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Variation in virulence of *Plasmodiophora brassicae* in Japan tested with clubroot-resistant cultivars of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Abstract

The differential hosts of Williams (1966) and the European Clubroot Differential (ECD) (Buczacki et al., 1975) have been used commonly to identify populations of *Plasmodiophora brassicae*, which causes clubroot disease in *Brassica* crops. However, some of these hosts showed intermediate and fluctuating scores to most populations from Japan. Therefore, these hosts could not be used to provide a clear classification in Japan. We have tried to clarify the genetic diversity in pathogenicity of *P. brassicae* in Japan using Japanese clubroot-resistant (CR) F_1 hybrid (F_1) cultivars and lines of *Brassica rapa*. The responses of some CR F_1 cultivars were very clear. Four groups of field populations in Japan were recognized using the CR F_1 cultivars. The clear response obtained here may depend largely on the genetic purity of the F_1 cultivars. Moreover, it is possible to classify some of these Japanese populations into the same race using the Williams set and ECD 01 to ECD 05. The present differential hosts may be useful in the study of European populations of *P. brassicae*. The response of the differential hosts suggests that there are several major CR genes in *B. rapa*. It is suggested that pyramiding CR genes would be useful in breeding CR cultivars that can overcome the breakdown of the present CR cultivars of Chinese cabbage.

Introduction

Clubroot disease, caused by *Plasmodiophora brassicae* Wor., is one of the most damaging diseases of cabbage (*Brassica oleracea* var. *capitata*), Chinese cabbage (*B. rapa* L. ssp. *pekinensis*) and other cruciferous crops throughout the world (Voorrips, 1995). Karling (1968) reported that resting spores of *P. brassicae* remained viable in the soil for at least 7 years. Agricultural practices, in particular the application of calcium, increase the pH of the soil and thus may reduce the damage, but their effects often are insufficient to keep the crop healthy (Voorrips, 1995). The control of the disease with agrochemicals is also often insufficient in the case of clubroot and other soil-born diseases.

The breeding of resistant cultivars is an effective approach to eliminate the use of fungicides and minimize crop losses. Since no highly resistant materials were found among cultivars of Chinese cabbage, clubroot-resistant (CR) lines of Chinese cabbage were bred by introducing a resistance gene from a CR European fodder turnip (Yoshikawa, 1981). Subsequently, more than 50 CR F_1 hybrid (F_1) cultivars of Chinese cabbage have been released in Japan. However, most have now become susceptible in some cultivation areas of Japan. For breeding of new CR lines, studies on the diversity in pathogenicity of this pathogen are indispensable. Using the differential series of Williams (1966) and the European Clubroot Differential (ECD) set (Buczacki et al., 1975), differences in pathogenicity of populations of *P. brassicae* have been studied extensively, mainly in Europe (Crute et al., 1980; Voorrips, 1995). However, the response of these differential hosts to the

populations in Japan was not clear in our preliminary experiment.

The aim of the present study was to assess the genetic diversity in pathogenicity of populations of *P. brassicae* in Japan using Japanese CR commercial cultivars and lines in *Brassica rapa*. The differential hosts of Williams (1966) and the ECD (Buczacki et al., 1975) were used for comparison.

Materials and methods

Plant materials

The following were used: two CR accessions of fodder turnip, Siloga and Gelria R, 5 CR F_1 cultivars of Chinese cabbage, CR Kanko (Nippon Norin Seed Co., Ltd., Tokyo), CR Kukai 65 (Takii Co., Ltd., Kyoto), CR Ryutoku (Watanabe Seed Co., Ltd., Kogota), CR Utage 70 (Nozaki Seed Co., Ltd., Nagoya) and CR W-1116 (Watanabe Seed Co., Ltd., Kogota), Chinese cabbage parental line No. 4 (CCPL No. 4), 5 ECD differential hosts, ECD 01 to ECD 05, and 4 Williams' differential hosts, Jersey Queen, Badger Shipper, Laurentian and Wilhelmsburger. The susceptible F_1 cultivar of Chinese cabbage, Muso (Takii Co., Ltd., Kyoto), was used as a negative control for resistance to clubroot. CR CCPL No. 4 was developed at NIVOT by introducing a resistance gene from the CR European fodder turnip Siloga. Club root resistant CCPL No. 1 has been developed at NIVOT by introducing a resistance gene from the CR European fodder turnip 77b, and was used in a preliminary test.

