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The use of IAA to overcome interspecific hybrid inviability in reciprocal crosses between *Nicotiana tabacum* L. and *N. repanda* Willd.

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Summary. Flowering hybrid plants were obtained from reciprocal crosses between *N. tabacum* L. (2n=48) and *N. repanda* Willd. (2n=48), in which cross incompatibility and hybrid inviability were manifested. Stylar pollination and ovule culture were used to overcome the cross incompatibility of stylar barriers and ovular death. It was shown that application of 2 mg/l indole-3-acetic acid (IAA) during the growth period, from the five- to six-leaf stage to the flowering stage, is a useful and easy method to overcome hybrid inviability.

Key words: Hybrid inviability – IAA – Interspecific hybridization – Reciprocal cross – Tobacco

Introduction

Interspecific or intergeneric hybridization is important for introducing certain desirable genes into crops. *N. repanda* has long been of interest to breeders, because it is resistant to more diseases than any other species in the genus (Stavely et al. 1973).

Three kinds of sexual barriers have been observed in interspecific hybridization of *N. tabacum* L. with wild *Nicotiana* species (DeVerna et al. 1987). The first barrier is the failure of flower fertilization after pollination because pollen tubes stop growing in the styles. When the wild species *N. repanda* was used as a male parent, stylar barrier in *N. tabacum* was found. Stylar pollination was successfully used to overcome the stylar barrier in this cross (Suda et al. 1989).

The second barrier is that even if fertilization occurs, embryos and endosperms degenerate within several days. To overcome this type of cross incompatibility, ovule culture during early ovule stage is considered to be a useful method (Reed and Collins 1978). In reciprocal crosses between *N. tabacum* and *N. repanda*, embryos could be rescued from abortion by the ovule culture method (Shintaku et al. 1985; Suda et al. 1989).

The third barrier is that the obtained hybrid seedlings die in the young stages of development (Reed and Collins 1978; DeVerna et al. 1987). This phenomenon is considered to be a manifestation of hybrid inviability.

Various attempts have been made to overcome the hybrid inviability in the reciprocal crosses between *N. tabacum* and *N. repanda*. Pittarelli and Stavely (1975) reported that sesquidiploid F_1 flowers were obtained when autotetraploid *N. repanda* was pollinated with diploid *N. tabacum*. Shintaku et al. (1988) obtained mature hybrids by using either the pollen irradiation technique or the egg cell irradiation technique, i.e., pollination after the destruction of chromosomes causing inviability in *N. tabacum* pollen grains or in *N. repanda* egg cells. In another study, Suda et al. (1989) and Suda and Marubashi (1990) obtained mature hybrids in the reciprocal crosses between *N. tabacum* and *N. repanda* only when the hybrids were kept at constant high temperature (30°–36°C).

Various significant effects of auxin on the control of plant growth are well-known. There has been no detailed study on the effects of auxin upon the growth of interspecific hybrids in which hybrid inviability is manifested. In this study, we investigated the effect of IAA on the growth of hybrid plants and showed that exogenous IAA made it possible to overcome hybrid inviability in reciprocal crosses between *N. tabacum* and *N. repanda*. Also, the roles of exogenous and endogenous auxin in hybrid plants are discussed.

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Materials and methods

Nicotiana tabacum L. cv Red Russian (2n=48) and *Nicotiana repanda* Willd. (2n=48) were used for the experiments. Seeds were supplied by Japan Tobacco, Inc., and plants were grown in the greenhouse at the University of Tokyo.

Stylar pollination

When *N. repanda* was used as a male parent, fertilization did not occur because pollen tubes did not reach the bases *N. tabacum* styles. In vitro pollination was then applied, using the procedures for stylar pollination and placenta culture as described by Niimi (1976), except that the medium for placenta culture consisted of 0.8% agar, 8% sucrose, the mineral salts of Murashige and Skoog (1962), and the vitamins of Nitsch and Nitsch (1969), albeit with a concentration of nicotinic acid of 0.5 mg/l. The petridishes (9 cm in diameter) were kept under continuous illumination (about 3,000 lx) at 28 °C.

