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BACTERIAL AND PHYTOPLASMA DISEASES

Molecular epidemiology of *Pseudomonas syringae* pv. *syringae* strains isolated from barley and wheat infected with bacterial black node

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Abstract A repetitive sequence-based (rep)-polymerase chain reaction (PCR) and inter-simple sequence repeat (ISSR)-PCR were used to molecular type *Pseudomonas syringae* pv. *syringae* (PSS) strains isolated from barley and wheat plants with bacterial black node symptoms grown in 22 different locations and six different seed-production districts in Japan. Eighteen genomic fingerprinting (GF) genotypes were obtained from the combined results of BOX-, REP-, and GTG$_5$-PCR, indicating that the PSS population in Japan has high genetic diversity. The result based on logistic regression indicated that the population of GF genotype A was significantly related to a seed-producing district and that the epidemic of PSS strains in fields originated mainly from seed infection. This study will be applicable to future studies of the molecular epidemiology of bacterial plant diseases that have multiple infection routes.

Keywords Bacterial black node of barley and wheat ·
Pseudomonas syringae pv. *syringae* · Molecular
epidemiology · rep-PCR · ISSR-PCR

Introduction

Bacterial black node, a disease caused by *Pseudomonas syringae* pv. *syringae* (PSS) van Hall 1902 [=*P. syringae*

pv. *japonica* (Mukoo 1955) Dye et al. 1980], is the most important bacterial disease of barley and wheat in Japan (Bull et al. 2010; Fukuda et al. 1990; Mukoo 1955). Bacterial black node has been reported only in Japan (Kawaguchi 2013; Mukoo 1955; Oba et al. 1990; Yamashiro et al. 2011), although the pathogen is known to cause basal kernel blight of barley throughout the world (Braun-Kiewnick et al. 2000). The disease appeared on a large scale in 1947 in different regions of the central area of Japan, causing increasing damage every year through 1955 (Mukoo 1955). In recent years, bacterial black node has also emerged in the western regions of Japan (Kawaguchi 2013; Yamashiro et al. 2011). Although there are reports that bacterial black node is transmitted via seed (Goto and Nakanishi 1951; Fukuda et al. 1990), the ecology and epidemiology of bacterial black node of barley and wheat in commercial fields are still unclear, and the disease is hard to control.

Inoue and Takikawa (2006) designed group-specific polymerase chain reaction (PCR) primers based on the DNA sequences of *hrpZ* for *P. syringae* pathovars and reported that the *hrpZ* sequences contained insertions, deletions, and base substitutions, with consequent changes in the amino acids. Kawaguchi (2013) previously reported the use of differences in *hrpZ* sequences determined by PCR-restriction fragment length polymorphism (RFLP) to investigate the molecular epidemiology of PSS strains isolated from diseased barley and wheat plants in Okayama Prefecture, Japan. Although many strains isolated from barley and wheat belonged to various PCR-RFLP groups of PSS strains, we did not find that specific PCR-RFLP groups were distributed in a specific area or were isolated from specific cultivars. On the other hand, repetitive sequence-based (rep)-PCR and inter-simple sequence repeat (ISSR)-PCR are fast and inexpensive genotyping techniques utilizing variations among repetitive sequences and microsatellites.

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For example, *Clavibacter michiganensis* subsp. *michiganensis* (the causal agent of tomato bacterial canker) and *Xanthomonas arboricola* pv. *pruni* (the causal agent of peach bacterial spot) collected in Japan were fingerprinted by rep-PCR and ISSR-PCR and the genetic diversity of each was clarified (Kawaguchi 2013; Kawaguchi and Tanina 2014).

Thus, the objective of this study was to classify the genetic diversity of 149 strains of PSS obtained from diseased barley and wheat plants by ISSR-PCR and rep-PCR and to clarify the molecular epidemiology of PSS strains collected in the western region of Japan.