Test for clubroot-resistance

The test for resistance to clubroot was carried out as described by Yoshikawa (1981). Resting spores were isolated from the clubs and used for infection. The infected soil was adjusted to pH 5.5–6.0. The spore load was 5×10^6 per g of dry soil. Eight-centimeter pots were filled with non-infected soil. A block (3 cm depth × 6 cm length × 2 cm width) of the infected soil was inserted in the center of the pot. For each pot, ten seeds were sown on the infected soil and covered with non-infected soil in late May 1993, 1994 and 1995, excluding CCPL No. 4. CCPL No. 4 was inoculated in late May 1992, 1993 and 1994. Two pots of each genotype were used in each test. After 6 weeks, the symptoms of each plant were rated on a scale of 0 to 3, where 0 = no clubs, 1 = a few small clubs, 2 = moderate clubbing and 3 = severe clubbing (Figure 1). The disease index (ID) in a test was calculated from the results as the mean value for 10 to 20 seedlings, and the ID for a line was expressed as the mean from 3 tests. The differences between ID of each accession and ID of accession with minimum or maximum ID were tested by the Wilcoxon method (1945), which is a non-parametric significance

Figure 1. Result of seedling test. 0 = no clubs, 1 = a few small clubs, 2 = moderate clubbing, 3 = severe clubbing.

test between two populations. The accession, which was not significantly different from accession with min. ID, was determined as resistant. The accession showing no significant difference from accession with max. ID was determined as susceptible. The accession, which was significantly different from both of accessions with min. ID and max. ID, was treated as partially resistance. The disease incidence (IC) was also calculated for each line as follow: (Number of diseased plants)/(number of inoculated plants) $\times$ 100.

Populations of P. brassicae

Severely infected roots of CR Chinese cabbage cultivars were collected from 36 cultivated areas from Hokkaido Prefecture to Yamaguchi Prefecture in Japan in 1990 to 1993. In a preliminary test, these 36 population were used for inoculation of a cultivar Muso and a line CCPL No. 1. Although the latter had been bred as a resistant line, this line is already severely infected in many cultivation areas in Japan. Twelve populations showing ID values from 2.5 to 3.0 in both hosts were thought to be agriculturally important and selected as aggressive pathogens. These 12 lines were further used for inoculation of 5 to 10 CR F_1 cultivars of Chinese cabbage (Kuginuki et al., 1994). Most of the pathogens showed intermediate scores, while three populations, Date-01, Rokunohe-01 and Yuki-01, showed clear differences in their pathogenicity (Figure 2). Therefore, these three populations were finally selected as representatives of pathogenicity. Another population, Ano-01, collected from a root of the susceptible cultivar Muso at NIVOT in 1989, was also used for comparison. Ano-01 derived from Hiratsuka-01, was collected in Kanagawa Prefecture in 1977 and has been used in our breeding program since 1977. The population Date-01 was collected from severely infected roots of CR Kukai 65 in Hokkaido Prefecture, Rokunohe-01 from CR CCPL No. 1 in Aomori Prefecture and Yuki-01 from CR Ryutoku in Ibaraki Prefecture. These four severely infected roots of Chinese cabbage were propagated in one cycle using the susceptible cultivar Muso. Clubs were stored at $-20\,^{\circ}$C until use.

