Features in Stem Blight Resistance Confirmed in Interspecific Hybrids of *Asparagus officinalis* L. and *Asparagus kiusianus* Makino

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We investigated the resistance to stem blight disease (*Phomopsis asparagi* (Sacc.)) in the progeny of two combinations of interspecific crosses between *Asparagus officinalis* (sensitive) and *Asparagus A. kiusianus* (resistant) in an effort to produce resistant cultivars. The progeny showed different degrees of disease severity, depending on the combination of crosses. Most of the hybrids derived from AO0060 (*A. officinalis*) × AK0501 (*A. kiusianus*) showed high disease resistance comparable to that of *A. kiusianus*. The results indicate that disease resistance could be introduced from *A. kiusianus* into *A. officinalis*, and that the selection of an appropriate cross combination is important for the production of disease-resistant cultivars. We analyzed the parents and hybrids of reciprocal crosses between *A. officinalis* and *A. kiusianus* using derived cleaved amplified polymorphic sequence markers to investigate the inheritance of the chloroplast genome, whose inheritance and genetic characteristics are not yet known. The chloroplast DNAs were inherited from the maternal parent, indicating that no major genes related to stem blight resistance were found in the chloroplast DNA.

Key Words: chloroplast DNA, dCAPS marker, maternal inheritance, *Phomopsis asparagi*, SNP.

Introduction

Stem blight in asparagus (*Asparagus officinalis* L.) caused by *Phomopsis asparagi* (Sacc.) is a disease that kills the host plants and substantially reduces crop production in many countries, including the USA, Australia, New Zealand, China, Greece, and Brazil (Davis, 2001; Elena, 2006; McKirdy et al., 2002; Reifsneider and Lopes, 1982; Udayanga et al., 2011). Primary infection occurs via soil. In plants infected by *P. asparagi*, small lesions or spots initially form on the stem surface a short distance above the ground. As the lesions expand, pycnidia usually appear in the central part of the lesion and become secondary sources of infection (Sakai et al., 1992; Sonoda et al., 1997). Infected stems eventually wilt, and the plant dies.

In Japan, asparagus is widely cultivated, and stem blight is a serious problem in almost all production areas, especially in open-field culture in warm regions. In these regions, asparagus is grown under shelter from the rain, and chemical control is used; these difficult and expensive measures are the main solutions to avoid this disease (Kobayashi and Shinsu, 1990). Breeding of resistant cultivars is, therefore, urgently required. Sonoda et al. (1997, 2001) reported that several hermaphroditic species in the genus *Asparagus* (which includes 100 to 300 species), such as *A. densiflorus*,
A. virgatus, A. asparagoides, and A. macowanii, have strong resistance to stem blight, although all commercial A. officinalis cultivars are sensitive.

Although interspecific hybrids offer one way to improve resistance of A. officinalis cultivars, crosses between resistant wild species and A. officinalis have not been obtained because of the distant relationship among members of this genus (Sonoda et al., 2001). Interspecific hybrids, produced by cell fusion technique using electro pulsation from protoplasts of A. officinalis and A. macowanii, did not grow into adult plants, since they might have an abnormal genome composition (Forsthoefel et al., 1992) and in A. kiusianus, some reports have been examined (Nakatate et al., 2016), but there have been no reports about polymorphic markers for chloroplast DNA (cpDNA). Although cpDNAs are generally inherited from the maternal parent in angiosperms (Corriveau and Coleman, 1988), some reports have documented a surprising amount of variation in inheritance patterns, suggesting that cpDNA could be maternally or paternally inherited, as in Medicago (Forsthoefel et al., 1992) and in Actinidia (Jung et al., 2003; Testolin and Cipriani, 1997), or paternally inherited in interspecific crosses as in the Passifloraceae (Hansen et al., 2007). cpDNA inheritance in interspecific hybridization between A. officinalis and A. schoberioides was shown to be maternal by using restriction-fragment-length polymorphism analysis (Ito et al., 2007), but the mechanism of inheritance between A. officinalis and A. kiusianus remains unknown. A second objective of this study was, therefore, to resolve the inheritance of cpDNA in hybrids between A. officinalis and A. kiusianus to provide insights into the possible involvement of cpDNA genes in stem blight resistance.