Materials and methods

Bacterial strains

The PSS strains used in this study are listed in Table 1. We collected barley and wheat plants grown from seeds, produced from various places in Okayama and Hiroshima Prefectures in western Japan from 2008 to 2016. Barley and wheat stems with black node symptoms were randomly collected from the fields, and one strain was isolated from one symptomatic stem. The PSS strains were identified by *P. syringae* group III-specific PCR (forward primer 5′-AGC TGG CCG AGG AAC TGA TG-3′, reverse primer 5′-AAC TGG TCA AGA TCC TGA GC-3′) (Inoue and Takikawa 2006). All strains isolated from barley and wheat were tested for pathogenicity on each host plant (barley or wheat) with the needle-prick method previously reported (Kawaguchi 2013).

rep-PCR and ISSR-PCR

DNA fingerprint analysis of the collected PSS strains was carried out using ISSR-PCR or rep-PCR method. Based on the DNA fingerprints, phylogenetic relationship of the PSS strains was analyzed. ISSR-PCR was performed using primers $GTGC_4$, $GAGC_4$ and GTG_5 (5′-GTG GTG GTG GTG GTG GTG-3′) according to the procedure of Alves et al. (2004). Rep-PCR was performed using primer sets BOX, BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′); ERIC, ERIC1R (5′-ATG TAA GCT CCT GGG TAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′); REP, REP1R-I (5′- ICG ICG ICA TCI GGC-3′) and REP2-I (5′-ICG ICT TAT CIG GCC TAC-3′), according to procedures previously described (Hulton et al. 1991; Kawaguchi 2014a; Kawaguchi and Tanina 2014; Versalovic et al. 1994). Bacterial cells from colonies on PSA were suspended in 20 µL of sterilized Milli-Q water (sMW). The suspension was heated at 95 °C for 10 min, then cooled for 5 min on ice. It was then centrifuged at 11,183×*g* for 2 min, and

the resulting supernatant used as a template for PCR. All DNA samples were stored at −20 °C until use. PCR amplifications were conducted in a total volume of 25 µL of the following reaction mixture: 1 µL of DNA template, 2 µL (20 pmol/µL) of each primer set ($GAGC_4$, $GTGC_4$, GTG_5), (BOX/BOXA1R, ERIC1R/ERIC2), or (REP1R-I/REP2-I), 12.5 µL of 2×QIAGEN Multiplex PCR Master Mix (Qiagen, Hilden, Germany), and 9.5 µL of sMW. Cycling conditions were as follows: initial denaturation step at 95 °C for 14.5 min; 35 cycles of denaturation at 95 °C for 30 s; annealing at either 40 °C for 90 s for REP-PCR, or at 50 °C for 90 s with BOX-, $GAGC_4$-, GTG_5-, and ERIC-PCR, or at 56 °C for 90 s in $GTGC_4$-PCR; extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. The amplification reaction was performed in a Takara PCR thermal cycler MP (Takara Bio, Otsu, Japan). These PCR experiments were performed three times as an independent replication.

Amplified PCR products were separated using gel electrophoresis to detect DNA fragments. Each DNA fragment with a distinct electrophoretic mobility was assigned a position number and scored either 1 or 0 depending on the presence or absence of the fragment, respectively, at the position. In this study, the PCR data by ERIC, $GAGC_4$, and $GTGC_4$ primer sets were not used because the results were not reliable. The scores obtained from each PCR method were combined for analysis to determine the genomic fingerprint (GF) genotypes. A dendrogram was constructed from similarity coefficient data (BOX-, REP-, and GTG_5-PCR) by the unweighted pair group method with arithmetic average (UPGMA) clustering using MEGA6 software (http://www.megasoftware.net/; Tamura et al. 2013). UPGMA trees were constructed, and the strength of the internal branches from the resulting tree was tested by bootstrap analysis using 2000 replications.