Results

In three inoculation experiments, the resistant cultivars Siloga and Gelria R showed nearly complete resistance. Their IC were 0 to 20% (data not shown) and their ID values were 0.0 to 0.5 with standard deviations (SD) of 0.0 to 0.4, respectively (Figure 2). The IC and ID of the susceptible cultivar Muso and the European differential ECD 05 were 100% and nearly 3.0 with SDs of 0.0 to 0.1, respectively. These results demonstrate the high degree of reproducibility of this scoring system. Some of the Williams or the ECD differential hosts showed intermediate scores for ID (Figure 2) and IC. For example, the ID of ECD 01 was 1.8 for the population Date-01 and the ID of Wilhelmsburger was 1.5 for the population Rokunohe-01 (Figure 2). The SD of the ID in some of these hosts was higher than that in the CR F_1 cultivars, except for CR W-1116 (Figure 2). The results indicated these differential hosts cannot classify the Japanese populations clearly.

The Japanese CR F_1 cultivars showed clear resistance profiles. These cultivars can be classified into three groups. (1) CR Ryutoku showed resistance to Ano-01 (ID $=$ 0.2) and Rokunohe-01 populations (ID $=$ 0.3), but were susceptible to Date-01 and Yuki-01. CR Kukai 65, CR Kanko and CCPL No. 4 showed similar responses as CR Ryutoku, but CR Kukai 65 showed partial resistance (PR) to Rokunohe-01, CR Kanko showed PR to Date-01 and Yuki-01, and CCPL No. 4 showed PR to Rokunohe-01. (2) CR Utage 70 showed a different profile. It was resistant to Ano-01 and Yuki-01 populations and susceptible to Rokunohe-01 and Date-01. (3) CR W-1116 showed a distinct response to the pathogen. This cultivar showed partial resistance to all the populations tested. Moreover, the response seemed to fluctuate.

CCPL No. 4 was partially resistant to the population Rokunohe-01 and was susceptible to Date-01 and Yuki-01 (Figure 2). The cultivar had been developed at NIVOT by introducing a resistant gene from Siloga. However, Siloga itself was resistant to all pathogens here. The results suggest that CCPL No. 4 has only a part of CR genes in Siloga.

Discussion

Use of commercial F_1 hybrid cultivars for the inoculation test

The differential hosts of Williams (1966) and the ECD (Buczacki et al., 1975) have been used commonly to analyze populations of *P. brassicae*, mainly in Europe and North America (Williams, 1966; Buczacki et al., 1975; Crute et al., 1980; Voorrips, 1995). However,

