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Production of Crown-Rot Resistant Strawberry by Concentration of Indigenously Exisiting Resistant Cells through Tissue Culture

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Synopsis

Tissue culture was used for selecting and enhancing the resistant variant cells which indigenously exist in leaf tissues of the susceptible cultivar in order to establish an efficient system for producing crown-rot resistant lines of strawberry. Leaves of the resistant and susceptible cultivars were inoculated with the crown-rot pathogen by needle-prick inoculation method. Two types of lesions (small necrotic spots and extensively expanding lesions) were formed on leaves of both the resistant and susceptible cultivar. The resistant cultivar was characterized by higher percentage of the small necrotic spots. The cells forming the resistant-type lesion were amplified by inducing callus tissues from leaf explants of the susceptible cultivar, and regenerants showing the higher frequencies of the resistant-type lesion were selected. The amplification of these cells to the level of the resistant cultivar was achieved by repeating callus induction and plant regeneration from the selected regenerants. The regenerants finally obtained in the present system showed the resistance in the field inoculation test.

Key words : strawberry, crown-rot disease, resistance selection

Introduction

Genetic variations are frequently induced in plant tissue cultures as a result of spontaneous or mutagen-induced gene mutations or chromosomal abnormalities (Evans and Sharp 1983, Sunderland 1977). These variations have been utilized as a new gene source for improving major crop plants as commercially important traits including disease resistance (Larkin and Scowcroft 1981, Toyoda and Ouchi 1991). The somaclonal variation for the disease resistance was first selected *in vitro* in the presence of a model phytopathogen toxin (Carlson 1973), and more specifically in the pressure of host specific toxins produced by the pathogens (Brettell and Ingram 1979). This methodology has been also applied to the diseases in which the mechanisms for the pathogenecity or host resistance have not become obvious or in which the toxins are not essentially involved. Actually, the resistant variants have been obtained by improving the methods for selection in viral (Murakishi and Carlson 1982, Toyoda et al. 1989b), bacterial (Toyoda et al. 1989a), and fungal diseases (Shahin and Spivey 1986, Heath-Pagliuso et al. 1988, Toyoda et al. 1991) of various crop plants. These results imply that this mothodology could be further applicable to selection of useful variations which indigenously exsisted in tissues as well as somaclonal variations induced during tissue culture.

A few cultivars of strawberry have been extensively cultivated in Japan, and the high yields of

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strawberry fruits have been mainly obtained from the cultivar Nyoho (Oda 1991). However, this cultivar is highly susceptible to the crown-rot disease, and the yield loss due to this disease is a serious problem in strawberry cultivation. Therefore, the production of the resistant lines has been an urgent matter. We have an additional Japanese cultivar (Hokowase) resistant to the disease, but cultivation acreage of this cultivar has declined recently, mainly because of the rapid softening of harvested fruits. To solve such post-harvest fruit quality and commercial problems, the following strategies could be used for improving strawberry; i) a classical breeding by crossing between the resistant and susceptible cultivars, and ii) isolation of genetic variation as to the disease resistance through tissue culture of the susceptible cultivars. An efficent system for tissue culture of strawberry has been elaborated in our laboratory (Toyoda et al. 1990) and Fusarium wilt-resistant strawberry lines have been selected (Toyoda et al. 1991). Herein, we report a possible application of tissue culture for the selection and amplification of crown-rot resistant cells existed in strawberry leaf tissue, and examine the feasibility of this methodology for producing disease resistant plants.

Materials and Methods

Plants. Three Japanese cultivars, 'Nyoho', 'Toyonoka' and 'Hokowase' of strawberry (*Fragaria*×*ananassa*) were used in the present study. The two cultivar Toyonoka and Nyoho are susceptible and highly susceptible, respectively, and Hokowase is resistant to the crown-rot disease. The cultivars Toyonoka and Hokowase were used as a control for evaluating susceptibility and resistance after inoculation, respectively. These plants were grown in a greenhouse controlled at 26℃ and vegetatively propagated by runners. Younger leaves were harvested from one-month-old runner (daughter) plants and used for inoculation with the pathogen or for callus induction.