PCR-RFLP

According to procedures previously described (Kawaguchi 2013; Inoue and Takikawa 2006), PCR-RFLP assay was performed using *P. syringae* group III specific primer sets described above and a restriction enzyme *Hha*I (New England BioLabs Japan, Tokyo, Japan). The PCR products (753 bp), including the partial sequence of the *hrp* genes, amplified by PCR with the group III primer sets using the cell lysate as a template (Kawaguchi 2013; Inoue and Takikawa 2006) were digested with *Hha*I according to the manufacturer's protocol. PSS strains investigated in this study by PCR-RFLP with *Hha*I separated into two different PCR-RFLP groups A and B (Kawaguchi 2013).

Table 1 Populations of *Pseudomonas syringae* pv. *syringae* strains tested and their genetic groups

Host	Cultivar	Genomic fingerprint (GF) genotype	Target of logistic regression	Seed-producing district		Location		Year	No. of strains
				Field[a]	Prefecture	Field[a]	City, P refecture		
Wheat	Fukuhonoka	A	T	a	Okayama	A	Tamano, Okayama	2008	2
Wheat	Fukuhonoka	A	T	a	Okayama	B	Okayama, Okayama	2008	2
Wheat	Fukuhonoka	A	T	a	Okayama	C	Soja, Okayama	2008	2
Wheat	Fukuhonoka	A	T	a	Okayama	D	Soja, Okayama	2008	2
Wheat	Fukuhonoka	A	T	a	Okayama	E	Soja, Okayama	2008	2
Wheat	Fukuhonoka	A	T	a	Okayama	F	Soja, Okayama	2008	3
Wheat	Fukuhonoka	A	T	a	Okayama	G	Tsuyama, Okayama	2008	2
Wheat	Fukuhonoka	A	T	a	Okayama	H	Akaiwa, Okayama	2010	2
Wheat	Fukuhonoka	A	T	a	Okayama	G	Tsuyama, Okayama	2010	2
Wheat	Fukuhonoka	A	T	a	Okayama	I	Tsuyama, Okayama	2010	2
Wheat	Fukuhonoka	A	T	a	Okayama	H	Akaiwa, Okayama	2011	5
Wheat	Fukuhonoka	A	T	b	Hyogo	G	Tsuyama, Okayama	2011	5
Wheat	Fukuhonoka	A	T	a	Okayama	K	Okayama, Okayama	2011	8
Wheat	Fukuhonoka	A	T	c	Hiroshima	L	Akaiwa, Okayama	2011	5
Wheat	Fukuharuka	A	–	c	Hiroshima	T	Fukuyama, Hiroshima	2008	2
Wheat	Fukuhonoka	B	T	a	Okayama	H	Akaiwa, Okayama	2011	2
Barley	Sainohoshi	B	–	e	Gunma	Q	Akaiwa, Okayama	2011	3
Barley	Sukai Golden	K	–	f	Tochigi	Q	Akaiwa, Okayama	2011	4
Barley	Sainohoshi	K	–	e	Gunma	Q	Akaiwa, Okayama	2011	2
Barley	Miharu Gold	L	–	–	Unknown	M	Shoo, Okayama	2010	6
Barley	Ichibanboshi	L	–	a	Okayama	V	Shoo, Okayama	2016	3
Barley	Miharu Gold	N	–	–	Unknown	M	Shoo, Okayama	2010	3
Wheat	Fukuhonoka	P	T	b	Hyogo	J	Kagamino, Okayama	2011	3
Wheat	Fukuhonoka	P	T	b	Hyogo	I	Tsuyama, Okayama	2011	2
Wheat	Fukuhonoka	Q	T	b	Hyogo	J	Kagamino, Okayama	2011	1
Wheat	Fukuhonoka	Q	T	b	Hyogo	I	Tsuyama, Okayama	2011	6
Wheat	Fukuhonoka	R	T	b	Hyogo	J	Kagamino, Okayama	2011	2
Barley	Sukai Golden	C	–	f	Tochigi	Q	Akaiwa, Okayama	2011	6
Wheat	Fukuharuka	D	–	c	Hiroshima	L	Akaiwa, Okayama	2011	5
Wheat	Fukuharuka	D	–	c	Hiroshima	T	Fukuyama, Hiroshima	2008	2
Barley	Ichibanboshi	D	–	c	Hiroshima	U	Fukuyama, Hiroshima	2016	3
Barley	Miharu Gold	E	–	–	Unknown	N	Okayama, Okayama	2011	3
Barley	Miharu Gold	E	–	–	Unknown	R	Shoo, Okayama	2011	2
Barley	Miharu Gold	E	–	–	Unknown	S	Shoo, Okayama	2011	4
Barley	Sainohoshi	E	–	e	Gunma	Q	Akaiwa, Okayama	2011	2
Barley	Sainohoshi	F	–	e	Gunma	Q	Akaiwa, Okayama	2011	2
Barley	Oumiyutaka	F	–	–	Unknown	O	Soja, Okayama	2011	3
Wheat	Fukuhonoka	F	T	b	Hyogo	J	Kagamino, Okayama	2011	1
Barley	Sukai Golden	G	–	f	Tochigi	Q	Akaiwa, Okayama	2011	3
Barley	Houshun	H	–	d	Fukuoka	Q	Akaiwa, Okayama	2011	6
Barley	Houshun	I	–	d	Fukuoka	Q	Akaiwa, Okayama	2011	2
Barley	Sukai Golden	J	–	a	Okayama	P	Akaiwa, Okayama	2011	13
Barley	Houshun	J	–	d	Fukuoka	Q	Akaiwa, Okayama	2011	5
Barley	Sukai Golden	M	–	f	Tochigi	Q	Akaiwa, Okayama	2011	1
Wheat	Fukuhonoka	O	T	a	Okayama	H	Akaiwa, Okayama	2011	3

