells of plant tissues under mechanical or chemical stresses are released from the restraint of maternal plant tissues, resulting in development of somatic embryos from these cells having predetermined embryogenic competency. However, it is not clear how plants can regulate somatic embryo development in the normal growth condition.

Intact zygotic embryos at various developmental stages of ginseng were cultured on hormone-free Murashige and Skoog agar medium. Immature or dormant zygotic embryos being unable to germinate

produced somatic embryos directly, while normally germinating zygotic embryos never formed somatic embryos [9]. By the 2,4-D treatment [8] or macrosalt treatment [16], direct somatic embryo formation from ginseng zygotic embryos was reversely related to normal growth of embryo axis. From the various excision or wounding treatments to zygotic embryos of ginseng, cotyledon explants without embryo axis or excised zygotic embryos with removed plumule and radicle tip produced somatic embryos [9, 10]. These observations suggest that the embryo axis, especially plumule and radicle tips might regulate somatic embryo formation. To clarify the regulation, excised plumule or radicle tip was placed in direct contact with the basal cut-end of cotyledons [9]. The adhesion of excised plumule or radicle tips strongly suppressed somatic embryo formation from cotyledon explants [9]. When an agar block containing exudation from excised plumule or radicle tips was placed in contact with the cut end of cotyledons, a similar strong inhibition was observed [9]. These results indicate that the apical tips of embryo axis synthesize an agar-diffusible substance under the normal growth condition, which suppress somatic embryo formation.

2. Factors leading to induce somatic embryos

In direct somatic embryogenesis, it has been suggested that plant tissues had already predetermined embryogenic competency, thus some treatments such as auxin or stress treatments only stimulate the embryonic development [26]. However, it is still unclear on the factors leading to the reprogramming events for direct somatic embryo development.

We observed the ultrastructural changes of cells during early initiation of somatic embryo development from cotyledon epidermis of ginseng. Electron microscopic observation [13] revealed that dramatic changes of cotyledon cells were observed before initiation of embryogenic cell division. Before culture, epidermal cells of cotyledons were filled with reserved materials, among them lipid bodies were the most abundant cellular organelles. After 3 days of culture, starch grains were accumulated in plastids, but the size of lipid bodies was slightly decreased. All of vacuoles were filled with protein-like matrixes. Numerous mitochondria and microbodies were observed. Cytoplasm was filled with free ribosome. First embryogenic cell division was observed from these epidermal cells of cotyledons after about 5 days of culture. In contrast, during the normal germination of zygotic embryos, structural changes of cotyledon epidermis were highly different to the process of somatic embryogenesis. Epidermal cells of cotyledons were rapidly vacuolated together with rapid disappear of all the reserved materials but only chloroplast development was prominent event. These results indicate that epidermal cells of cotyledons are rapidly reprogrammed into the embryogenically determined cells before embryonic cell division is initiated.

It is still question what are the factors leading to the process into embryogenically determined cells. We observed that reserve material deposition in the cotyledon tissues of ginseng is closely related to the frequency of somatic embryo formation [13]. Cells of cotyledons at the abaxial side were filled with reserved materials, while those at the adaxial sides contained sparse reserved materials with large vacuole. Somatic embryos were developed at much higher frequency from abaxial side of cotyledons than from adaxial one [13]. In contrast, during germination of zygotic embryos, these reserved materials were rapidly disappeared, simultaneously resulting in rapid decrease of somatic embryo formation [13]. The reserve materials deposited in cotyledon tissues may play a role for energy sources for reprogramming into embryogenically determined cells and early embryo development.

From morphological observation, polar somatic embryo development occurred because somatic embryos always developed directly near the excised portions of cotyledon base [10, 14]. This probably indicates auxin polar accumulation toward the basal portion of cotyledon segments may play a role for inducer of somatic embryos. When 2,3,5-triiodobenzoic acid, an auxin polar transport inhibitor was applied to cotyledon explants, direct somatic embryogenesis was strongly suppressed and somatic embryos were developed sporadically on the all surface of cotyledon [14]. Therefore, polar development of somatic embryos in the cotyledon explants can be explained by polar accumulation of endogenous auxins toward the basipatal sides of cotyledon segments. However, the endogenous polar auxin accumulation is not directly related to only somatic embryogenesis by the following experiments. When the cotyledon explants of ginseng were cultured on medium with different balance of ammonium and nitrate ions, adventitious root or somatic embryo formation was observed as alternative morphogenetic event [12]. Adventitious roots or somatic embryos was developed near the basal excised region of cotyledons. However, in moderate or high concentration of ammonium ion (over 20 mM), somatic embryos were developed. While in no or low concentration of ammonium ion (less than 4 mM), adventitious roots formed [12]. This result indicates that endogenous auxin polar accumulation stimulate the reprogramming of cotyledon cells for the adventitious root or

