Characterization of a New Anastomosis Group (AG-W) of Binucleate *Rhizoctonia*, Causal Agent for Potato Stem Canker

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Abstract

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Two binucleate *Rhizoctonia* (BNR) isolates were recovered from potato cankered stems in Heilongjiang Province, northeastern China. Their cultural appearance on potato dextrose agar remained whitish as the cultures aged. White monilioid cells formed in the fluffy aerial hyphae, whereas no sclerotia appeared during the incubation. The two isolates could anastomose with each other, but they failed to anastomose with reference strains of BNR from AG-A to AG-Q, and AG-U. Analyses of restriction fragment length polymorphism (RFLP) of internal transcribed spacer of ribosomal DNA (rDNA-ITS) regions confirmed that these two isolates

differed from the reference strains. The phylogenetic tree based on the sequences of rDNA-ITS regions showed that they were located in a distinct clade from other BNR AGs. These collective results suggested that the isolates recovered from potato in this study belonged to a new BNR AG designated as AG-W. Pathogenicity tests under glasshouse conditions revealed that both isolates were able to cause brown, dry, and slightly sunken lesions on potato subterranean stems. To our knowledge, this is the first report of the AG-W causing potato disease in China as well as worldwide.

Binucleate *Rhizoctonia* (BNR; teleomorphs: *Ceratobasidium* spp.) are distributed worldwide in both agricultural and forest soils (González et al. 2006). On the basis of hyphal fusion, 22 anastomosis groups (AGs) have been described. Ogoshi et al. classified BNR isolates from Japan into 17 anastomosis groups (from AG-A to AG-Q) (Ogoshi 1985; Ogoshi et al. 1979). In North America, Burpee et al. (1980) assigned BNR isolates to seven *Ceratobasidium* anastomosis groups (from CAG-1 to CAG-7). Ogoshi et al. (1983) pointed out that CAG-1 isolates were able to anastomose with members of AG-D, while CAG-2 corresponded to AG-A, CAG-4 corresponded to AG-F, and CAG-3 and CAG-6 corresponded to AG-E. Sneh et al. (1991) designated CAG-5 and CAG-7 as new AGs of AG-R and AG-S, respectively. In 2005, Hyakumachi et al. reported two new binucleate anastomosis groups (AG-T and AG-U) isolated from cut-flower roses. Recently, BNR isolates collected from taro and ginger in Yunnan Province of China were assigned to a new anastomosis group, AG-V (Dong et al. 2013). Since AG-J and AG-N isolates have been excluded from *Rhizoctonia* spp. (Cubeta et al. 1991; Sharon et al. 2008), representative isolates of AG-M were lost (Sneh et al. 1991), and AG-T isolates were confirmed to belong to AG-A (Sharon et al. 2008); only 18 BNR AGs are currently known.

Based on other characteristics such as frequency of anastomosis, cultural morphology, pathogenicity, and sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA genes (rDNA), AG-B has been further divided into AG-Ba, AG-Bb, and AG-B(o) (Sneh et al. 1991), while AG-D was subdivided into AG-D I, AG-D II, and AG-D III (Hayakawa et al. 2006; Toda et al. 1999). It was suggested that subgroups might also exist in AG-F (Sharon et al. 2007) and AG-P (Sharon et al. 2008).

Although the classical hyphal fusion method is valid for the identification of *Rhizoctonia* spp. into anastomosis groups, it is insufficient because some isolates of certain AGs failed to anastomose with certain isolates of the same AG. For example, several isolates within AG-P had low fusion frequency (Sharon et al. 2008). On the other hand, some isolates belonging to different AGs were able

to anastomose with each other as Ogoshi reported that AG-I isolates could anastomose with some isolates of AG-C (Sneh et al. 1991). Compared with the conventional hyphal fusion method, restriction fragment length polymorphism (RFLP) analysis of the rDNA-ITS regions can provide quick and reliable results (Liu and Sinclair 1993), which has been used to differentiate subgroups within BNR AG-D (Toda et al. 1999) and *R. solani* AG-1 (Priyatomojo et al. 2001). Currently, DNA sequence analysis of rDNA-ITS region has been recognized as the most appropriate method for classification of *Rhizoctonia* spp. (Gonzalez et al. 2001; Sharon et al. 2006).