T target, – nontarget

[a]A field is a farm unit

Logistic regression

All the PSS strains isolated from the wheat plant cv. Fuku-honoka were used in the molecular epidemiological study (Table 1). The PSS strains were considered to be adequate to perform the statistical analysis due to the large sample size (64 strains) and because they were isolated from one specific cultivar, various locations, and had a clear seed-production history. The PSS strains isolated from wheat plants cv. Fukuhonoka were binary-coded as either 1 (belonging to genetic fingerprinting (GF) genotype A) or 0 (belonging to GF genotypes other than A). The proportion of PSS strains belonging to GF genotype A was represented as the odds of an infection, defined as:

$$\text{Odds} = P/(1 - P),$$

where P is the proportion of rep-PCR genotype A strains isolated from wheat plants cv. Fukuhonoka. The years, locations, and seed-producing districts, when/where PSS strains were isolated, were also coded using a binary scale (1 or 0). The logistic regression model was defined as:

$$\ln\{P/(1 - P)\} = \alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n,$$

where α is the y-intercept and β_n is the coefficient associated with predictor variable x_n. According to procedures previously described (Kawaguchi 2014b), this analysis was performed using the EZR (Kanda 2013) graphical user interface for R software (R Foundation for Statistical Computing, version 2.14.0).

The stepwise selection of the explanatory variables was based on the value of Akaike's information criterion (AIC) instead of using P values; AIC can be used to select among models on the basis of an optimal combination of parsimony (limiting the model to the smallest number of parameters needed to explain the data) and goodness of fit (Akaike 1973). In this approach, lower values of AIC indicate a better model. AIC was defined as:

$$\text{AIC} = -2\ln L + 2k,$$

where L is the maximum likelihood and k is the number of parameters (Akaike 1973). The AIC stepwise procedure provided by R uses the EZR.

In addition, the relationship between GF genotypes and factors of year or field was calculated as an odds ratio (OR). An OR was defined as:

$$\text{OR} = [P_s/(1 - P_s)]/[P_o/(1 - P_o)],$$

where P_s is the proportion of GF genotype A strains in a specific year or field, and P_o is the proportion of GF genotype A strains in other years or fields (except the previous specific year/field). An OR is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared with the odds of the outcome occurring in the absence of that exposure. In the present study, a high OR indicates a high probability of appearance of GF genotype A strains in a specific year or field, and a low OR indicates a low probability of appearance of GF genotype A strains in that specific year or field.

