Formation of Style- and Stigma- Like Structures by Ovule Culture in Tobacco

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Summary

The factors inducing the formation of style- and stigma-like (SSL) structures by ovule culture in tobacco were investigated. In juvenile ovules which were excised from flower buds containing pollen grains at unicellular stage, the key to the formation of these structures seems to be isolation of the ovules with placenta from surrounding tissues and organs. Plant growth substances were not necessary for these formations in the ovules. However, kinetin was necessary for these formations in more advanced ovules which were excised from flower buds containing pollen grains at bicellular stage. In media containing kinetin, ovule-like structures were also observed on the basal part of style-like structures.

The ability of pollen germination on the stigma-like tissues and pollen tube penetration into style-like structures was confirmed. This observation seems to indicate that these structures have the same functions as normal stigmas and styles. Moreover, the morphological characteristics of the SSL structures were similar to those of normal tissues.

Introduction

In the course of our work to establish an effective method to induce haploids from unpollinated ovules of tobacco plants, it was observed that ovules with placenta in a wide range of stages developed into carpelloids (Hicks and McHughen, 1974) consisting of SSL and ovule-like structures. These structures were also observed in in vitro studies on the stamen primordia (Hicks, 1975), the stigma of a flower bud (Matsuzaki et al., 1984), receptacles (Katoh and Iwai, 1991), and ovules (Haccius et al., 1974; Hicks and McHughen, 1974). In the case of receptacles, stigmas, and stamen primordia, cytokinin is necessary for the formation of these structures. However, in ovules, the factors inducing the formation of SSL structures were not fully understood. If the factors are clarified, we can provide a useful information for designing experimental plans for inducing haploids from ovules, and a broader understanding of carpelloid morphogenesis of some tobacco floral tissues and organs. We studied how plant growth substances affect the ovule at various stages of development with regard to the formation of SSL structures, and the morphology and the function of these structures.

Materials and Methods

Plants of tobacco (*Nicotiana tabacum* cv. BY-4) were grown in a greenhouse. After preliminary experiments with buds of various sizes, we finally used ovules with placenta from Stage A; bud length is 8–15 mm, pollen

grains are unicellular and ovules contain megaspore mother cell at the prophase of meiosis, Stage B; bud length is 25–30 mm, pollen grains are bicellular and ovules contain megaspore mother cells in meiosis to megaspores in tetrad. The flower buds were surface-sterilized with 70% ethanol for 30 sec, 1% sodium hypochlorite for 10 min and rinsed with sterilized water. Ovary wall was removed and placenta with ovules was divided into four segments. Then, ovules with placenta were cultured on an agar-solidified medium in the test tube 3 cm in diameter, 10 cm in height. The NN medium supplemented with major and minor salts, and organic compounds was prepared according to Nitsch and Nitsch (1969). Sucrose (2%) was also included. The pH of the medium was adjusted to 5.7 with 0.1 N KOH before autoclaving. IAA and kinetin were added individually or in combination to the basal medium. 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) were also used. Cultures were kept at 28 °C on a photoperiod of 12 hr light (white fluorescent lamps, 6 W · m⁻²) and 12 hr dark for 5 weeks.

The ability of pollen germination on the stigma-like tissues and pollen tube elongation in style-like structures were investigated according to Katoh and Iwai (1991). For examination under the scanning electron microscopy, the samples were prepared as described in Katoh and Iwai (1993).

Results

Effect of concentration of kinetin and IAA

The effects of various concentrations of plant growth substances on morphogenesis of ovules were studied (Table 1). In stage A, altered morphogenesis of ovules

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Table 1. Effects of IAA and kinetin on morphogenesis of ovules.

Kinetin (mg/l)	Stage A				Stage B			
	IAA (mg/l)				IAA (mg/l)			
	0	0.1	1	10	0	0.1	1	10
0	D ^z	D	D	D	A	A	A	A
0.1	E	E	E	E	E	E	E	E
1	E	E	E	E	E	E	E	E
10	F	F	F	F	F	F	F	F

Twenty segments of placentae with ovules were cultured for 4 weeks.

^z Alphabets in table represent types of the growth patterns in Fig. 1. A; Shrunk and browned ovules on placenta, D; stigma-like and style-like structures, E; stigma-like, style-like and ovule-like structures, F; placenta-like structures with ovule-like structures. Reproduced from Katoh and Iwai(1993).

