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Evaluation of Stable Resistance Expression in Self-pollinated Progenies of Bacterial Wilt Resistant Regenerants Obtained from Leaf Callus of Tomato

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Abstract

The bacterial wilt resistant line LNSR-7 of tomato was isolated from self-pollinated progenies of leaf-callus derived regenerants by directly inoculating a bacterial wilt pathogen *Pseudomonas solanacearum* into injured roots of tested plants. The subsequent self-pollinated progenies of the line were examined for their fruit quality and resistance expression under natural cultivation conditions in a pathogen-infested tomato field. During three generations of progenies, the tomato plants showing both the bacterial wilt resistance and the high fruit qualities comparable to the parental cultivar were selected in order to fix commercial characteristics of the line. The stable inheritance of the resistance in the subsequent self-pollinated progenies was further examined by directly inoculating the pathogen into the roots of test plants. Inoculated plants were planted in soil heavily infested with the pathogen to ensure exposure to the pathogen. Under these artificial inoculation conditions, the selected line was shown to be highly resistant to the disease. The resistance mechanism in the line was analyzed by examining multiplication and translocation of the pathogen *in planta*. The precise monitoring of infection behavior of the pathogen was successfully achieved using the genetically marked *P. solanacearum*. Consequently the present line LNSR-7 strictly limited secondary multiplication and translocation of the pathogen and suppressed the wilt induction by the pathogen.

1. Introduction

Somaclonal variation in plant tissue cultures is a useful genetic source for crop improvement [1, 2]. One of the major approaches in plant biotechnology has been an effective isolation and utilization of useful somaclonal variations for producing disease resistant lines in important crop plants [3, 4]. Actually, some investigators have successfully isolated somaclonal variants for viral [5, 6], bacterial [7, 8], and fungal disease resistance [9-11]. However, these variations are frequently unstable and therefore, it is essential to evaluate stable propagation of isolated variations in progeny plants. From this point of view, we have examined expression of fungal wilt resistance in self-pollinated or runner-multiplied progenies of the isolated tomato [12] and strawberry lines [11, 13], respectively, and discussed the mechanisms for acquired resistance in their tissue cultures [4]. Also in the tomato regenerants selected for bacterial wilt resistance, a similar analysis would be necessary to produce commercially available, disease-resistant tomato cultivars.

Bacterial wilt disease caused by *Pseudomonas solanacearum* is a typical soil-borne disease of *Solanaceae* plants, and the protection of major crops from the disease has been an urgent matter in agriculture. Unfortunately, in Japan effective resistant lines of tomato have not been produced by conventional breeding techniques. Successful isolation of somaclonal variation for bacterial wilt resistance from leaf callus derived regenerants of tomato provided a promising program for production of disease resistant cultivars [8].

In the case of tomato, high yield and high fruit quality are commercially important in addition to disease resistance. These qualities of tomato plants were shown to be frequently varied when objective

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plants originated from tissue cultures [14]. In the present study, therefore, the experiments were designed to evaluate both fruit productivity and stable propagation of the resistance in self-pollinated progenies of the isolated tomato line in order to produce a new tomato cultivar resistant to bacterial wilt disease.

2. Materials and Methods

2. 1 Tomato plants

The bacterial wilt-resistant tomato line (LNSR-7R2) selected among the pathogen-inoculated R1 progenies (self-pollinated progenies) of regenerants obtained from leaf callus of tomato (*Lycopersicon esculentum* Mill, cv. Fukuju No. 2) [8] was used in the present study (**Fig. 1**). Additionally, two susceptible cultivars (Fukuju No. 2 and Ponderosa) of tomato were used as controls for evaluating infectivity of the pathogen.

2. 2 Selection of tomato plants on the basis of fruit qualities

One-month-old seedlings of the LNSR-7R2 and parental cultivar (Fukuju No. 2) were transplanted to the tomato field of Kanagawa Horticultural Experimental Station and cultivated for 3-4 months until tomato fruits of the sixth flower cluster matured. The number of flowers and fruits per flower cluster, Brix indices of tomato fruits, and some growth properties (growth rate, plant appearance) were examined on the basis of selection criteria for commercially available tomato qualities [14]. The cultivation was repeated for the subsequent self-pollinated progenies (R3 and R4) of the LNSR-7 in order to fix fruit qualities of the line (**Fig. 1**).