Ovule culture

Six days after stylar pollination of *N. tabacum* × *N. repanda*, enlarged ovules on placenta were removed and transferred into ovule culture medium. Details of the method are described by Reed and Collins (1978), except that medium for the ovule culture consisted of mineral salts and vitamins of Nitsch and Nitsch (1969), 8% sucrose, and 0.8% agar. After approximately 25 days of incubation, germinated seedlings (ca. 0.5 cm long) were transferred to a rooting medium. Murashige and Skoog (1962) medium with one-fourth the concentration of inorganic salts (¼ MS) was used. The plants were kept under continuous illumination (about 3,000 lx) at 30 °C.

Nutrition of hybrid plants

Hybrid plants bearing five to six leaves were potted in vermiculite and supplied with ¼ MS containing 2 mg/l IAA. The plantlets bearing 12–13 leaves were transferred to a culture room (30 °C day/25 °C night, 6,000 lx, 18 h day/6 h night).

Cytological observation

For cytological analysis, the pollen mother cells of the flowers of the hybrid plants were observed. The flowers were fixed in Carnoy's fluid and stained with acetocarmin.

Results

Production of hybrids in N. repanda × N. tabacum

Within 15 days of ovule culture, germination occurred. Approximately one-half of the obtained hybrid seedlings had poor root systems and retarded growth, and finally died. The other seedlings, however, had good root systems and grew vigorously and survived until they bore five to six leaves. The subsequent growth of the hybrids was retarded, however and these leaves turned pale and died in the end. In order to investigate the effect of IAA on the further developmental stages of hybrids, plantlets with five to six leaves were transplanted into pots and divided into two groups: one was supplied with ¼ MS solution or 1/1,000 Hyponex solution, and the other was supplied with 2 mg/l IAA in addition to ¼ MS solution. The former did not develop any vegetative parts and died within several weeks, however the latter continued to grow vigorously and finally bore flowers.

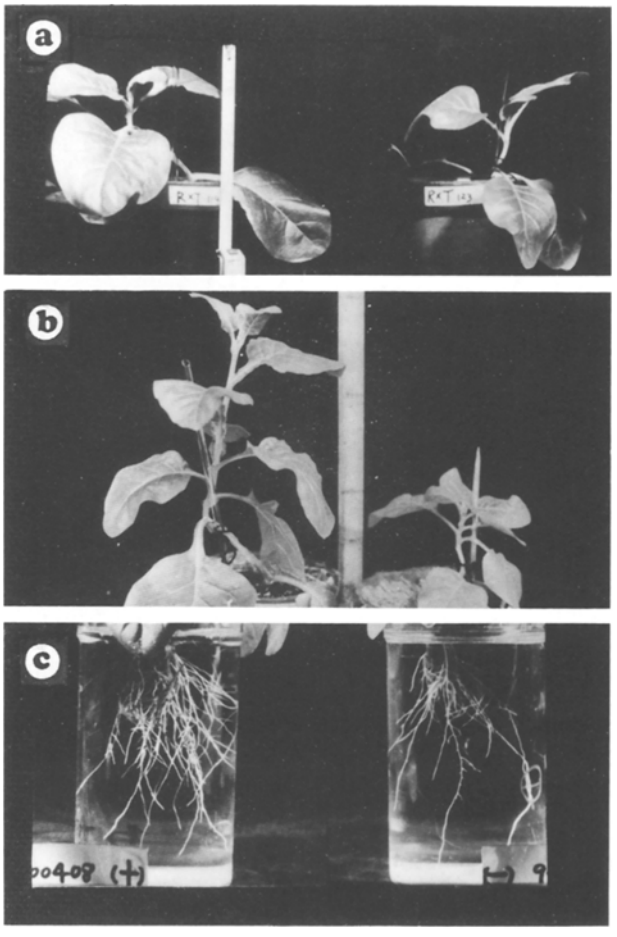

Fig. 1 a–c. Growth response of hybrid between *N. repanda* × *N. tabacum* to the nutrition with 2 mg/l IAA (*left*) and without IAA (*right*): **a** hybrid plants with five- to six-leaf stage before treatment; **b** hybrid plants 10 days after treatment; **c** morphology of roots of hybrids 10 days after treatment