Materials and Methods

Inoculation test

Two interspecific crosses, AO0042 × AK0102 and AO0060 × AK0501, between A. officinalis ‘Mary Washington 500W’ (the AO accessions) as the female parent and A. kiusianus (the AK accessions) as the male parent, were performed at Tohoku University, Japan. The parents, three hybrids of AO0042 × AK0102, and eight hybrids of AO0060 × AK0501, were used for inoculation tests. Four A. officinalis accessions (AO-1, AO-2, AO-4, and AO-6) and three A. kiusianus accessions (AKN-2, AKN-4, and AKN-6) were also tested to compare the resistance among populations of A. officinalis, A. kiusianus, and hybrids.

The inoculation test was conducted at Kyushu University from October to November 2012. Phomopsis asparagi strain P1, obtained from the Saga Prefectural Agricultural Experiment Station (Saga, Japan), was cultured on potato sucrose agar medium at 25°C. After sporulation, we prepared a spore suspension adjusted to an inoculum density of 2 × 10⁶ spores·mL⁻¹. Inoculation of P. asparagi and evaluation of disease resistance to stem blight followed the methods described by Iwato et al. (2014). In summary, 4- to 6-year-old plants were propagated by division and planted in pots to allow control of temperature, light intensity, and humidity in a growth chamber. Shoots of these plants were cut back to ground level to promote new shoot emergence. Newly emerged shoots (one per individual), 2 or 3 weeks after cutting, were inoculated. The main stems were wrapped with cotton that had been soaked in the spore suspension at a height of 2 to 5 cm above the lowest branching node, and were then covered with vinyl tape to prevent desiccation. The plants were incubated at 25°C and 90% relative humidity under 40000 lx light for 3 days after inoculation, and they were then kept at 25°C and 60 to 70% relative humidity under 40000 lx in a growth chamber after removal of the cotton and vinyl tape. Disease severity was determined weekly for 5 weeks as a disease severity grade (DSG): 0 = no lesion; 1 = small lesion (<1 cm); 2 = spread lesion; 3 = large lesion (>half the plant) or defoliation; and 4 = death of the aerial parts.

Fig. 1. Asparagus kiusianus Makino in natural habitat.
Identification of cpDNA polymorphism

DNA was isolated from young cladophylls of *A. officinalis* ‘Welcome’ (WC-114L) and *A. kiusianus* (Nijinomatsubara line AKN-1m) planted in Fukuoka, Japan. It was extracted by the modified CTAB method (Stajner et al., 2002). The DNA concentration was adjusted to approx. 20 ng·μL⁻¹. Polymerase chain reaction (PCR) amplification of the *rbcL* region of the cpDNA was performed in a total volume of 50 μL containing 48 ng of template DNA, 0.5 μM forward and reverse primers, 5 μL of 10x Taq polymerase PCR buffer (Takara, Kusatsu, Japan), 0.2 mM of each dNTP (Takara), and 1.25 U Takara Ex Taq HS polymerase (Takara). The primer sequence designs were based on a previous report (Shinozaki et al., 1986): *rbcl_F*, 5'-TTGCGCATCTCCGAGTAA-3' and *rbcl_R*, 5'-TCT CCTAAAGTTCCTCCACC-3'.

Amplification was performed in a Takara PCR Thermal Cycler Dice TP-600 (Takara) with initial denaturation for 5 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 62°C, and 1.5 min at 72°C; and a final extension for 7 min at 72°C. *rbcL* fragments were subcloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into competent high *Escherichia coli* DH5α (Toyobo, Osaka, Japan). After culture on LB plates containing 100 μg·mL⁻¹ ampicillin, 100 μg·mL⁻¹ X-Gal, and 23.83 μg·mL⁻¹ isopropyl-β-d-thiogalactopyranoside, target clones were selected, and then plasmids containing the inserts were extracted using a LaboPass Plasmid Mini Purification Kit (Hokkaido System Science, Sapporo, Japan).