somatic embryos, but the fate leading to somatic embryo or adventitious root formation is depended on the salt composition of medium [12]. Therefore, basic salt composition of medium is more essential factor for determining the cell fate leading to the process of somatic embryogenesis. In general, reduced nitrogen sources is important for somatic embryo formation [20].

In zygotic embryogenesis, embryo sac is physiologically isolated state from the surrounded ovular tissue by absence of plasmodesmata. In somatic embryogenesis, however, it is not clear whether the isolation of cells plays an important role for somatic embryo induction. When cotyledons of ginseng were pre-plasmolyzed by 1.0 M sucrose for one to three days, somatic embryos were developed from the all surfaces of cotyledons and the number of somatic embryo per cotyledon highly increased [11]. From ultrastructural observation, cell walls of pre-plasmolyzed cotyledon tissue became highly thicker then those of non-treated cotyledon [11]. In addition, disruption of plasmodesmatal strands between the cells was observed. The enhanced somatic embryo formation from ginseng cotyledon after plasmolysis pretreatment indicates that physiologically isolated state of cells is an important factor for inducing somatic embryos. However, the necessity of cell isolation before somatic embryo development is somewhat controversial because embryogenesis originated from multiple cells is common event in direct somatic embryogenesis [30].

Based the above data, we suggest that various factors are involved on the direct somatic embryogenesis. Probably the polar accumulation of auxin or cell-cell isolation by plasmolysis preatment might be grouped into stimulating factor for somatic embryo induction. The physiological conditions of explants and the medium composition might be grouped into determining factor for the process of somatic embryogenesis.

Cell-cell interaction on the origin and development of somatic embryos

Contrary to zygotic embryogenesis, origin and developmental pattern of somatic embryos is various. In direct somatic embryogenesis, somatic embryos are derived from single cells [22] or multiple cells [30]. The factor leading to different cellular origin of direct somatic embryos is poorly understood. Histological observation of somatic embryo development from ginseng cotyledon revealed that single and multiple cell origin of somatic embryo was depended on the differentiated state of maternal ginseng cotyledon [6, 7]. In immature cotyledon constituted with homogenous and meristematic cells, somatic embryos were originated from multiple cells of epidermis and subepidermis. In fully mature cotyledon constituted with heterogeneous cells, somatic embryos were developed from single epidermal cells [7]. These data suggest that the differentiated state of cotyledons is closely related to the cellular origin. Williams and Meheswaran [30] suggested that the factors affecting the origin of somatic embryos correspond to the coordinating behavior of cells participating in embryonic development. However, it is not easy to explain why cells of immature cotyledon appear to coordinated behavior for embryonic development. Probably, the coordinating behavior of cells might be caused from cell-cell relationship. It has been known that plasmodesmata play an important role for cell-cell communication [3]. Temporary plasmolysis has been frequently used to induce abnormal pattern of development in lower plants such as algae, mosses, ferns and liverworts [3]. In carrot embryogenic cells, plasmolysis pre-treatment enhance the somatic embryo formation [29]. When the immature cotyledon tissues of ginseng were pretreated with 1.0 M sucrose for 3 days and then transferred to MS agar medium, this treatment strongly enhanced the single-cell derived somatic embryos [11]. This result indicate that the multiple and single cell origin of somatic embryos is determined by the degree of cell-cell isolation within tissues.

Efficient plant regeneration system in Panax ginseng

Efficient plant regeneration is an important protocol both for micropropagation and genetic transformation. In *Panax ginseng*, callus-derived somatic embryogenesis was reported extensively [1, 2, 4, 24, 27]. Lately we reported the plant regeneration via adventitious shoot formation [15] and via embryo axis-like shoot and root formation from cotyledon explants [19]. In addition to the above protocols, direct somatic embryogenesis of ginseng on hormone-free medium can be applied to a technique for plant regeneration. This method has some advantages due to the rapid propagation and to the low frequency of genetic mutation compared to calli-derived embryogenesis. In carrot somatic embryos formed on hormone-free medium, the conversion rate of somatic embryos into plantlets is significantly higher than the embryos induced by exogenous auxin [23]. Based on our previous studies, we tried to set up an efficient plant regeneration protocol using direct somatic embryogenesis on hormone-free medium.