To determine the effect of IAA on maturation, plantlets with 12–13 leaves were treated in two ways. One group was supplied only with ¼ MS solution, and the other was supplied with ¼ MS containing 2 mg/l IAA (Fig. 1). The first groups exhibited very slow expansion of leaves, but the second grew vigorously and developed new vegetative parts. The internode length of the second group was noticeably longer than that of the first (Fig. 1 b), and the roots of the latter became thicker and longer than those of the former (Fig. 1 c). In the latter case, a flowering plant was finally obtained after 1 month. On the other hand, the plants that were supplied with only ¼ MS solution exhibited retarded growth and died without producing any flower buds. To examine the effect of IAA on the flowering of hybrids, immediately after meiosis the nutritional solution was changed from ¼ MS solution containing 2 mg/l IAA to ¼ MS solution

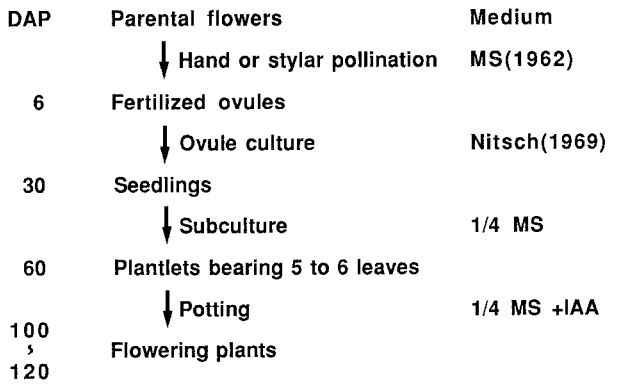

Fig. 2. Scheme of hybrid production between *N. tabacum* and *N. repanda* (DAP: days after pollination)

Table 1. Effect of IAA on survival rate of hybrids in the reciprocal crosses between *N. tabacum* and *N. repanda*

Cross combination		IAA (mg/l)	
♀	♂	0	2
N. repanda × *N. tabacum*		0[a] (0/16)	6.3 (3/48)
N. tabacum × *N. repanda*		0 (0/44)	9.8 (4/41)

[a] Percentage of hybrids that survived to bear flowers (survived hybrids/total hybrids with five to six leaves)

without IAA. The flower buds stopped expanding and then died without flowering. This indicates that exogenous IAA was necessary throughout the term of maturation, from the five- to six-leaf period to flowering. The survival rate of the hybrid plantlets bearing five to six leaves was 6.3% when IAA was supplied (Table 1). In total, it took 100–120 days to establish hybrid plants from hand pollination to the flowering stage (Fig. 2). In the crosses where IAA was not supplied, flowering plants could not be obtained (Table 1).

Production of hybrid plants in N. tabacum × N. repanda

In the reciprocal cross, we investigated whether or not flowering hybrid plants could be obtained in the same way. Enlarged ovules fertilized by stylar pollination were peeled from placenta and transferred to ovule culture medium. The germinated seedlings were transferred to ¼ MS medium without IAA for rooting. When the plants on the rooting medium developed five to six leaves, they were potted in vermiculite and supplied with ¼ MS solution containing 2 mg/l IAA. To investigate the role of IAA in the regulation of maturation, IAA was omitted during the various terms of maturation in the same way as in the reciprocal cross. None of the plants that were supplied with only ¼ MS could reach flowering stage. Thus, also in the reciprocal cross, the addition of IAA was found to be necessary for maturation of hybrids. The