Sequences were analyzed with a BigDye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). *rbcL* sequences were aligned in v. 3.2 of DNASIS software (Hitachi Software Engineering, Tokyo, Japan) to detect single-nucleotide polymorphisms (SNPs). We examined whether the SNPs could be converted into CAPS markers. If not, then we designed dCAPS primers. PCR of two regions (*rbcl_A* and *rbcl_B*) that included a SNP was conducted with initial denaturation for 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 40°C (*rbcl_A*) or 42°C (*rbcl_B*), and 1.5 min at 72°C; and a final extension for 15 min at 72°C. We then digested the PCR products (10 μL) in 2.0 μL of CutSmart buffer (New England Biolabs, Ipswich, MA, USA) and 1.0 μL (5 U) of *Acl* restriction enzyme (New England Biolabs) (*rbcl_A*) or 0.5 μL (10 U) of *SacI* restriction enzyme (New England Biolabs) (*rbcl_B*) for 3 h at 37°C. The digested DNA was electrophoresed in 3.0% agarose gels, which were stained with Midori Green Advance DNA Stain Solution (Nippon Genetics, Tokyo, Japan), and visualized under LED 100 illumination (AMZ System Science, Osaka, Japan).

Inheritance of cpDNA

Reciprocal crosses between *A. officinalis* and *A. kiusianus* were made at Tohoku University and Kyushu University in Japan (Table 2). The parents and offspring were used for investigation of their cpDNA haplotypes. DNA extraction and confirmation of polymorphism were performed as described in the previous section.

Results and Discussion

Inoculation test

The aerial parts of *A. officinalis* died 3 or 4 weeks after inoculation, whereas *A. kiusianus* survived (Table 1). In *A. kiusianus*, the symptoms of stem blight did not spread, although symptoms appeared on the stems within 1 week after inoculation. These results agree closely with those of Iwato et al. (2014). The progeny of each cross combination showed different degrees of disease resistance even when *A. kiusianus* strains with similar degrees of the resistance were used as the pollen parents. The DSGs of all of progeny of AO0042 (*A. officinalis*) × AK0102 (*A. kiusianus*) resembled those of the female parent, since the aerial parts of two individuals were dead and one had a large lesion that covered more than half of the plant (Table 1). No hybrids showed strong disease tolerance in this cross combination. On the other hand, most of the eight hybrids of AO0060 (*A. officinalis*) × AK0501 (*A. kiusianus*) had high disease resistance similar to that of their pollen parent, *A. kiusianus* (Table 1). They formed only a small lesion by 5 weeks after inoculation. The hybrids obtained from the cross with AK0501 had stronger disease resistance than the hybrids from the cross with AK0102, indicating that AK0501 has greater potential as a pollen parent to produce disease-resistant cultivars. The difference in disease resistance between the two cross combinations might be caused by several factors, including the high heterozygosity of *A. kiusianus* and polygenic inheritance of the resistance trait. These results suggest that the strong disease resistance of *A. kiusianus* can be introduced into *A. officinalis* using resistant accessions of *A. kiusianus*, and that selection of parents whose progeny expressed strong resistance will be necessary for the breeding of disease-resistant cultivars.

Identification of cpDNA polymorphism

Sequence analysis revealed two SNPs, at 40 bp (T/C) and 136 bp (C/A), in WC-114L/AKn-1m (Fig. 2). As there were no restriction enzyme recognition sites at the SNPs, we tried to introduce sites by adding a 1-bp substitution (T→C) in the forward primer of *rbcl_A* and another (A→G) in that of *rbcl_B* for *Acl* and *SacI*-HF digestion, and successfully converted the SNP marker into a dCAPS marker (Fig. 2). The sizes of the PCR products were 280 bp in *rbcl_A* and 268 bp in *rbcl_B* in both WC-114L and AKN-1m (Fig. 3A). Amplicons...
of rbcL_A and rbcL_B could be digested by AciI and Sacl-HF, respectively, only in A. officinalis (WC-114L); that is, the 250-bp fragment digested from the 280-bp amplicon in rbcL_A and the 238-bp fragment digested from the 268-bp amplicon in rbcL_B were not digested with the restriction enzymes in A. kiusianus (AkN-1m). Thus, cpDNA polymorphism between A. officinalis (WC-114L) and A. kiusianus (AkN-1m) could be identified by using the dCAPS marker.

Inheritance of cpDNA

PCR products of 280 bp (rbcL_A) and 268 bp (rbcL_B) were identified in all of the parents and their progeny. Polymorphism between A. officinalis and A. kiusianus was confirmed in both rbcL_A and rbcL_B in all cross combinations by means of dCAPS analysis.