2. 3 Pathogen

The strain K-101 of *Pseudomonas solanacearum* [15] was used for the present study. The bacterium

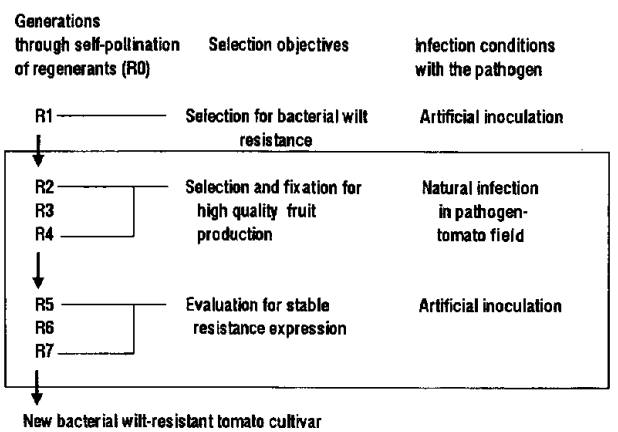

Fig. 1 Breeding program for selection and fixation of fruit productivity and bacterial wilt resistance in the isolated tomato line LNSR-7.

was shake-cultured in a PCG medium (10g peptone, 1g casamino acids and 10g glucose in 1 liter of water) at 26°C for 48 hr and collected by centrifugation. The bacterial pellet was suspended in distilled water so as to give the final density of 10^6 cells per ml and used for an inoculum suspension.

2. 4 Evaluation of stable inheritance of resistance

The seedling of the LNSR-7R5 were planted in autoclaved soil (without pathogen) of pots and grown in a temperature-controlled greenhouse (24-28°C) until the first fruit of the first flower cluster matured (approximately 40 days). By repeating this procedure, the R6 and R7 seeds of the LNSR-7 line were similarly obtained. One month-old seedlings of the R5, R6 and R7 of the line and the susceptible cultivars were inoculated with the pathogen (K-101) by a 'root-and soil inoculation' method used for inoculation of a soil-borne fungal wilt pathogen *Fusarium oxysporum* f. sp. *fragariae* into strawberry plants (11)(**Fig. 1**). The seedlings were gently pulled out from soil of pots and their roots were rinsed with water and then dipped in an inoculum bacterial suspension for 1 min. Root-inoculated seedlings were planted in a pathogen-infested soil of a bed (90×90×180 cm) in which soil was repeatedly amended with 5 *l* of bacterial suspension (10^8 cells/m*l*) at 4-day intervals for one month in order to maintain high population (10^6-10^7 cells/g. soil) of the pathogen. Disease symptoms (partial and systemic wilting) in the leaves of inoculated plants were recorded on a daily basis for one month. Wilted plants were cut at the stem and placed in distilled water to examine exudation of the pathogen which had multiplied in host vessel system.

2. 5 Monitoring of the pathogen by introduced marker genes

The strain K-101 was genetically marked with some selected genes by introducing them into bacterial chromosome through conjugation according to the method described previously [16]. Namely, the plasmid pUCD623 [17] which contain the transposon Tn4431 carrying *lux* operon genes and tetracycline-resistance gene (tet^r) was biparentally transferred to the recipient K-101 from the donor bacterium (*Escherichia coli* HB101). The transconjugants of the pathogenic bacterium were isolated in the presence of tetracycline and examined for their bioluminescence production. Insertion of the marker genes into chromosome was confirmed by a Southern hybridization analysis. In the present study, the transconjugant (K-101/lux8) emitting the strongest bioluminescence was isolated and used for inoculation. Our preliminary study confirmed that the K-101/lux8 showed the strong pathogenicity comparable to the original