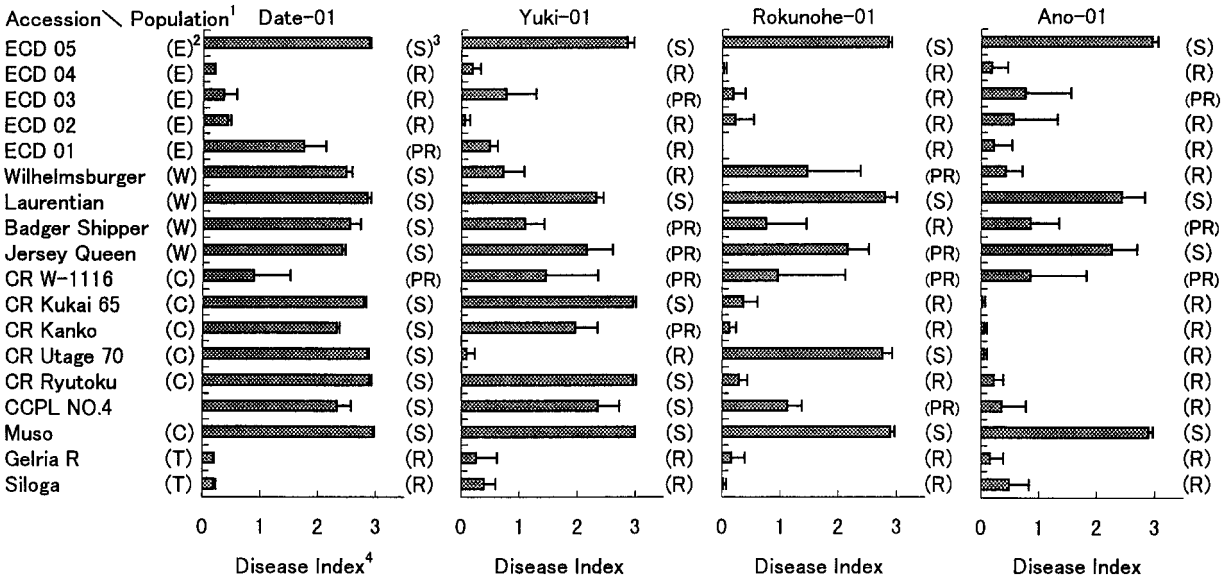

Figure 2. Responses of the accessions of 4 populations of *Plasmodiophora brassicae*. The inoculation tests for the cultivars, other than CCPL No. 4, were carried out simultaneously, and repeated three times. That for CCPL No. 4 was carried out twice at the same time and once independently. Tests were repeated three times. ⊣: Standard deviation. [1]Diseased roots were collected from four cultivated areas in Japan, Ano-01, Rokunohe-01, Yuki-01 and Date-01. [2](E): ECD's differential host, (W): Williams' differential host, (C): F_1 cultivars of Chinese cabbage, (T): European fodder turnip, CCPL No. 4: Chinese cabbage parental line No. 4. [3]When an accession was scored 0.00 to 0.77 for ID, it was treated as resistant, partially resistant for ID = 0.78–2.22 and susceptible for ID = 2.23–3.00, according to $LSD_{0.05} = 0.77$. [4]Disease Index: 0 = no clubs, 1 = a few small clubs, 2 = moderate clubbing and 3 = severe clubbing.

our results show that some Japanese populations cannot be classified clearly by these hosts. Genetic heterogeneity of the populations of *P. brassicae* may cause such unclear results (Jones et al., 1982). However, our inoculations were repeated three times using different samples of resting spores from the same stock of clubs, and obtained reproducible results in some F_1 cultivars. Taking account of the above findings, the observed fluctuating results with Williams and ECD differential hosts were not believed derive from the pathogen itself. The genetic heterogeneity of the differential hosts may cause the fluctuation. These hosts are obtained and maintained by mass selection because of self-incompatibility, and were thought to have some heterogeneity for CR genes. Therefore, we tried to use commercial CR F_1 cultivars of Chinese cabbage for differential lines in the inoculation tests. The response of the CR F_1 cultivars to different populations could be classified clearly in comparison with those of the differential hosts of Williams and the ECD. The clear response obtained here may largely depend on the genetic purity of the F_1 cultivars. The results show the advantage of using CR F_1 cultivars to classify the pathogenicity in *B. rapa*. However, genetic backgrounds or parentage of the CR F_1 cultivars are not disclosed. Therefore, they can not be maintained except by the plant breeders who developed them. Moreover the parentage is sometimes changed without any notice by the seed companies. We already have some doubled haploid (DH) lines with CR genes through microspore culture (Kuginuki et al., 1997). Genetically pure differential lines can be bred through microspore culture of the CR cultivars, and will be helpful to establish a more reliable screening system for the populations of *P. brassicae*.

Populations of Plasmodiophora brassicae

As mentioned above, Williams and the ECD hosts showed intermediate and fluctuating scores. Since these differential systems did not refer to the intermediate scores (Williams, 1966; Buczacki et al., 1975), the present populations of clubroot pathogen could not be classified. However, if differential hosts showing ID from 0.0 to 1.9 and from 2.0 to 3.0 were regarded as resistant and susceptible, respectively,

Date-01 was classified into race 4 and Yuki-01, Rokunohe-01 and Ano-01 into race 3 according to Williams' hosts. All 4 populations used here were classified as race 16 using ECD 01 to ECD 05. Ano-01 had previously been classified as race 2 according to Williams' hosts (Yoshikawa et al., 1981; Kuginuki et al., 1994). This discrepancy came from the fluctuating ID in one of Williams' hosts, Badger Shipper. The results suggest that the populations studied here would not be classified by the hosts of Williams and the ECD.