Many BNR isolates have been reported as economically important plant pathogens causing various diseases on a wide range of agricultural crops (Carling et al. 1999; González et al. 2006; Sneh et al. 1991; Wang and Wu 2012). On potato (*Solanum tuberosum* L.), BNR isolates of AG-A, AG-F, AG-K, and AG-U were reported to cause moderate stem canker symptoms (Miles et al. 2013; Yang and Wu 2013; Yang et al. 2014). In 2014, two binucleate-like *Rhizoctonia* isolates were recovered from cankered subterranean potato stems that were collected from two separate fields in Kedong county, the traditional potato growing region in Heilongjiang Province of China. These two isolates failed to anastomose with BNR tester strains representing AG-A to AG-Q and AG-U. The objective of this study was to characterize the two isolates by describing their (i) nuclear conditions; (ii) anastomosis reactions among reference strains of known BNR AGs; (iii) morphological characteristics including hyphal diameter, radial growth rate of colonies, and cultural appearance on potato dextrose agar (PDA); (iv) RFLP pattern and sequence data of the rDNA-ITS regions; and (v) virulence on potato plants.

Materials and Methods

Sample collection and fungal isolation. In 2014, subterranean potato (cv. Kexin 13) stems with brown and sunken lesions were collected from commercial fields in Kedong county (latitude 48°09' N; longitude 126°12' E) of Heilongjiang Province, northeastern China. Samples were gently washed under running tap water to remove adhering soil and debris, and then were cut into pieces (about 5 × 5 mm) from the margins of the healthy and diseased tissues. Surface disinfection was performed with 70% ethanol for 30 s, followed by soaking in 0.5% sodium hypochlorite for 3 min and rinsed three times with sterile distilled water. After drying on sterilized filter paper, the fragments were placed on 2% water agar (WA) containing 50 µg/ml streptomycin sulfate and incubated at 25°C for 24 to 72 h. Two fungal isolates with characteristic right angle branches, a septum near the

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branch, and a slight constriction at the branch base were preliminarily identified as *Rhizoctonia* spp. and were designated as HL-CZ and HL-ZA. The hyphal tips were transferred onto PDA plates for purification. Following sufficient growth, culture plates were kept at 4°C for short-term storage. For long-term preservation, these isolates were colonized on sterile barley grains and maintained at 4°C.

Nuclear condition. Isolates HL-CZ and HL-ZA were characterized as either binucleate or multinucleate by the method of Ahvenniemi et al. (2009). A 7-mm-diameter disk of PDA removed from the margin of an actively growing colony was placed on sterilized microscope slides overlaying 2% WA plate in 9-cm-diameter petri dishes and incubated at 20°C in the dark for 24 to 48 h. Developed mycelia were stained with 1 µg/ml 4'-6-diamidino-2-phenylindole (DAPI) for 10 min in the dark and the slides subsequently rinsed with sterile distilled water. Nuclei of at least 20 cells of more than one hypha were counted under a fluorescence microscope at 400× magnification in order to determine the nuclear status of each isolate. Simultaneously, diameters of mature hyphae were measured.

AG determination. The clean-slide method (Kronland and Stanghellini 1988) was used to determine AG identities of the isolates HL-CZ and HL-ZA by observing hyphal anastomosis. Reference strains FuK-600 (AG-A), C484 (AG-Ba), C-350 (AG-Bb), Ao-1-2 (AG-C), Ayu-WP-1 (AG-DI), TAK-14KT (AG-DII), KOU04-12FW (AG-DIII), RH-155 (AG-E), AH-6 (AG-Fb), AH-9 (AG-G), STC-11 (AG-H), AV-2 (AG-I), 55D45 (AG-K), FKO-2-16 (AG-L), FKO-6-2 (AG-O), C-578 (AG-P), C-620 (AG-Q), and MWR-20 (AG-U) were kindly offered by N. Kondo (Graduate School of Agriculture, Hokkaido University, Sapporo, Japan). Seven millimeter diameter disks of each tested isolate were placed on microscope slides with reference strains separated by 2 to 3 cm. When the hyphae from two sides overlapped, the fungal disks were gently removed from the slide and then the overlapping hyphae were stained with safranin O (Sigma) and 3% KOH according to the method reported by Bandoni (1979). The stained area was observed under bright-field optics at 40× magnification to search for potential fusion events, which were confirmed at higher magnification (100× and 400×). Anastomosis reactions were classified according to Carling (1996) as C0, no interaction; C1, hyphal contact without fusion; C2, cell wall fused while anastomosing and adjacent cells die; C3, fusion of cell wall and membrane, with no cell death.