strain. One-month-old seedlings of the R7 and the parental cultivars were removed from soil and their roots were gently dipped in an inoculum suspension (10^6 cells/m*l*) of K-101/lux8 ('root dip inoculation' method) [18] and grown in autoclaved soil of pots in a temperature-controlled greenhouse. Inoculated plants were gently removed from soil at the various periods after inoculation and roots, lower stems, upper stems and leaves were detached for examination of multiplication and translocation of the pathogen. Detached plant materials were homogenized in distilled water and clarified by low-speed centrifugation. The supernatant was spread onto a selective medium (PCG medium containing 10 mg/m*l* tetracycline) and incubated at 28 °C for 48 hr. The capability of tetracycline-resistant bacteria to produce bioluminescence was examined by exposing luminiferous bacterial colonies to an X-ray film overnight in the dark according to the method described previously [16]. Colony hybridization of bacteria growing on the selective medium was conducted according to the standard protocols [19]. The *lux* C gene (located in the forefront of the *lux* operon) [20] was excised from the original vector, labeled with horseradish peroxidase, and used as a probe for Southern and colony hybridization.

3. Results and Discussion

In our previous work, we have successfully established an efficient tissue culture condition for callus induction and plant regeneration in major tomato cultivars [21, 22] and pointed out the possible selection of somaclonal variations induced during tissue culture for producing disease resistant tomato lines [12, 23]. The bacterial wilt-resistant tomato line (LNSR-7) used in the present study was isolated by directly inoculating an inoculum bacterial suspension into razor-injured roots of self-pollinated progenies (R1) of regenerants (R0) which were obtained from leaf-callus of tomato [8]. To breed commercially available tomato lines, however, it is essential for tomato plants to produce high yield and high quality of fruits in addition to disease resistance. For this purpose, we adopted the breeding program of the isolated line, as shown in **Fig. 1**. According to the program, we first planted self-pollinated progenies (LNSR-7R2) of the R1 in a pathogen-infested tomato field in which the susceptible tomato cultivars had been repeatedly cultivated to cause frequent natural infection and maintain a high population of the pathogen. The present cultivation system enabled us to simultaneously evaluate both resistance to natural infection by the pathogen and agriculturally important characteristics of tested tomato plants. Under the present condition, approximately 60 % of the parental susceptible plants were heavily infected and completely withered within an experimental period. On the other hand, the LNSR-7R2 line showed normal growth and fruit production without any accompany-

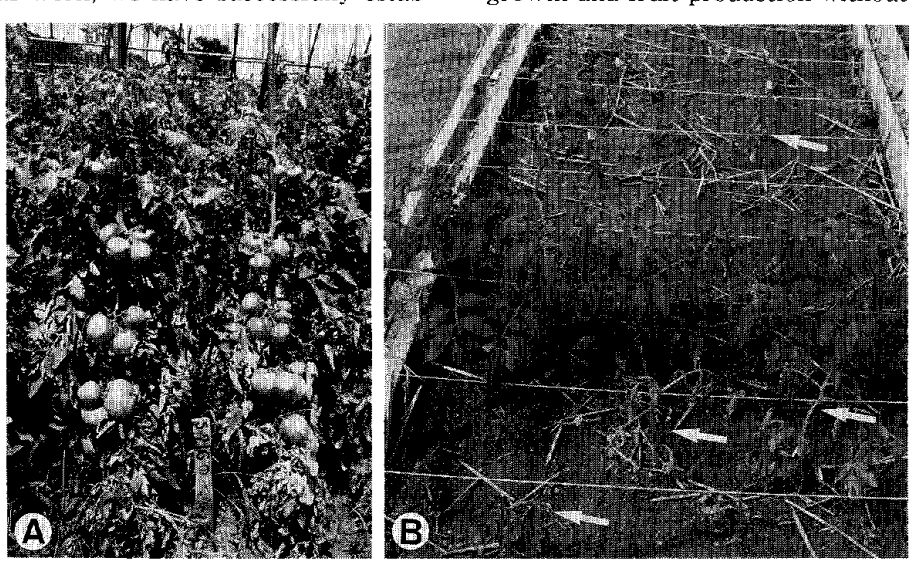

Fig. 2 Evaluation of fruit production (A) and bacterial wilt resistance (B) in self-pollinated progeny of the LNSR-7 line.
The fruit productivity of the LNSR-7R2 was examined by cultivating tomato plants in a pathogen-infested tomato field till fruit of the sixth flower cluster were harvested (for 3-4 months). The stable inheritance of bacterial wilt resistance in the subsequent self-pollinated progeny plants (R5-R7) was examined using a 'root-and soil inoculation' method. Note sever wilting of susceptible plants (arrows) planted next to the vigorously growing plants of the LNSR-7R7.