Fungus and inoculation. The crown-rot pathogen, *Glomerella cingulata* (*Colletotrichum gloeosporioides*) which was isolated and kindly gifted by Dr. K. Okayama, (Nara Agricultural Experiment Station, Japan), was cultured with liquid Czapek medium (without agar) at 26℃ for 3-4 weeks. Newly produced conidiospores were washed with sterilized water and collected by centrifugation. The conidia were suspended in sterilized water to give 10^6 spores/mℓ and used as inoculum. Leaves were harvested from 8-15 petioles per plant, and one of trifoliolate leaves on each petiole was used for inoculation. Excised leaves were placed in a moisten Petri dish and punctured with a bundle of sterilized sewing needles. A drop (100$\mu\ell$) of the conidial suspension was placed onto needle-pricked portions of leaves, and inoculated leaves were incubated at 28℃ for 6 days under a continuous illumination of 4,000 lux ('a needle-prick inoculation method'). Necrotic lesions formed around the inoculation sites were classified into two types; a small necrotic spot (Fig. 1A) in the resistant cultivar and a rapidly and extensively expanded lesion (Fig. 1B) in the susceptible cultivars. In the resistant-type necrotic lesions (RL), the pathogen was strictly limited around the inoculation sites, whereas in the susceptible-type lesions (SL) the pathogen elongated mycelia and produced numerous progeny conidiospores 5 or 6 days after inoculation. Our preliminary study confirmed that no necrotic lesion was formed when water was dropped onto the punctured sites. It was clarified that the necrotic lesion formation was due to the pathogen infection, but not the mechanical injury.

Callus induction and plant regeneration. Callus induction and plant regeneration were carried out according to the method reported previously (Toyoda et al. 1990). Young leaves newly developed from

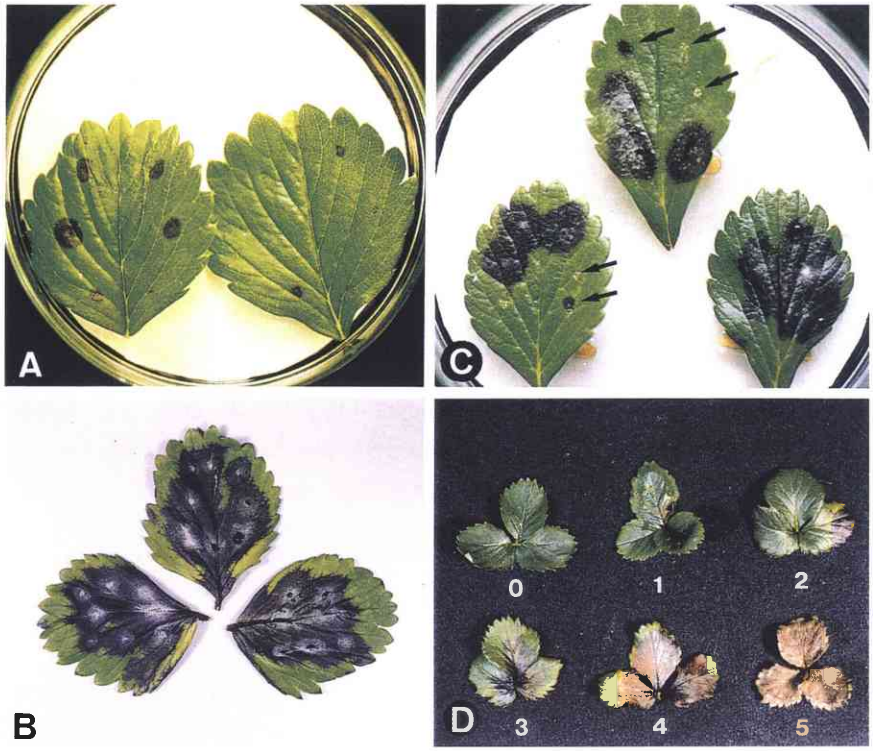

Fig. 1. Disease symptoms on strawberry leaves inoculated with the crown-rot disease pathogen by needle-prick inoculation method (A-C) and spray inoculation method (D). A, small necrotic spots (RL) formed on inoculated leaves of the resistant cultivar (Hokowase); B, rapidly expanded and fused necrotic lesions (SL) accompanied by numerous progeny conidiospores on the susceptible cultivar (Nyoho) 6 days after inoculation; C, simultaneous formation of RL (arrows) and SL on the same leaves of Nyoho; D, classification determining disease severities (0-5) of infected trifoliolate leaves of regenerants obtained from leaf-calli of Nyoho (two weeks after inoculation). 0 and 5 correspond to the disease severities of Hokowase and Nyoho, respectively.