Morphological characterization. The morphological characteristics (color, size, and shape of sclerotia and colony pigmentation) of the two isolates growing on PDA at 25°C in the dark were continuously examined and recorded every 3 days for at least 3 weeks. Three plates of each isolate were randomly arranged in an incubator. The experiment was repeated to ensure the reproducibility of results.

Radial growth rates. Growth rates of the two isolates were determined at 0, 5, 10, 15, 20, 25, 30, 35, and 40°C on PDA. Fungal disks (7 mm in diameter) from the margin of an actively growing colony were transferred to PDA in a 9-cm-diameter petri dish. Colony radius was measured at 24-h intervals until the colony reached the edge of the petri dish. Each treatment was replicated three times, and the experiment was repeated once.

Induction of teleomorphs. Attempts were made to induce perfect stage of the two isolates on 2% WA, 2% V8 juice agar (18 g of agar plus 20 ml of V8 juice per liter), and with the soil overlay technique (Ogoshi 1976). For each isolate, 10 petri dishes were prepared and incubated at 20 to 23°C for 3 weeks. Cultures were examined every second or third day for the presence of hymenia and basidiospores under the microscope.

DNA extraction. Isolates HL-CZ and HL-ZA and reference strains mentioned above were grown on a cellophane disc placed on PDA media. After 3 to 5 days, mycelia were collected, freeze-dried, and ground to fine powder in liquid nitrogen. The mycelial powder was suspended with 700 µl CTAB extraction buffer (100 mM TrisHCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 20 mM β-mercaptoethanol, and 2% w/v cetyltrimethylammonium bromide) in 2-ml microcentrifuge tubes and incubated at 65°C for 30 min (with intermittent mixing of tube contents). The solution was extracted with an equal volume of chloroform-isoamyl alcohol

(24:1, v/v) and centrifuged at 12,000 rpm for 10 min at room temperature. The resulting supernatant was incubated with 40 µg/ml of RNase A at 37°C for 30 min. The solution was re-extracted with an equal volume of chloroform-isoamyl alcohol (24:1, v/v) and centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with an equal volume of isopropanol and 1/10 volume of 3 M sodium acetate (pH 4.8) to precipitate genomic DNA. After being centrifuged at 12,000 rpm for 10 min at room temperature, the resulting pellet was washed twice with 70% ethanol. Then the pellet was dried under vacuum and dissolved in 50 µl ddH₂O. To ensure that each DNA sample was of adequate quality for PCR, UV spectrophotometry (Nanodrop 2000, Thermo, Wilmington, DE) was used to determine the concentration and purity of DNA.

PCR amplification and product purification. The rDNA-ITS regions were amplified from genomic DNA with universal primers ITS1 and ITS4 (White et al. 1990). A total volume of 50 µl reaction mixture contained 22 µl ddH₂O, 25 µl Premix Ex Taq (Version 2.0, TaKaRa, containing 0.625 U DNA polymerase, 200 µM dNTP, and 1.5 mM Mg²⁺), 1 µl each of primer (10 µM), and 1 µl DNA (100 µg/ml). Samples containing the same reagents but without DNA were used as negative controls. Amplification was performed in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany) with the following program: initial denaturation at 94°C for 3 min; followed by 30 cycles of denaturing at 94°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 1 min; one cycle of extension at 72°C for 10 min and final incubation at 4°C. After being examined on a 1% agarose gel, PCR products were washed with 50 µl of chloroform, precipitated with 5 µl of 3 M sodium acetate and 125 µl of 99.5% ethanol at -80°C for 30 min, and centrifuged. Pellets were washed in 70% ethanol, dried under vacuum, resuspended in 50 µl of Tris-EDTA (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0) buffer, and stored at 4°C until use.

RFLP of rDNA-ITS. The purified PCR product of each isolate was digested separately with restriction enzymes *EcoRI*, *HaeIII*, *HapII*, *HhaI*, *HinfI*, and *MboI* in accordance with the manufacturer's recommendations (TaKaRa Bio Inc., Dalian, China). The restriction fragments were separated by electrophoresis on 2% agarose gel and visualized under UV light after staining with ethidium bromide. A molecular marker (20- and 100-bp DNA ladder; TaKaRa) was used as a reference in each gel.