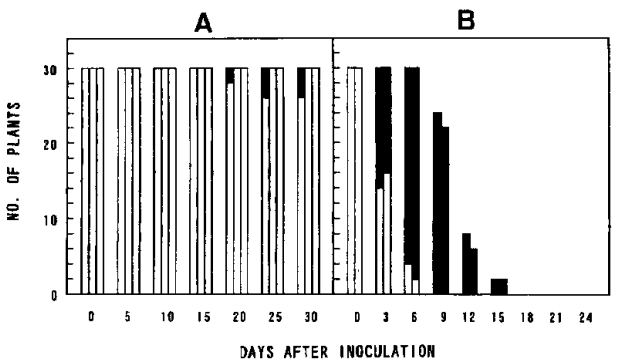

Fig. 3 Response of resistant (A) and susceptible (B) tomato plants to the inoculation with *P. solanacearum* K-101 by a 'root-and soil inoculation' method.
A, self-pollinated progenies R5 (left), R6 (middle), and R7 (right column) of the LNSR-7 line ; B, the susceptible cultivars Fukuju No.2 (left) and Ponderosa (right column). One-month-old seedlings of tomato were dipped in an inoculum bacterial suspension and planted in soil which had been infested with the pathogen. The shaded column represents the plants showing partial wilting.

ing wilt disease symptoms. **Fig. 2A** shows normally growing tomato plants of the present line with successful fruit production under a pathogen-infested field condition. In the present study, we selected tomato plants which showed Brix indices and fruit production percentages similar to those of Fukuju No. 2 (data not shown) and obtained the LNSR-7R3 seeds by self-pollination. In order to fix these fruit qualities of the line, the self-pollination and pathogen-infested soil cultivation were repeated. Finally, the LNSR-7R5 was obtained in the present study.

To further confirm stable propagation of bacterial wilt resistance in the progenies, the R6 and R7 plants were produced by subsequent self-pollination of the LNSR-7R5 and examined for their resistance expression to the pathogenic bacteria (K-101) inoculated. In this experiment, the plants were inoculated with the pathogen by a 'root-and-soil inoculation' method in order to avoid infection escape by the pathogen (**Fig. 3**). An infection efficiency by the pathogen could be evaluated by assessing symptom appearance in two susceptible cultivars (Fukuju No. 2 and Ponderosa). Partial wilting of upper young leaves appeared in approximately 50 % of inoculated susceptible plants in 3-4 days and in all plants 9 days after inoculation. These plants caused severe systemic wilting within a few days after the partial wilting appeared in leaves. There was no significant difference in appearance of partial and systemic wilting between the two susceptible cultivars. In all of the systemically wilted plants, the bacterial exudation, a typical syndrome of infected plants applied for identification of the bacterial wilt disease by *P. solanacearum* [24], was detected. These results indicated that the present inoculation method was effective enough to avoid infection escape by the pathogen and therefore to evaluate resistance expression in the resistant line. An inoculation analysis of the progeny plants of the LNSR-7 line revealed that all of the tested plants were highly resistant to the pathogen inoculated, although a few plants of the R5 showed limited wilting in one or two leaves which did not expand to sever wilting. Judging from these results, we concluded that the resistance induced in the tissue cultures could be successfully passed on to the regenerated plants and their progeny through self-pollination. Thus, the LNSR-7R7 would be commercially available as a new tomato cultivar resistant to the bacterial wilt.