runners of strawberry (Nyoho) were harvested and surface-sterilized with 70% EtOH and 2% sodium hypochlorite. After rinsing several times with sterilized water, leaves were cut into small pieces (1×1 ㎝) and put onto a Murashige-Skoog (MS) (1962) medium supplemented with growth regulators, solidified with 0.2% Gellan gum, and adjusted to pH6.0 with NaOH before autoclaving. The growth regulators used were 0.1㎎/ℓ 2,4-dichloro-phenoxyacetic acid (2,4-D) for auxin and 1.5㎎/ℓ 6-benzylaminopurine (BA) for cytokinin. Culture bottles were tightly sealed and incubated at 26℃ under a constant illumination of 4,000 lux until callus tissues were induced from the leaf explants (for 30-40 days). Induced callus tissues were subcultured for several passages at a two-week interval. The shoot developed from callus tissues in the presence of 0.1㎎/ℓ 2,4-D and 1.5㎎/ℓ BA. The shoot formation was well synchronized after the tissues were subcultured for two or three passages with the fresh same medium. After leaflets were well developed, shoots were transferred to growth regulator-free MS medium for root initiation. Regenerated plants were transplanted to soil and acclimated for 3-4 days in a moist chamber. These plants grew normally under field conditions.

Field inoculation test. The regenerated and parental strawberry plants were multiplied through runner, planted in pots, and cultivated for one month in a glass house controlled at 28℃ and 70-100% of relative humidity. For inoculation, the plants were sprayed with the conidial suspension (10^6 spores/㎖) of the pathogen so as to entirely cover leaf surface of test plants with inoculum droplets and incubated for two weeks. As shown in Fig. 1D, the appearance and expansion of necrotic lesions on

leaves were classified into six types: O, no necrotic lesion on trifoliolate leaves; 1 and 2, smaller (less than half) and larger (more than half of leaf surface area) necrotic lesion on one of trifoliolate leaves, respectively; 3 and 4, smaller and larger necrotic lesions on all of trifoliolate leaves, respectively; 5, entire necrosis of all of trifoliolate leaves. On the basis of the classification, the scores (0 to 5) were given to total trifoliolate leaves of each plant and the disease severity of inoculated plants was determined by calculating the average of scores.

Results and Discussion

In the present study, the experiments were designed to select the crown-rot resistant cells which pre-existed in strawberry leaf tissues, and to produce the resistant strawberry plant through tissue culture. For this purpose, the following two steps were considered essential; 1) an easy and effective inoculation for precisely evaluating the resistant or susceptible responses, and 2) an establishment of a highly efficient system for callus induction and plant regeneration. Since the tissue culture system of strawberry had been established in our laboratory, the present study first focused on the responses of the resistant and susceptible cultivars after inoculation.

Fig. 1 shows the typical resistant (A) and susceptible (B) necrotic lesions formed on the leaves by the present inoculation method. The appearance of these necrotic lesions in both the susceptible and resistant cultivars was given in Table 1. The frequencies of the lesions were considerably different among these cultivars. In the resistant cultivar, the RL was formed in approximately 90% of the total inoculated leaves, whereas in the susceptible cultivars the SL was found in approximately 80 and 90% of the inoculated leaves in Toyonoka and Nyoho, respectively. These results indicated that the frequencies of RL reflect the degree of resistance or susceptibility of the cultivars. In the present study, however, some inoculated leaves showed the simultaneous occurrence of different types (RL and SL) of necrotic lesion on the same inoculated leaves (Table 1), as shown in Fig. 1C. These results indicate that different types of cells with different responses to the pathogen exist chimerically in the same leaf tissue as well as in the same plants. Judging from these results, we deduced that a resistant cultivar could be produced by enchancing the frequency of RL in the susceptible cultivar through tissue culture.