Sequencing and phylogenetic analysis of rDNA-ITS region. Purified PCR products from the isolates HL-CZ and HL-ZA were cloned into the vector pMD19-T (TaKaRa) according to the manufacturer's instructions. The cloning reaction mixture was transformed into competent cells of *Escherichia coli* MC1022 by heat shock at 42°C for 90 s, which were then cultured on Luria-Bertani broth media containing 50 µg/ml ampicillin. Colonies verified by PCR were sent to Beijing Sunbiotech Co., Ltd (Beijing, China) for sequencing. The resulting sequences of HL-ZA and HL-CZ were deposited in GenBank as accession numbers KP281807 and KP281808, respectively.

Phylogenetic analysis was performed on the basis of rDNA-ITS sequences of the two BNR isolates from this study and representative strains of known BNR AGs (Table 1) retrieved from GenBank (Copes et al. 2011; Gonzalez et al. 2001; Sharon et al. 2008). *Athelia rolfsii* isolate FSR-052 (GenBank Accession No. AY684917) was used as the outgroup (Fang et al. 2013; Sharon et al. 2007, 2008). All sequences were aligned using the Clustal W algorithm (Thompson et al. 1994) and obvious errors were adjusted manually. A phylogenetic tree was constructed using MEGA5 software (Tamura et al. 2011) with maximum likelihood (ML) method. Bootstrap support was estimated based on 1,000 pseudoreplicates and only bootstrap values ≥70% are shown in the phylogenetic tree. The percent sequence identity of the rDNA-ITS region was calculated by direct pairwise comparisons within BioEdit software (Hall 2011).

Pathogenicity tests. The pathogenicity of the two isolates was tested using a method modified from Lehtonen et al. (2008). Wheat seeds were moistened with sterile distilled water (60% v/w) in 250-ml flasks and sterilized by autoclaving twice at 121°C for 1 h, with a 24 h interval. The seeds were cooled at room temperature and spread on the 4-day-old actively growing mycelium of BNR isolates on PDA. The fungus was allowed to colonize the seeds for 3 days at room temperature in the dark.

Visually asymptomatic potato seed tubers (cv. Favorita) sprouted at room temperature in the dark until the sprouts were about 3 mm long. One sprouted tuber was placed in a sterile plastic pot (1 liter) filled to 3 cm depth of sand and sawdust mixture (1:2 v/v, dry heat sterilization at 161°C for 4 h before use). The amount of inoculum was standardized as one wheat seed for each plant. The wheat seed was placed 10 mm above the uppermost sprout tip and then the pot was filled with the sand-sawdust mixture mentioned above. Control plants were inoculated with uninfested autoclaved wheat seeds. The experiment

consisted of three replicates per isolate and 20 plants per replicate. The pots were arranged in randomized block design. After 3 weeks of incubation in a glasshouse maintained at 25 to 27°C with a 14 h/10 h light/dark cycle, all potato plants were harvested and assessed for disease incidence and disease index was calculated. Based on the relative size of necrotic area on subterranean stems, the disease rating scale was as follows: 0 = no disease, 1 = less than 10%, 2 = 10 to 50%, 3 = 50 to 100%, and 4 = plants dead. The disease incidence and the disease index were calculated as follows: disease incidence =

Table 1. Information of representative binucleate *Rhizoctonia* isolates for anastomosis testing and phylogenetic analysis