In a previous paper, we observed that single and multiple cell origin of somatic embryo depended on the differentiated state of maternal ginseng cotyledon

[7]. We compared the developmental pattern and plant conversion rate of embryos derived from multiple and single cells. In the culture of immature cotyledons, somatic embryos derived from multiple cells developed into abnormal multiple embryos fused each other and fused to maternal explants, and these embryos regenerated into only multiple shoots [17]. While, in cotyledons from fully matured zygotic embryos, somatic embryos were originated from single cells and developed into single independent state [17]. These single embryos developed into normal plantlets with both shoots and roots. Therefore, direct single embryogenesis derived from single cells is highly important for normal plant regeneration in ginseng [17]. However, to induce single embryos, only cotyledons from fully matured zygotic embryos should be sampled as explant. When immature cotyledons of ginseng were pretreated with 1.0 M sucrose for 3 days, single cell-derived single embryos formed at a high frequency and these embryos were regenerated into normal plantlets with both shoots and roots [18].

Plant conversion from ginseng somatic embryos was not achieved because somatic embryos of ginseng represented a tendency of dormancy [18]. GA_3 or chilling treatment was necessary for germination [17, 18]. In addition, high content of ammonium ion of medium suppressed the root growth of plantlets, therefore, transferring of plantlets to medium with low content of ammonium ions was necessary for the balanced shoot and root growth [17]. Using above procedures, high frequency of plant production of ginseng through direct somatic embryogenesis was accomplished [17, 18]. Therefore, we developed new protocol of efficient regeneration of ginseng using direct single embryogenesis.

Genetic transformation of Panax ginseng

Korean ginseng plant is difficult to cultivate compared to the other economically important plants. Cultivation of this plant requires a period of more than three years to produce seeds. In addition, this plant is extremely sensitive to direct sun light (probably from photooxidation damage), thus should be grown under shade condition. Disease caused from fungus is serious problem. Therefore, genetic transformation technique is an important method for the breeding of ginseng.

Agrobacterium rhizogenesis-mediated transformation of ginseng had reported for hairy root production [21, 31]. Transgenic plants of ginseng were produced using *Agrobacterium tumefaciens*-mediated transformation haboring GUS genes [25]. This little progress of genetic transformation of ginseng might be due to difficult regeneration protocol and unsuitable transformation technique. We compared the frequency of genetic transformation of ginseng using direct somatic embryogenesis and calli-derived somatic embryogenesis. Using direct somatic embryogenesis, induction of transgenic plant is very rapid and identification of transgenic embryos was easy to be detected because only transgenic embryos developed directly from cotyledon surface on selection medium. While, in callus-derived somatic embryogenesis, several months required for inducing transgenic plants, and early identification of transgenic embryos was difficult among callus together with preexisting embryos. However, transgenic calli were maintained and proliferated for long time, which is an advantage using call-derived embryogenesis. Using above methods, transgenic ginseng plantlets harboring GUS, herbicide resistant and *rolC* genes were constructed. Now we are planning to construct the photooxidation resistant transgenic ginseng plant.

Future prospects

Ginseng zygotic embryos or somatic embryos can produce flowers in vitro culture condition, and these flowers are derived from pre-existing dormant axilary buds of cotyledon base of embryos [5, 24]. Although the transgenic plants of ginseng are successfully transferred to soil, three years of cultivation required for getting seeds. Therefore in vitro flower formation is a promising method for the early seed setting of ginseng especially in transgenic plants. When the zygotic or somatic embryos were cultured on medium containing 5 mg/L GA_3 and 2 mg/L BAP, flowers formed from axilary dormant buds within 2 months of culture. Adventitious buds and supernumerary buds after decapitation of embryonic shoots produced flowers. Maturation process of these flowers normally proceeds until early stage in vitro condition but did not succeed to in vitro seed setting. This suggests that maternal influence is an important factor for further maturation of flower. Therefore, if the young flower buds are grafted to the natural ginseng plants, we suppose that the growth of in vitro formed flowers will grow more normally. Now we are planning to graft the in vitro formed flower buds of transgenic plants of ginseng to the field cultivated seed- derived plants.

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