To produce the strawberry plants responding to the pathogen only with the RL, we tried to concentrate these cells by repeating the tissue culture and selection by inoculation, using the leaves showing different types of necrotic lesion. Nevertheless, it would be difficult to estimate this type of

Table 1. Formation of two types of necrotic lesions in strawberry leaves inoculated with the crown-rot pathogen (*G. cingulata*) by needle-prick inoculation method[a)]

Cultivars	Percentage of inoculation sites with		
	RL	RL + SL	SL
Hokowase (resistant)	90.9 (2.7[b)])	9.1 (3.2)	0
Toyonoka (moderately susceptible)	0	19.4 (1.6)	80.6 (3.3)
Nyoho (highly susceptible)	0	8.3 (0.6)	91.7 (3.8)

[a)] Ten plants of each cultivar were runner-multiplied and used for inoculation. Twenty leaves per plant were harvested at random and an inoculum solution was dropped at six needle-injured sites of each leaf. Resistant-type small necrotic spots (RL) and highly susceptible-type necrotic lesions (SL) formed at the inoculation sites were recorded 6 days after inoculation.

[b)] Standard deviation.

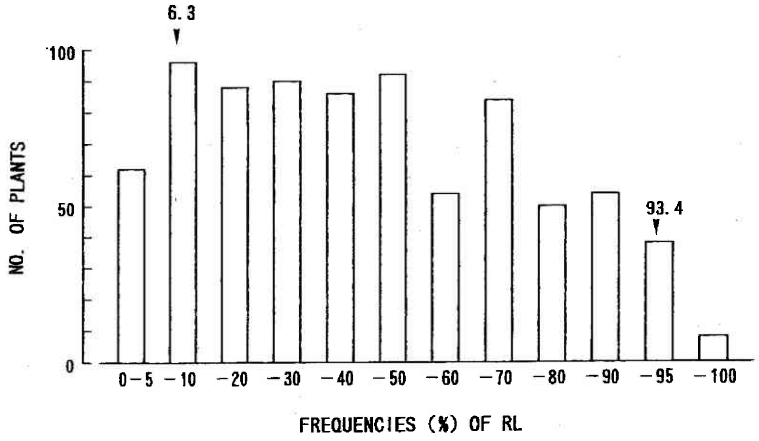

Fig. 2. Distribution of regenerated plants derived from leaf-calli of strawberry cultivar (Nyoho) on the basis of freqencies of resistant-type lesion (RL) after inoculation with the crown-rot pathogen by needle-prick inoculation method. The scores given in the figure represent percentages of RL in the resistant cultivar Hokowase (93.4%) and susceptible cultivars Toyonoka (12.7%) and Nyoho (6.3%).

Table 2. Formation of necrotic lesions on leaves of secondary regenerants [a] of strawberry after inoculation with the crown-rot pathogen

Secondary regenerants	No. of leaves with			No. of RL / total inoculation sites
	RL	RL + SL	SL	
N2-132R	8	1	0	48/54
N2-105R	12	2	0	81/84
N2-096R	7	0	0	42/42
N2-101R	9	1	0	59/60
N2-157R	13	0	0	78/78
N2-171R	6	2	0	33/36

[a] The secondary regenerants were obtained from callus tissues of the original regenerants with high frequencies of the RL. Leaves were harvested from 7-14 petioles of these secondary regenerants and inoculated with the pathogen by a needle-prick inoculation method.