AG	Isolate	GenBank accession number	Host	Origin	Collector
A	C-538	AB196640	Potato	Japan	Oniki
A	AH-1	AB196639	Peanut	Japan	Ogoshi
A	FuK-600	AB196663	Rose	Japan	Hyakumachi
Ba	C-484	AB196641	Rice	Japan	Oniki
Ba	Scl-2	AB286930	Rice	Japan	Ogoshi
Bb	C-455	AF354087	Rice	Japan	Oniki
Bb	C-350	AB122144	Rice	Japan	Oniki
Bo	RU89-1	DQ102431	Soil	USA	Sneh
Bo	C-302	AB219143	Soil	Japan	Oniki
C	OR706	AJ242894	<i>Gymnadenia</i>	Japan	Tsutsui
C	55D25	AB290021	Sugar beet	Japan	Uchino
C	Ao-1-2	AB290020	Soil	Japan	Ogoshi
DI	YC-SDS-1	AB198699	<i>Zoysia</i>	Japan	Tanaka
DI	Ayu-WP-1	AB198703	<i>Agrostis</i>	Japan	Tanaka
DII	YG-EF-1	AB198704	<i>Zoysia</i>	Japan	Tanaka
DII	MW-EF-1	AB198708	<i>Zoysia</i>	Japan	Tanaka
DII	TAK-14KT	AB214367	<i>Zoysia</i>	Japan	Hayakawa
DIII	KAG-9R	AB198709	<i>Zoysia</i>	Japan	Hayakawa
DIII	KOU04-12FW	AB198713	<i>Zoysia</i>	Japan	Hayakawa
E	Oc-1	AB290019	<i>Oxalis</i>	Japan	Ogoshi
E	BN74	AF354083	<i>Erigeron</i>	USA	Burpee
E	RH-155	Unknown	Beet	Japan	Ogoshi
Fa	PS-17	AB219144	Pea	Japan	Ogoshi
Fa	Str10	DQ102434	Strawberry	Israel	Sneh
Fb	AH-6	AB196645	Peanut	Japan	Ogoshi
Fb	FKO2-28	AB219145	Soil	Japan	Ogoshi
G	Str15	DQ102396	Strawberry	Israel	Sneh
G	AH-9	AB196646	Peanut	Japan	Ogoshi
H	STC-9	AF354089	Soil	Japan	Ogoshi
H	STC-10	AB196648	Soil	Japan	Ogoshi
H	STC-11	AB196649	Soil	Japan	Ogoshi
I	FKO-6-7	AB290022	Soil	Japan	Ogoshi
I	FKO-1-17	AB196651	Soil	Japan	Ogoshi
I	AV-2	AB196650	<i>Artemisia</i>	Japan	Ogoshi
K	AC-1	AB122145	Onion	Japan	Ogoshi
K	SH-10	AB196652	Soil	Japan	Ogoshi
K	55D45	Unknown	Sugar beet	Japan	Ogoshi
L	FKO-2-26	AB196653	Soil	Japan	Ogoshi
L	FKO-2-11	AB286934	Soil	Japan	Ogoshi
L	FKO-2-16	AB286933	Soil	Japan	Ogoshi
O	FKO-6-2	AF354094	Soil	Japan	Ogoshi
O	FKO-2-10	AB286936	Soil	Japan	Ogoshi
P	C-584	AB286938	Soil	Japan	Oniki
P	Xb-3	AB286939	Ginger	China	Yang
P	C-578	AB196654	Tea	Japan	Takaya
Q	C-620	AF354095	<i>Cynodon</i>	Japan	Oniki
R	BN-37	AB219146	Cucumber	USA	Burpee
R	X4-3	AB286942	Ginger	China	Yang
S	Bn 22	AF354084	<i>Pittosporum</i>	USA	Burpee
S	RhJN2073WCz5	HQ269814	Azalea	USA	Copes
U	MWR-20	AB196664	Rose	Japan	Hyakumachi
U	MWR-22	AB196666	Rose	Japan	Hyakumachi
	KMJ-14-1	KM387384	Ginger	China	Yang
	KMYT-2-8-1	KM275622	Cocoyam	China	Yang
	LDDL02-1	KF907736	<i>Brassica</i>	Vietnam	Hua
	B13	JX135076	Poplar	France	Didier-Lacercat
	CMH555	KF800644	Indoor air	USA	Rittenour
	R107	JQ676926	Canola	Canada	Zhou

$100\% \times (n_1 + n_2 + n_3 + n_4) / N$ and disease index = $100 \times (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) / (4N)$, where n_0 to n_4 is the number of plants in each disease rating category and N is the total number of inoculated plants. Isolates were made from all visible lesions on subterranean potato stems and the resulting *Rhizoctonia* isolates were identified by morphological and molecular methods mentioned above to fulfill Koch's postulates. The experiment was repeated once.

Results

Fungal isolation and anastomosis grouping. Two *Rhizoctonia* isolates designated as HL-CZ and HL-ZA were recovered from diseased potato stems that were collected from two separate fields in Kedong county of Heilongjiang Province. Nuclear staining with DAPI revealed that they had two nuclei per cell. When paired with BNR reference strains, isolates HL-CZ and HL-ZA gave C1 reactions with AG-A and AG-K, while they gave C0 reactions with all other AGs. When the two isolates were paired with each other, a C2 reaction was observed, indicating that they belonged to the same AG.