Results

Phylogenetic analysis by rep-PCR and ISSR-PCR

All PSS strains were confirmed as pathogenic strains on barley or wheat in a pathogenicity test. Genomic fingerprints were generated from 149 PSS strains by rep-PCR and ISSR-PCR, obtaining four fingerprint patterns by using BOX-PCR (a–d), nine fingerprint patterns by REP-PCR (a–i), and five fingerprint patterns by GTG$_5$-PCR (a–e) (Fig. 1). Eighteen GF genotypes (A–R) were obtained from the combined results of BOX-, REP-, and GTG$_5$-PCR (Fig. 1; Table 1). The PCR-RFLP group was divided into several GF genotypes, and in brief, the 10 GF genotypes (C–J, M and O) and eight GF genotypes (A, B, K, L, N, P, Q, and R) belonged to PCR-RFLP groups A and B. According to the phylogenetic tree based on genomic fingerprints, GF genotypes A to Q were divided into groups (A–J, K–Q, and R) at an 80% similarity level, though bootstrap values were slightly low (Fig. 1).

Logistic regression

Wheat plants cv. Fukuhonoka with black node symptoms grew in various fields in Okayama and Hiroshima Prefectures, and the seeds were collected from field a in Okayama, field b in Hyogo, and field c in Hiroshima (Table 1). Sixty-four PSS strains isolated from wheat plants cv. Fukuhonoka belonged to seven GF genotypes (A, B, F, O, P, Q, and R), and 44 strains belonged to GF genotype A (Table 1). A logistic regression with a stepwise selection method based on AIC was conducted, and a seed-producing district as a variable significantly correlated with the objective variable ($P < 0.001$, AIC = 47.523, Table 2). A logistic regression based on P values was also conducted, and field b in a seed-producing district was only selected as a variable (Table 3). The odds ratio, which was obtained from the logistic regression used to predict the proportion of GF genotype A strains isolated from wheat plants cv. Fukuhonoka, was 0.043 (Table 3). This result indicated that there is a significantly low probability of appearance of GF genotype A strains in field b as a seed-producing district ($P = 7.0.\text{E}{-}06$, Table 3).
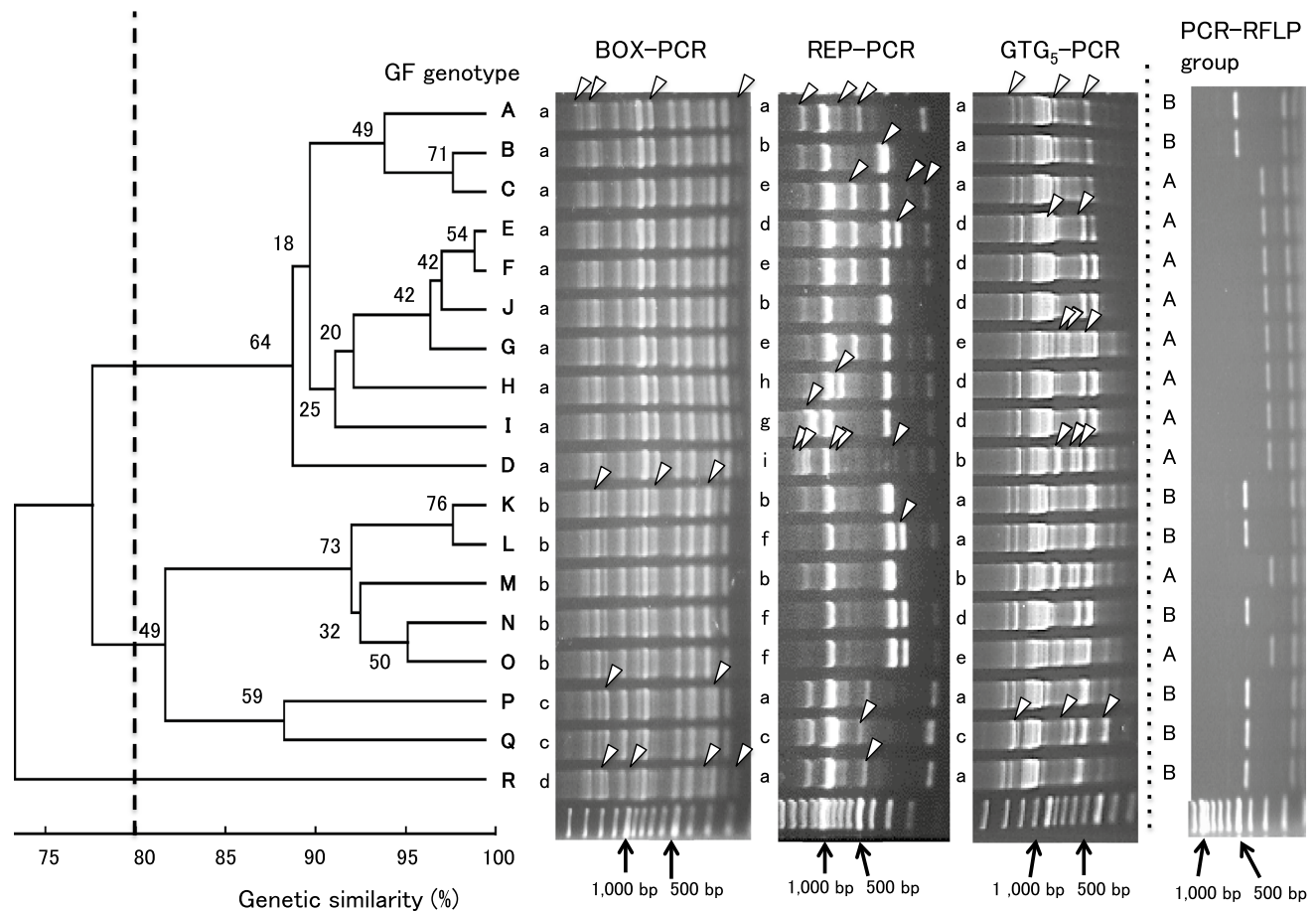
Fig. 1 Genetic diversity of *Pseudomonas syringae* pv. *syringae* (PSS) revealed by rep-PCR and ISSR-PCR analyses. *Values* on dendrogram branches represent the results of bootstrap analysis with 2000 iterations. *White arrowheads* indicate the difference among band patterns