heterogeneity prior to callus induction, because inoculated leaves become unsuitable for tissue culture due to deterioration of leaf tissues and contamination with the inoculated pathogen. In the present study, therefore, callus tissues were induced from leaf-explants harvested at random, and the regenerated plants obtained were inoculated with the pathogen as above. In this experiment, 802 regenerants were used for inoculation, and the results obtained were summarized in Fig. 2, where each plant was classified on the basis of the frequencies of the RL at total inoculation sites of tasted leaves. The strawberry plants regenerated from leaf-callus tissues were diverse in their responses; 66 regenerants showed the lower values and the remaining regenerants the higher values than that (6.3%) of the parental susceptible cultivar. Morever, scores higher than that (93.4%) of the resistant cultivar (Hokowase) were recorded of some regenerants. Judging from these results, it was considered that the 'resistant-type' cells (cells which respond with the RL-formation) were highly and efficiently concentrated in these high-score regenerants. In the following experiment, therefore, callus induction and plant regeneration were further conducted using leaf explants obtained from these high score

regenerants. As a result, some candidate regenerants successfully obtained from callus tissues were examined for their lesion formation after inoculation. Table 2 summarizes the types of lesion formation in each of candidate regenerant leaves. The data clearly indicated that all of the tested leaves of these secondary regenerants showed the RL and no secondary regenerant leaves responded with only the SL after inoculation. Thus, it was proved that the 'resistant-type' cells could be preferentially concentrated in callus-derived regenerants produced by the present culture system.

Although the mechanisms for lesion formation have not been elucidated in this study, the present results revealed that the tissue culture is effective for selection and amplification of variant cells concerning important traits. Some workers have successfully isolated somaclonal variation for disease resistance from strawberry tissue cultures, where *Fusarium* wilt resistance (Toyoda et al. 1991) or *Alternaria* black spot resistance (Takahashi et al. 1992) was isolated by combination of direct inoculation with the pathogens and the devised selection method. In principle, however, the present method would be applicable to every type of plant tissues that can be cultured for callus induction and plant regeneration, and provide a useful and practical technique for improving a broad range of plant species with respect to varieties of cultivation characteristics.

The present study demonstrated that the regenerated plants (N2-096R and -157R) obtained in this selection were resistant against the pathogen inoculated by needle-prick inoculation method. However, it is important and essential that these plants could express the resistance to the pathogen under field conditions for cultivation. For this purpose, the selected regenerants were multiplied through runner, grown in a temperature-controlled greenhouse, and sprayed with an inoculum solution. As pointed out in a previous work (Okayama 1993), a high humid and high temperature condition after inoculation was useful for achieving an effective infection of the pathogen into the susceptible cultivar. As shown in Fig. 3, the present condition for inoculation caused severe disease symptoms (between type 3 and 5 in Fig. 1D) in more than 80% of control susceptible plants and in almost all of the highly susceptible regenerants (N2-114S) formimg the SL at all infection sites of tested leaves by needle-prick inoculation method. On the other hand, the selected lines of secondary regenerants did not show any disease symptoms in leaves during the entire experimental periods (for 40 days) (Fig. 3), although necrotic spots were formed slightly in the stem and runner. These results suggest that the N2-096R and -157R may be availed as commercial strawberry lines for crown-rot resistance.

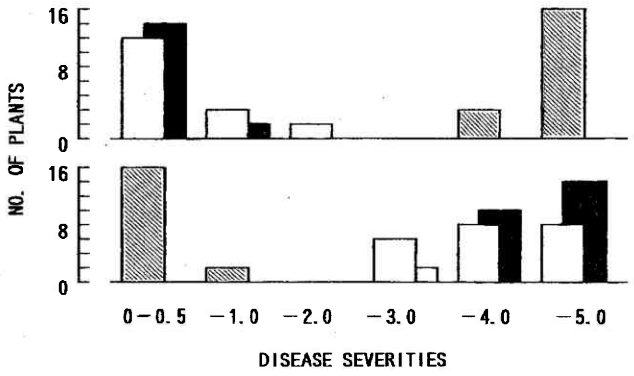

Fig. 3. Distribution of daughter plants runner-multiplied from the secondary regenerants (N2-096R and -157R) of Nyoho on the basis of disease severities after inoculation with the crown-rot pathogen by spray-inoculation method. Refer to the legend of Fig.1D for classification of disease severities of infected trifoliolate leaves of strawberry plants. Upper, resistant regenerants N2-096R (□) and N2-157R (■) and highly susceptible regenerant N2-114S (▨); lower, resistant cultivar Hokowase (▨) and susceptible cultivars Toyonoka (□) and Nyoho (■).

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