Morphological characteristics. The mean widths of 20 mature hyphae of isolates HL-CZ and HL-ZA were $4.54 \pm 0.48 \mu\text{m}$ (ranged from 3.88 to 5.52 μm) and $4.79 \pm 0.49 \mu\text{m}$ (ranged from 4.08 to 5.63 μm), respectively. The mycelia of two isolates growing on PDA were floccose and remained white after growing for 3 weeks. Concentric rings were visible in cultures of isolate HL-CZ (Fig. 1A), whereas this zonation was not observed in isolate HL-ZA (Fig. 1B). Both isolates produced white moniloid cells in their fluffy aerial hyphae, but no sclerotia formed during the incubation of 21 days at 25°C. Despite repeated attempts, neither of the two isolates could be induced to produce hymenia or basidiospores on 2% WA, 2% V8 juice agar, or on soil overlay dishes. Both isolates were able to grow from 5 to 30°C on PDA media (Fig. 2). The optimum temperature for hyphal growth of HL-ZA and HL-CZ was 25°C with mean radial growth of $19.8 \pm 0.2 \text{ mm/day}$ and $17.2 \pm 0.5 \text{ mm/day}$, respectively. The isolates grew very slowly at 35°C and stopped growing at 0 or 40°C.

rDNA-ITS RFLP and sequence analysis. PCR amplification of the rDNA-ITS regions with primers ITS1 and ITS4 produced a single fragment for each of binucleate *Rhizoctonia* isolates in this study. The length of the PCR products of the two isolates was estimated at 670 bp. When digested with enzymes *EcoRI*, *HaeIII*, *HapII*, *HhaI*, *HinfI*, and *MboI*, respectively, isolates HL-CZ and HL-ZA shared the same unique banding patterns that clearly distinguished them from all reference strains. The PCR products were not cleaved by enzyme *HapII* except for those of AG-C, AG-DI, AG-DIII, AG-I, and AG-K. The *EcoRI* digested PCR products of all isolates into two fragments of different lengths, indicating one restriction site in each isolate. *HhaI* digestion also indicated that all AGs had one restriction site, except for AG-Bb and AG-Q. The enzyme *HhaI* digested isolates of AG-Bb and AG-Q into three different-sized fragments. *HaeIII*, *HinfI*, and *MboI* digestion showed a variety of polymorphisms among all AGs.

The PCR products of isolates HL-ZA and HL-CZ were successfully sequenced and the resulting sequences deposited in GenBank as accession numbers of KP281807 and KP281808, respectively. Cluster analysis based on rDNA-ITS sequences of the isolates from this study and of known AGs retrieved from GenBank revealed that

Fig. 1. Cultural morphology of the two binucleate *Rhizoctonia* isolates after 14 days of incubation on potato dextrose agar in the dark. A, HL-CZ. B, HL-ZA.

most isolates belonging to the same AG were clustered individually in the phylogenetic tree with support of high bootstrap value (Fig. 3). A few isolates belonging to different AGs, such as AG-P and AG-U, isolates HL-ZA and HL-CZ were grouped into a distinct clade with unknown AGs of *Rhizoctonia* isolates B13 (JX135076), R107

Fig. 2. Effect of temperature on radial growth rate of isolates HL-ZA and HL-CZ grown on potato dextrose agar in the dark. Bar shows a standard error.

Fig. 3. Phylogenetic tree generated from the comparison of rDNA-ITS sequences of isolates HL-ZA and HL-CZ from this study and those of binucleate *Rhizoctonia* isolates from GenBank. Bootstrap values (1,000 replicates) greater than 70 are shown above the branches. Scale bar represents a genetic distance of 0.05 for horizontal branch lengths.

(JQ676926), and CMH555 (KF800644), which were from France, Canada, and the United States, respectively. The percent identity of the rDNA-ITS region between HL-CZ and HL-ZA was 99.2%. When compared with each of the other AGs, sequence similarity levels ranged from 73.2 to 90.0% (Table 2). AG-V isolates KMYT-2-8-1 (KM275622) and DL-YT-06-4-9 (HM623624) clustered with isolate LDDL02-1 (KF907736) from Vietnam, which was described as AG-Fc in GenBank. The groups of AG-Fc and AG-V closely related to AG-Fb in the ML tree.