After screening 36 populations of *P. brassicae* from Japan, 4 populations with clear differences in pathogenicity were identified. The pathogen was first described in 1892 in Japan (Naiki, 1987), and is thought to have been introduced from Europe. The present results show that genetic diversity in pathogenicity has already developed in Japan, as described mainly in Europe and north America (Williams, 1966; Buczacki et al., 1975; Crute et al., 1980). Among the populations tested here, the Date-01 population can infect all CR F_1 cultivars of Chinese cabbage, showing the highest pathogenicity. While Ano-01 did not show any virulence on CR cultivars of Chinese cabbage. Rokunohe-01 was virulent on CR Utage 70, but avirulent on CR Ryutoku. In contrast, Yuki-01 was avirulent on CR Utage 70, and virulent on CR Ryutoku. The results suggest that virulence of Rokunohe-01 and Yuki-01 populations was qualitatively differentiated.

The results may be explained by a gene-for-gene interaction, although the populations of the pathogen used here were not single-spore isolates and may be genetically heterogeneous (Jones et al., 1982; Crute and Pink, 1989; Some et al., 1996). Genetically uniform single-spore isolates (Buczacki, 1977; Haji Tinggal and Webster, 1981; Jones et al., 1982; Some et al.,1996) would be ideal to study host parasite interactions of this system.

CR genes in Brassica rapa

Yoshikawa (1981) reported that the CR of CCPL No. 4 was controlled mainly by a major gene and addtionally by some minor genes. CCPL No. 4 and CR Ryutoku have a similar resistance profile but CR Ryutoku was highly resistant to the Rokunohe-01. It is possible that CR Ryutoku has additional gene(s) with an effect on resistance to the Rokunohe-01 population. In contrast, the resistance profile of CR Utage 70 to various populations was distinct from CCPL No. 4 and CR Ryutoku. The results suggest that CR Utage 70 has at least one resistance gene not found in the others.

All Chinese cabbage accessions tested here, except for W-1116, showed clear susceptibility to the Date-01 population, while the fodder turnips Siloga and Gelria R were highly resistant to the four populations. All CR Chinese cabbage F_1 cultivars were developed by introducing CR genes from CR accessions of fodder turnip (Kuginuki et al., 1994). A possible explanation for the resistance in the turnips is an interaction of resistance genes in the CR commercial cultivars. The other possibility is that all resistance genes were not incorporated into the CR cultivars. These results suggest there are several CR gene in fodder turnips.

Breeding of CR cultivars in B. rapa

A number of CR cultivars of Chinese cabbage were bred using European fodder turnips as the source of resistance genes. However, most have now become susceptible in some cultivation areas of Japan. Field populations of *P. brassicae* exhibited clear diversity in pathogenicity in this study. This finding suggests that the erosion of resistance is probably due to selection for pathogenicity in populations of *P. brassicae*. It is noteworthy that CR European fodder turnips, including Siloga and Gerlia R, are still highly resistant to the pathogen isolated from a field where CR Chinese cabbage cultivars are infected. These results suggest that these fodder turnips are, therefore, still valuable sources of resistance genes. New CR cultivars of Chinese cabbage, which are more resistant than the present CR cultivars, should be developed by pyramiding resistance genes.

DNA markers are thought to be very useful in this field. Four reports have been published on the mapping of CR genes in *B. oleracea* (Figdore et al., 1993; Pink et al., 1994; Grandclement and Thomas, 1996; Voorrips et al., 1997) and Landry et al. (1992) reported the mapping of CR genes introduced to *B. oleracea* from *B. napus*. However, reports on the mapping of CR genes in *B. rapa* are rare. Kuginuki et al. (1997) recently found RAPD markers linked to a CR gene against the population Ano-01. Finding DNA markers linked to CR against other populations with different pathogenicity from Ano-01, would be a useful approach for finding other useful CR genes in CR turnips and for the pyramiding of these CR genes in cultivars of *Brassica* crops.

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