Fig. 1. Photographs of various types of ovular transformation. A) Shrunk and browned ovules on placental tissue. B) Ovules without changes. C) Elongated ovules without stigmas. D) Developed style- and stigma-like structures. E) Style- and stigma-like structures with ovule-like structures. F) Style- and stigma-like structures had not formed, but placenta-like structures were found with ovule-like structures. EO: elongated ovule, NO: normal ovule, OL: ovule-like structure, PL: placenta-like structure. Reproduced from Katoh and Iwai (1993).

was observed in most of segments in all media. A lot of ovules on excised placenta developed into SSL structure in media without kinetin (Fig. 1D, Type D). In media containing 0.1–1 mg/liter kinetin, ovule-like structures were also observed on the basal part of style-like structures (Fig. 1E, Type E). Then ovule-like structures developed into secondary SSL structures. When the concentration of kinetin was increased to 10 mg/liter, ovules formed no SSL structures. High concentration of kinetin inhibited the development of style-like structures, and ovules developed into ovule-like structures with placenta-like tissue (Fig. 1F, Type F). These results showed that kinetin was not necessary for inducing pistil-like structures but necessary for inducing ovule-like structures in young ovules.

In stage B, in the absence of kinetin, ovules did not develop into pistil-like structures. In this condition, ovules shrank and browned (Fig. 1A, Type A). In the media containing kinetin, however, the developmental features of ovules were almost the same as those in stage A. Altered morphogenesis of ovules was observed in most of the segments in the same way as seen in stage A. However, when ovules with placenta were cultured on the medium with 0.1 mg/liter kinetin and 0.1, 1.0 or 10.0 mg/liter IAA, the frequencies of segments forming SSL structures were low (15–30%). Using BA or zeatin instead of kinetin, it was possible to induce the same style-, stigma-, and ovule-like structures.

Effect of concentration of 2,4-D and MCPA

In stage A, the development of SSL structures was observed in most media containing only auxin. But the differentiation of SSL structures from ovules was inhibited by the addition of 10 mg/liter MCPA or 2,4-D (Fig. 1B, Type B).

In stage B, auxins did not stimulate the development of ovules into SSL structures. When 2,4-D and MCPA were added at 0.1–1 mg/liter and 1–10 mg/liter respectively, ovules elongated to about 3 mm without terminal stigma-like structures (Fig. 1C, Type C). When 2,4-D was added at 10 mg/liter, this elongation of ovules was not observed.

Function and morphological characteristics of SSL structures

Tobacco pollen grains germinated on stigma-like tips and pollen tubes penetrated into style-like structure. Scanning electron micrographs of the epidermal cells of SSL structures showed that stigma-like structures were accompanied by many papillae which were similar to those on normal stigma. The epidermal cells of style-like structures were rectangular; this was similar to that of normal styles. Longitudinal sections of ovule-like structures showed that the structures had embryo sacs and integuments which were similar to normal young ovules. These observations seem to indicate that the SSL structures have the same morphological characteristics

and functions as normal stigmas and styles.

Discussion

Our results showed that there was a difference in plant growth substance requirements for the formation of SSL structures between young and developed ovules. The difference may be caused by the different physiological states of ovules. At least two possible explanations can be considered for SSL structure formations in stage A. First, the young ovules retain the capacity to develop into a pistil-like carpel primordia but outgrowth of ovules is suppressed by the surrounding tissues. When ovules in stage A are isolated from the surrounding tissues, the suppression may be removed and the outgrowths of ovules are initiated. An alternative explanation is that ovules in stage A have enough endogenous cytokinin to induce carpelloid structures from ovules, whereas ovules in stage B do not contain sufficient amount of cytokinin.

Recently, in transgenic petunia and tobacco plants to which were introduced MADS box genes, the homeotic conversion of ovules into carpelloid structures was observed (Mandel et al., 1992; Angenent et al., 1995). These studies may provide the understanding for molecular biological mechanism of SSL structure formation by ovule culture in tobacco.

Many researchers reported that the SSL structures were transformed from floral parts of tobacco in the medium containing cytokinin. Moreover, in this report, it was shown that tobacco ovules with megaspores in tetrad having undergone meiosis maintain a developmental potential for carpelloid structures and this potential could be expressed by cytokinin. The morphogenesis of tobacco floral organs induced by cytokinin became more obvious by our results. In tobacco, by culturing on the medium containing cytokinin, terminal stigmas develop into only SSL structures (Matsuzaki et al., 1984), ovules form SSL structures and ovule-like structures (Haccius et al., 1974; Hicks and McHughen, 1974), whereas basal receptacles develop into SSL and ovule-like and phylloid structures (Katoh and Iwai, 1991). However, these structures do not form any flower buds. Flower buds are induced from floral axes (Tran Thanh Van, 1973). These results suggest that there is a hierarchical progression of the developmental potential expressed by cytokinin from stigma, ovule, receptacles and floral axis.

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