Pathogenicity determination. Pathogenicity tests were performed twice with similar results. Control plants remained asymptomatic (Fig. 4A) and no *Rhizoctonia* isolates were reisolated from them. On the other hand, potato plants inoculated with isolates HL-CZ and HL-ZA showed symptoms of brown, dry, and slightly sunken lesions (Fig. 4B and C), which were similar to those observed on diseased stems collected from the fields. The disease incidence caused by isolate HL-ZA was $28.70 \pm 4.24\%$, while that caused by isolate HL-CZ was $26.19 \pm 2.32\%$. The disease indexes caused by the two isolates were 7.59 ± 0.70 and 6.93 ± 1.04 , respectively. There was no significant difference in aggressiveness between the two isolates. BNR isolates were consistently reisolated from the artificially inoculated plants and their identities were confirmed by morphological and molecular characteristics as described above, fulfilling Koch's postulates.

Discussion

In this study, two BNR isolates were recovered from cankered subterranean potato stems that were collected from Heilongjiang Province, northeastern China. When paired with reference strains, they failed to anastomose with BNR isolates from AG-A to AG-Q and AG-U. However, the two isolates could anastomose with each other, indicating that they belonged to the same AG. RFLP analysis of the rDNA-ITS region revealed that they differed from these known BNR AGs. The RFLP banding patterns and fragment sizes of the reference strains in this research were similar with those reported by Toda et al. (1999) using the same enzymes. In the phylogenetic tree, the isolates HL-CZ and HL-ZA were clustered into a distinct group, which was closely related to AG-A and AG-K as they originated from one clade. All these results strongly indicated that the two isolates belonged to a new BNR AG. The sequence similarity of the rDNA-ITS region between the new AG and AG-R or AG-S was less than 80%. Since

isolates within the same AG or subgroup are likely to locate in a cluster in the phylogenetic tree and their rDNA-ITS regions are 95 to 100% similarity (Sharon et al. 2008), the isolates B13, R107, and CMH555 may also belong to this new BNR AG.

Twenty-two BNR AGs named with alphabetic characters (from A to V) have been reported (Dong et al. 2013; Hyakumachi et al. 2005; Ogoshi et al. 1983; Sneh et al. 1991). Among these AGs, AG-J, AG-M, AG-N, and AG-T have been confirmed to be invalid (Cubeta et al. 1991; Sharon et al. 2008; Sneh et al. 1991); AG-I and AG-C, AG-U and AG-P were suggested to combine into one AG, respectively (Sharon et al. 2008; Sneh et al. 1991); AG-V has not been officially published and the phylogenetic tree in this study showed that the represented isolates KMYT-2-8-1 (KM275622) and DL-YT-06-4-9 (HM623624) were closely related to AG-Fb. To avoid confusion,

Fig. 4. Symptoms of stem canker on potato subterranean stems caused by isolates HL-ZA and HL-CZ. **A**, Healthy control plants. **B and C**, Diseased stems induced by isolates HL-ZA and HL-CZ, respectively. Arrows indicate brown and sunken lesions.

Table 2. Matrix of percent sequence similarity of the internal transcribed spacer region between anastomosis groups and subgroups