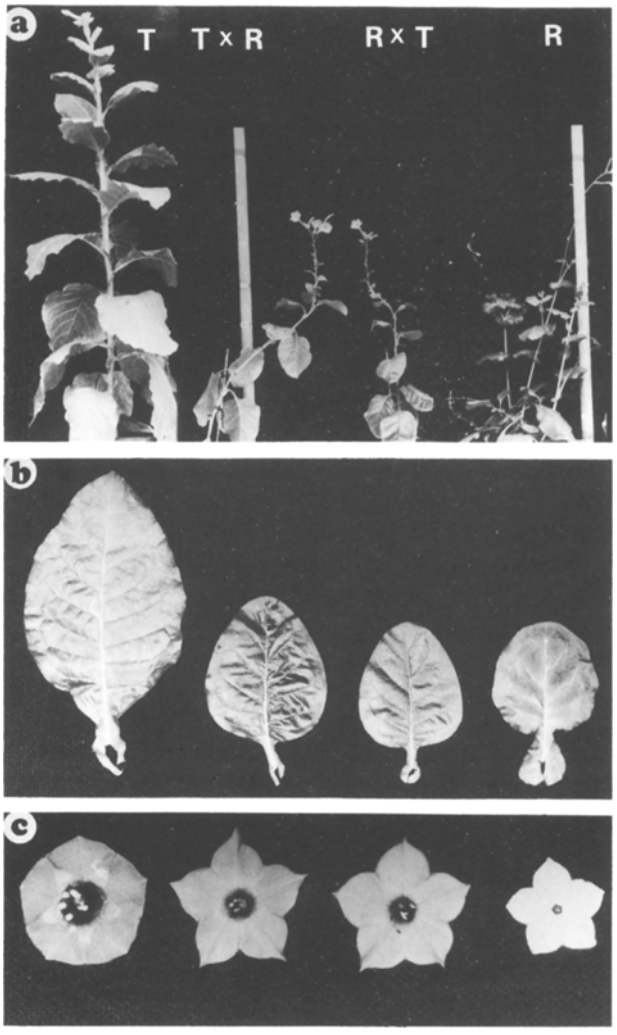

Fig. 3a–c. Morphological appearance of *N. tabacum* (T), *N. tabacum* × *N. repanda* hybrid (T × R), *N. repanda* × *N. tabacum* hybrid (R × T) and *N. repanda* (R), respectively (*left* to *right*): **a** plant type; **b** leaves; **c** flowers. Note: flower colors are carmine, pink, pink, and white, respectively (*left* to *right*)

survival rate of this cross was 9.8% (Table 1). As in the previous case, it took 100–120 days to establish hybrid plants from stylar pollination to the flowering stage (Fig. 2).

Characteristics of hybrids

In this study, we obtained many mature reciprocal hybrids. All hybrid plants appeared phenotypically uniform. With regard to branching type, both reciprocal hybrids were similar to *N. tabacum*, which showed apical dominance (Fig. 3a). The plant height of the hybrid plants was lower than that of both parents. Leaf shape and flower shape of the hybrids were intermediate between those of both parents (Fig. 3b and c). There was no apparent difference between reciprocal hybrids. The flower color of *N. repanda* was white and that of *N.*

tabacum was carmine, producing pink flowers in the hybrids.

All of the hybrids failed to produce fertile pollen and were completely self-sterile. Meiotic analysis was performed on metaphase I cells, and all of the cells examined had 48 univalents. These plants were confirmed to be real hybrids between *N. tabacum* (2n=48) and *N. repanda* (2n=48).

Discussion

In our study, it was shown that flowering hybrids from the reciprocal crosses between *N. tabacum* and *N. repanda* were obtained through stylar pollination, embryo (ovule) culture, rooting promotion, and application of IAA (Fig. 2). The final survival rates of young plantlets bearing five to six leaves in the reciprocal crosses between *N. tabacum* and *N. repanda* were 6.3 and 9.8% (Table 1). The growth period of hybrids was about 100–120 days, which was the same length as that of parents. These results indicate that application of IAA during the maturation period was a very useful, easy, and cheap method of overcoming hybrid inviability in this interspecific hybridization compared with the manipulation of ploidy level (Pittarelli and Stavely 1975), pollen or egg cell irradiation (Shintaku et al. 1988), or high temperature treatment (Suda et al. 1989 and Suda and Marubashi 1990).

The results showed that IAA was necessary throughout the period from the five- to six-leaf stage to the flowering stage. In contrast to this, it has been reported that the hybrids between *Solanum melongena* and *S. khasianum* are rescued from hybrid inviability manifested in the very young stages, by using a medium supplied with 0.1 mg/l IAA (Sharma et al. 1980). Also, the hybrids did not need exogenous IAA in the older stage and bore flowers. In our study, however, IAA had an inhibitory effect on the growth of seedlings and young plantlets before they bore five to six leaves (unpublished data). The phenomenon of hybrid inviability may vary with the species used.

Pittarelli and Stavely (1975) used tetraploids of *N. repanda* to obtain mature hybrids. Although they attempted to hybridize three diploid *N. tabacum* cultivars with the tetraploid, only one cultivar was successful in producing mature hybrid plants. Suda et al. (1989) and Suda and Marubashi (1990) obtained mature hybrids between *N. tabacum* and *N. repanda* with high temperature treatment. The plants needed to be kept at a high temperature in order to grow, and they died at temperatures below 28 °C even if they were supplied with IAA. We, however, obtained flowering hybrids at 25 °C when IAA was supplied (unpublished data). This may be due to the use of different cultivars of *N. tabacum*. The phenomenon of hybrid inviability may also vary with the cultivars used. The relationship between metabolism of IAA and high temperature is not clear.