Table 2 Parameter estimates[a] for the logistic regression model used to predict the proportion of genetic fingerprinting (GF) genotype A strains isolated from wheat plants cv. Fukuhonoka

Variable	Parameter estimate	Standard error	z value	P value[a]
Seed-produc-ing district[a]	–2.1	0.6	–3.394	<0.001
Location	–0.4	0.3	–0.007	0.219
Year	–17.0	2283.3	–1.227	0.994
y-Intercept	61.7	6850.1	0.009	0.993

AIC (Akaike's information criterion) = 47.523

[a] $P < 0.05$ was used for significant difference

Discussion

In this study, PSS strains were isolated from barley and wheat with bacterial black node symptoms that were grown in 22 different locations in Okayama and Hiroshima Prefectures and six different seed-production districts (Fukuoka, Gunma, Hyogo, Hiroshima, Okayama, and Tochigi Prefectures). Eighteen GF genotypes were obtained from the combined results of BOX-, REP-, and GTG$_5$-PCR, indicating that the PSS population in Japan is quite genetically diverse. In general, PSS has high genetic diversity as clarified by rep-PCR (Çepni and Gürel 2012; Kaluzna et al. 2010), and our result strongly supports previous reports.

The PCR-RFLP groups A and B, which were defined by our previous report (Kawaguchi 2013), were divided into 18 GF genotypes (A–R). However, the phylogenetic tree based on the GF genotype shows that PCR-RFLP groups A and B did not belong to independent clusters; that is, each cluster including GF genotypes A–J and K–Q represented a mixture of the PCR-RFLP groups A and B (Table 1; Fig. 1). However, many strains in PCR-RFLP groups A and B belonged to clusters that included GF genotypes A–J and K–Q, respectively (Table 1; Fig. 1). In the foreseeable future, we should clarify the relationship of location to the genomic sequence of pathogenicity-related genes and repetitive sequences and microsatellites in PSS using next-generation whole-genomic sequencing.