Table 1. Disease severity grade (DSG) in A. officinalis, A. kiusianus, and their interspecific hybrids inoculated with P. asparagi.

| Species/Hybrids (Parentage) | Accession | Sex | 1  | 2  | 3  | 4  | 5 |
|-----------------------------|-----------|-----|----|----|----|----|----|
| A. officinalis              | AO0042    | F   | 2  | 3  | 4  | 4  | 4 |
|                            | AO0060    | F   | 2  | 2  | 3  | 4  | 4 |
|                            | AO-1      |     | 2  | 3  | 3  | 4  | 4 |
|                            | AO-2      |     | 1  | 1  | 2  | 4  | 4 |
|                            | AO-4      |     | 2  | 3  | 4  | 4  | 4 |
|                            | AO-6      |     | 2  | 3  | 4  | 4  | 4 |
| **mean**                   |           |     | 1.8* | 2.5* | 3.3* | 4.0* | 4.0* |

| A. kiusianus               | AK0102    | M   | 1  | 1  | 1  | 1  | 1 |
|                            | AK0501    | M   | 1  | 1  | 1  | 1  | 1 |
|                            | AKN-2     | M   | 1  | 1  | 1  | 1  | 1 |
|                            | AKN-4     | M   | 1  | 1  | 1  | 1  | 1 |
|                            | AKN-6     | M   | 1  | 1  | 1  | 2  | 2 |
| **mean**                   |           |     | 1.0b | 1.0b | 1.0b | 1.2b | 1.2b |

| Hybrids (AO0042×AK0102)    | OK001     | F   | 2  | 4  | 4  | 4  | 4 |
|                            | OK002     | F   | 2  | 2  | 2  | 2  | 3 |
|                            | OK003     | M   | 2  | 2  | 3  | 4  | 4 |
| **mean**                   |           |     | 2.0* | 2.7* | 3.0* | 3.3* | 3.7* |

| Hybrids (AO0060×AK0501)    | OK007     | M   | 1  | 1  | 1  | 1  | 1 |
|                            | OK008     | F   | 0  | 0  | 0  | 0  | 1 |
|                            | OK009     | M   | 2  | 2  | 2  | 2  | 2 |
|                            | OK010     | M   | 0  | 1  | 1  | 1  | 1 |
|                            | OK011     | F   | 1  | 1  | 1  | 1  | 1 |
|                            | OK012     | M   | 1  | 1  | 1  | 2  | 2 |
|                            | OK014     | M   | 1  | 1  | 1  | 1  | 1 |
|                            | OK015     | F   | 1  | 1  | 1  | 1  | 1 |
| **mean**                   |           |     | 0.9b | 1.0b | 1.0b | 1.1b | 1.8b |

* F: Female plant; M: Male plant (Sexuality was investigated by the shape of the flowers.).
* Weeks after inoculation.
* Not investigated.

Different letters in a column indicate significant difference according to Tukey’s multiple range test ($P<0.05$).

Fig. 2. Chloroplast SNPs of rbcL region and their dCAPS conversion.
Table 2. dCAPS haplotypes of parents and offspring in four combinations of interspecific crosses.

| Interspecific hybrids | Parental haplotype | Number of offspring | Number of plants |
|-----------------------|--------------------|---------------------|-----------------|
|                       | Female (bp) | Male (bp) | rbcL_A | rbcL_B | Female (bp) | Male (bp) | rbcL_A | rbcL_B |
| A. officinalis (WC9) × A. kiusianus (AkN1) | 250 | 280 | 238 | 268 | 7 | 7 | 0 | 7 | 0 |
| A. officinalis (AO0060) × A. kiusianus (AK0501) | 250 | 280 | 238 | 268 | 6 | 6 | 0 | 6 | 0 |
| A. kiusianus (AkN5) × A. officinalis (WC5) | 280 | 250 | 268 | 238 | 5 | 0 | 5 | 0 | 5 |
| A. kiusianus (AkN7) × A. officinalis (WC93) | 280 | 250 | 268 | 238 | 5 | 0 | 5 | 0 | 5 |

(250 bp): (bp) 250 bp 280 bp 238 bp 268 bp

We found that stem blight resistance can be introduced from A. kiusianus into A. officinalis by interspecific crosses, and that the cpDNA of the hybrids of AO0060 (A. officinalis) × AK0501 (A. kiusianus), which had disease resistance similar to that of A. kiusianus, was the same as in AO0060 (A. officinalis). These results suggest that no major genes related to stem blight resistance were present in the cpDNA.

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