The reciprocal hybrids between *N. tabacum* and *N. repanda* were not complete auxin auxotrophs because, before they reached the five- to six-leaf stage, they could grow normally without exogenous IAA. In contrast to the characteristics of our hybrids, shoots that regenerated from auxin-auxotrophic variants of *N. plumbaginifolia* were indeed inviable when cultured *in vitro* in the absence of auxin, and they could not develop normal root systems even in the presence of auxin (Blonstein et al. 1988). Our hybrids must have the ability to produce auxin in the early stages since they were able to develop a root system. Dwarf shoot morphology of the hybrid plants without exogenous IAA (Fig. 1) was similar to that of auxin-resistant mutants *Dwf* (Mirza et al. 1984). *Dwf* was thought to have low concentrations of endogenous auxin or an altered sensitivity to auxins. In general, swelling, but not elongation, of stem is induced by exogenous IAA in intact plants because of the excess IAA in addition to endogenous auxin (Holm and Abeles 1968). In reciprocal hybrids between *N. tabacum* and *N. repanda*, elongation of internodes and roots was induced by exogenous IAA (Fig. 1 b and c). This result suggested that the hybrid plants with more than five to six leaves contained insufficient endogenous auxin for normal growth, and that exogenous IAA compensated for insufficient endogenous auxin. Although it is not clear why the low amount of endogenous auxin occurred, some possibilities include the insufficient synthesis of auxin, quick turnover of auxin, and a defect in auxin binding protein. The hybrid inviability in this case might be manifested partly because of insufficient usable endogenous auxin.

References

Blonstein AD, Vahala T, Koornneef M, King P (1988) Plants regenerated from auxin-auxotrophic variants are inviable. Mol Gen Genet 215:58–64

DeVerna JW, Myers JR, Collins GB (1987) Bypassing prefertilization barriers to hybridization in *Nicotiana* using *in vitro* pollination and fertilization. Theor Appl Genet 73:665–671

Holm RE, Abeles FB (1968) The role of ethylene in 2,4-D-induced growth inhibition. Planta 78:293–304

Mirza JI, Olsen GM, Iversen TH, Maher EP (1984) The growth and gravitropic responses of wild-type and auxin-resistant mutants of *Arabidopsis thaliana*. Physiol Plant 60:516–522

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Niimi Y (1976) Effect of "stylar pollination" on *in vitro* seed setting of *Pentunia hybrida*. J Jpn Soc Hortic Sci 45:168–172

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Pittarelli GW, Stavely JR (1975) Direct hybridization of *Nicotiana repanda* × *N. tabacum*. J Hered 66:281–284

Reed SM, Collins GB (1978) Interspecific hybrids in *Nicotiana* through *in vitro* culture of fertilized ovules. J Hered 69:311–315

Sharma DR, Chowdhury JB, Ahuja U, Dhankhar BS (1980) Interspecific hybridization in genus *Solanum*. Z Pflanzenzuecht 85:248–253

Shintaku Y, Yamamoto K, Nakajima T (1985) Overcoming hybrid inviability in interspecific cross between *Nicotiana repanda* Willd. and *N. tabacum* L. Jpn J Breed 35:76–79

Shintaku Y, Yamamoto K, Nakajima T (1988) Interspecifc hybridization between *Nicotiana repanda* Willd. and *N. tabacum* L. through the pollen irradiation technique and the egg cell irradiation technique. Theor Appl Genet 76:293–298

Stavely JR, Pittarelli GW, Burk LG (1973) *Nicotiana repanda* as a potential source for disease resistance in *N. tabacum*. J Hered 64:265–271

Suda M, Marubashi W (1990) Effects of culture temperatures and IAA on the growth of hybrid seedlings of *Nicotiana repanda* Willd. × *N. tabacum* L. Jpn J Breed 40 [Suppl. 1]:58–59

Suda M, Marubashi W, Onozawa T (1989) Increase in fertility through "stylar pollination" and production of interspecific hybrid between *Nicotiana tabacum* L. and *N. repanda* Willd. Jpn J Breed 39 [Suppl. 2]:84–85