Table 3 Odds ratio obtained from the logistic regression used to predict the proportion of genetic fingerprinting (GF) genotype A strains isolated from wheat plants cv. Fukuhonoka

Variable	Odds ratio (OR)	95% Confidence interval		*P*-value
		Lower	Upper	
Field b in a seed-producing district	0.043	0.011	0.169	7.0.E−06
y-Intercept	7.800	3.070	19.800	2.0.E−05

Pathogenicity on host plants and elicitation of a hypersensitive response on nonhost plants are fundamental features controlled by *hrp* genes in plant pathogenic bacteria (Bonas 1994). Although the PCR-RFLP is based on one of the pathogenicity-related genes named *hrpZ*, all the strains used were pathogenic against barley and wheat, and the degree of virulence of the strains isolated from barley and strains from wheat did not differ in the needle-prick test on nodes of barley and wheat (Kawaguchi 2013). In this study, GF genotypes do not differ in the degree of virulence either. In the near future, we also should investigate the degree of virulence using another pathogenicity test and evaluate them.

In the results of the stepwise regression analysis focusing on the PSS strain isolated from cultivar Fukuhonoka, only the variable seed-producing district was selected as a significantly correlated parameter explaining the GF genotype A population (Table 2). This result indicates that GF genotype A population is significantly related to a seed-producing district. Also, there is a significantly low probability of appearance of GF genotype A strains in field b as a seed-producing district (Table 3). Although GF genotype A strains were isolated from wheat plants in field G from seeds that were produced in field a (Okayama) in 2008 and 2010, other strains of GF genotype A were isolated from wheat planted in field G (Tsuyama, Okayama) in 2011, and the seeds were produced in field b (Hyogo) (Table 1). These results indicated that the strains of GF genotype A in 2011 might remain in field G and inoculum that infected wheat plants was not from seeds but rather from soil or a secondary infection pathway (e.g., rain and wind). This result thus indicates that many PSS strains of GF genotype A that infected wheat plants should be from seeds produced in fields a and c. Moreover, the four GF genotypes (F, P, Q, and R) were isolated from wheat plants in two different fields I (Tsuyama, Okayama) and J (Kagamino, Okayama) in 2011, and seeds were produced in field b (Table 1). Because the first outbreak of bacterial black node occurred in two fields (I and J) in 2011 when the seeds were introduced from field b, the four GF genotypes (F, P, Q, and R) might originate from seeds.

On the other hand, PSS strains belonging to GF genotype K were isolated from two different barley plants cv. Sky Golden and Sainohoshi in the same field, Q (Akaiwa, Okayama) in 2011, and seeds were produced in two different fields e (Gunma) and f (Tochigi) (Table 1). This result indicates the possibility that the strains of GF genotype K might have remained in field Q and infected barley plants not from seeds but other unknown infection pathways in 2011. Thus, we could presume that bacterial black node might occur not by seed infection but by a secondary infection pathway according to circumstances. According to the result of the logistic regression with a stepwise selection method based on AIC, although there was no significant difference (*P* = 0.219), "location" was secondarily selected as a variable (Table 2).

In conclusion, rep-PCR and ISSR-PCR separated PSS strains isolated from barley and wheat plants diseased with bacterial black node into 18 different GF genotypes that showed genetic diversity of this pathogen. The result based on logistic regression indicated that epidemics of PSS strains in commercial fields originated mainly from seed infection. Thus, to manage bacterial black node disease, seed disinfestation is important. On the other hand, there were some cases in which PSS might have infected barley and wheat plants from a source other than seeds. We should investigate in detail how PSS infect barley and wheat plants in fields in the foreseeable future. Also, this study will be applicable to future studies of the molecular epidemiology of bacterial plant diseases that have various infection routes.

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