In order to clarify the mechanisms for resistance in the LNSR-7 line, we analyzed multiplication and translocation of the pathogen at the various stages after inoculation. For precisely isolating and identifying the pathogen, the genetically marked pathogen (K-101/lux8) was utilized for inoculation, and infection behavior of the pathogen was traced on the basis of expression of the integrated marker genes. Actually, the use of the K-101/lux8 enabled us to distinguish the inoculated pathogen from coexisting non-pathogenic bacteria in the presence of tetracycline and moreover from non-pathogenic tetracycline-resistant bacteria by the production of bioluminescence. The luminiferous bacteria growing on the selective medium were confirmed to be the gene-marked *P. solanacearum* by a colony hybridization analysis. Using this method, the pathogen attached to roots of inoculated plants was first recovered, because an attachment of the phytopathogenic soil-borne bacteria to root surface of host plants is an initial infection process followed by entry and multiplication of the pathogens [24]. The result is given in **Table 1**. The present analysis indicated that the pathogen could adhere to roots of both resistant and susceptible plants at the similar levels. The subsequent initial multiplication of the pathogen was detected in both resistant and susceptible plants 3 days after inoculation. The increase of the pathogen at this stage was considered to be due to multiplication of the pathogen which entered into root tissues, because the similar levels of the pathogen could be recovered even after roots were surface-sterilized (data not shown). There was no significant difference in the initial increase of the pathogen between the resistant and susceptible plants. In the susceptible cultivars, however, the pathogen multiplied prominently and translocated to stem regions during the subsequent three days, whereas in the resistant line the bacterial multiplication and translocation were strictly limited during the entire

Table 1.
Detection of the gene-marked *P. solanacearum* K-101/lux8 in detached parts of inoculated tomato plants.

Detached Plants parts	Number of bacteria/g. fresh weight*1				
	0	3	6	9	12*2
Roots					
LNSR-7R7	$1.6(0.3)\times10^2$	$4.3(0.5)\times10^4$	$6.9(1.8)\times10^2$	$3.2(1.0)\times10^3$	$1.9(0.6^{*3})\times10^2$
Fukuju No. 2	$2.2(0.4)\times10^2$	$6.3(0.9)\times10^4$	$4.7(0.6)\times10^5$	$5.8(1.1)\times10^7$	$1.4(0.3)\times10^7$
Ponderosa	$1.5(0.3)\times10^2$	$3.8(0.3)\times10^4$	$6.3(1.2)\times10^5$	$2.2(0.9)\times10^8$	$1.3(0.2)\times10^7$
Lower stems					
LNSR-7R7	ND*4	$<10^2$	$<10^2$	$<10^2$	$<10^2$
Fukuju No. 2	ND	$4.8(0.8)\times10^5$	$4.8(0.6)\times10^8$	$6.2(0.7)\times10^8$	$10^9<$
Ponderosa	ND	$3.3(0.7)\times10^4$	$6.9(1.1)\times10^7$	$2.3(1.2)\times10^8$	$10^9<$
Upper stems and leaves					
LNSR-7R7	ND	ND	ND	ND	ND
Fukuju No. 2	ND	$<10^2$	$5.3(1.3)\times10^4$	$6.8(1.8)\times10^6$	$9.2(2.3)\times10^8$
Ponderosa	ND	$<10^2$	$3.8(0.7)\times10^4$	$7.3(1.4)\times10^7$	$8.2(1.5)\times10^8$

*1 Thirty plants were tested and the values were given as means of three separate replications. The methods for inoculation and detection of the pathogen were given in the text.
*2 Days after inoculation.
*3 Standard error.
*4 Not detected.

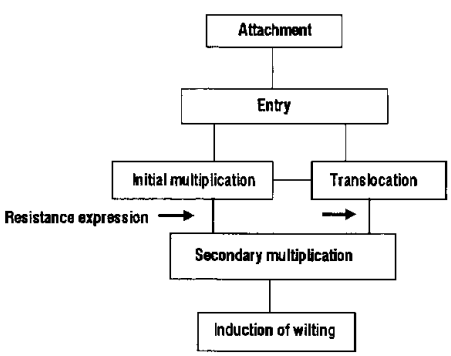

Fig. 4 Infection events of the bacterial wilt pathogen *P. solanacearum* and postulated sites (bold arrows) for resistance expression in the LNSR-7R7 line.

periods of cultivation. The partial and systemic wiltings were observed 6-8 days and 10-14 days after inoculation, respectively, but not induced in the resistant line. Judging from these results, we summarized infection events of the pathogen in **Fig. 4**, where the resistance expression by the LNSR-7R7 was denoted as host responses to inhibit secondary multiplication of the pathogen in root and stem tissues of tomato plants. Although the substantial nature of the resistance reactions has not been elucidated in the present study, the results suggest that the resistant line could statically suppress the growth of the pathogen, since viable bacteria could be recovered from the resistant plants at the stages when the resistance reactions had been expressed. The further analysis of gene expression for the resistance is underway.

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