	ZA	CZ	A	Ba	Bb	Bo	C	DI	DII	DIII	E	Fa	Fb	G	H	I	K	L	O	P	Q	R	S	U
CZ	99.2																							
A	88.7	88.8																						
Ba	78.4	78.5	79.7																					
Bb	75.2	75.3	74.9	84.2																				
Bo	79.0	79.1	80.2	92.6	84.0																			
C	78.8	79.1	80.0	81.9	77.0	83.1																		
DI	73.6	73.7	75.1	77.8	76.9	79.9	79.0																	
DII	74.6	74.6	75.6	77.2	76.0	79.9	78.6	85.6																
DIII	73.2	73.3	73.9	76.6	74.3	79.0	78.0	91.9	83.1															
E	79.1	79.2	79.2	77.0	74.1	77.4	76.7	73.7	73.1	72.6														
Fa	79.1	79.0	79.3	77.4	74.7	78.1	77.2	73.9	73.2	73.1	90.2													
Fb	78.8	78.9	77.9	77.0	75.2	77.8	76.6	74.5	73.1	73.7	87.7	88.2												
G	84.1	84.3	84.5	79.3	75.4	79.5	81.1	76.5	75.1	75.5	81.5	81.3	81.7											
H	78.6	78.7	80.4	81.0	76.4	82.3	92.5	78.7	77.5	77.3	76.0	77.0	76.6	79.7										
I	78.5	78.6	79.5	81.5	77.0	82.5	95.0	78.6	78.2	77.6	76.1	76.7	76.6	80.7	92.1									
K	89.9	90.0	92.2	79.0	75.7	79.0	78.2	75.0	75.7	73.8	79.6	78.7	77.2	82.8	78.1	78.1								
L	84.8	85.0	85.9	80.5	76.5	80.5	81.2	77.0	75.9	75.8	81.5	81.7	82.8	95.9	80.7	81.0	84.3							
O	84.7	84.9	85.6	79.5	75.9	80.0	81.2	77.0	75.1	75.8	82.1	82.0	82.8	95.9	80.1	80.5	83.8	97.4						
P	77.5	77.5	77.2	74.8	73.1	75.4	75.6	73.0	71.9	72.3	85.5	86.1	86.3	79.6	74.7	75.2	77.1	79.8	80.6					
Q	75.3	75.3	76.1	87.0	84.0	90.9	79.3	76.9	77.1	74.8	74.5	74.9	75.9	75.6	78.8	79.0	75.6	76.6	76.0	72.8				
R	75.8	75.7	73.9	74.0	73.2	74.0	74.0	71.9	70.7	71.4	83.2	83.7	84.4	77.2	72.9	73.5	74.5	78.4	78.3	87.3	73.6			
S	76.6	76.5	75.7	74.5	73.7	74.3	74.3	71.9	70.9	71.4	85.1	85.2	84.8	79.2	73.6	74.2	76.3	80.0	80.3	88.1	73.1	90.8		
U	77.5	77.4	77.4	74.7	73.2	75.2	75.2	72.7	71.4	72.1	85.3	86.1	86.0	79.5	74.3	74.7	76.9	79.6	80.5	97.5	72.8	88.0	87.9	
V	78.0	78.2	78.1	77.2	76.0	77.8	77.0	75.0	72.9	72.8	88.4	88.7	91.2	81.3	76.5	76.8	77.0	82.1	82.5	85.5	76.2	83.7	84.1	85.7

AG-W is being proposed for the AG of new BNR. Considering only the three letters X, Y, and Z are available in this naming system for potential new BNR AGs in the future, it is necessary to reorganize BNR AGs, although this work needs stronger international cooperation.

Environmental conditions may influence the prevalence of certain AGs (Virgen-Calleros et al. 2000). For example, as causal agents of potato stem canker, *R. solani* isolates of AG-4 and AG-5 are common in warm environments, whereas AG-3 prefers cooler and drier conditions (Anguiz and Martin 1989). Compared with BNR isolates of AG-A and AG-K recovered from cankered potato stems throughout China (Yang et al. 2014), AG-W isolates were only obtained from Kedong county in Heilongjiang Province, where the temperature ranged from 9 to 28°C during the potato growing season. Our research showed that the isolates HL-CA and HL-ZA grew very well from 10 to 30°C with optimum temperature at 25°C. The mycelia grew very slow when temperature was higher than 35°C and stopped growing at 40°C, indicating that AG-W isolates may prefer cool conditions.

Isolates assigned to different anastomosis groups may vary in host range (Sneh et al. 1991). It was reported that some AGs and subgroups of *R. solani* were shown to be host-specific. For example, AG-3 isolates are specialized to solanaceous crops (Banville et al. 1996; Bartz et al. 2010), and AG-8 predominantly infects cereals causing the “bare patch” disease (Ogoshi et al. 1990; Roget, 1995; Smiley and Uddin 1993). BNR AG-B isolates also showed host specificity as they infect species belonging to the family Gramineae (Sneh et al. 1991). Of the isolates clustering within one clade, isolates HL-CZ and HL-ZA were confirmed to cause brown, dry, and slightly sunken lesions on underground potato stems, which were similar to those induced by other BNR isolates (Yang et al. 2014). Isolate CMH555 was recovered from indoor air, and isolates B13 and R107 originated from poplar and canola, respectively, but their pathogenicity on these two hosts is unclear. Whether the isolates HL-CZ and HL-ZA (AG-W) are host species-specific or not warrants further research.

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