Quantification of *Alternaria brassicicola* infection in the *Arabidopsis thaliana* and *Brassica rapa* subsp. *pekinensis*

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Black spot caused by *Alternaria brassicicola* is an important fungal disease affecting cruciferous crops, including Korean cabbage (*Brassica rapa* subsp. *pekinensis*). The interaction between *Arabidopsis thaliana* and *Alt. brassicicola* is a representative model system, and objective estimation of disease progression is indispensable for accurate functional analyses. Five strains caused black spot symptom progression on Korean cabbage and *Ara. thaliana* ecotype Col-0. In particular, challenge with the strains Ab44877 and Ab44414 induced severe black spot progression on Korean cabbage. Ab44877 was also highly infective on Col-0; however, the virulence of Ab44414 and the remaining strains on Col-0 was lower. To unveil the relationship between mycelial growth in the infected tissues and symptom progression, we have established a reliable quantification method using real-time PCR that employs a primer pair and dual-labelled probe specific to a unigene encoding *A. brassicicola SCYTALONE DEHYDRATASE1* (*AbSCD1*), which is involved in fungal melanin biosynthesis. Plotting the crossing point values from the infected tissue DNA on a standard curve revealed active fungal ramification of Ab44877 in both host species. In contrast, the proliferation rate of Ab44414 in Korean cabbage was 3.8 times lower than that of Ab44877. Massive infective mycelial growth of Ab44877 was evident in Col-0; however, inoculation with Ab44414 triggered epiphytic growth rather than actual *in planta* ramification. Mycelial growth did not always coincide with symptom development. Our quantitative evaluation system is applicable and reliable for the objective estimation of black spot disease severity.

INTRODUCTION

*Alternaria brassicicola* is a necrotrophic fungal pathogen that causes black spot disease in many cruciferous vegetables, such as broccoli, cabbage, cauliflower, radish and turnip (Nowicki et al., 2012; Otani et al., 1995). A recent survey reported that vegetable crop losses caused by this fungus might be 20–80% worldwide (Nowicki et al., 2012). In addition to its economic importance, the interaction between this pathogen and several ecotypes or mutants of *Arabidopsis thaliana* has been used as a model system to investigate functions of fungal virulence factors and plant genes and signals modulating disease progression. Despite many accomplishments, no major gene for resistance has been identified for black spot disease.

Accurate and objective disease evaluation is a critical step in the evaluation of disease-resistant germplasm and effect of fungicides. Traditionally, black spot progression has been evaluated through scoring of visual symptoms. Although this method is the easiest to perform and remains useful for achieving some objectives, it does not produce quantitative values. Macroscopic symptom assessment is not applicable in chronological estimation of disease progression especially in the early period. More seriously, this technique does not measure fungal proliferation but rather identifies symptoms on the host. To avoid these problems, several alternative approaches have been designed, such as microscopic observation (Jarnagin & Harris, 1985), chemical detection and quantification of pathogen-specific constituents (Martin et al., 1990), and immunological detection (Harrison et al., 1990; Thornton et al., 2010). However, these technical approaches are usually laborious and not sensitive enough, and do not
provide objective and quantitative results. During the past three decades, a PCR-based strategy has been developed and used for evaluation of fungal biomass within plant tissue (Bretagne et al., 1995; Entz et al., 2005; Färber et al., 1997; Jurado et al., 2006; Nicholson et al., 1997). Although this technique has many advantages due to its broad spectrum, simplicity, sensitivity and time- and labour-efficiencies, it is basically qualitative and not quantitative because the amount of amplicon does not reflect the copy number of template DNA if the amount of each sample’s amplified DNA has reached saturation level prior to the checking point. More recently, crossing point (CP) value-dependent quantitative real-time PCR has been introduced to solve this problem (Lievens et al., 2006; Selma et al., 2008). As the CP value is able to correctly reflect the logarithm of the input copy number or the amount of template DNA, objective and quantitative measurement of fungal DNA within a certain amount of sample DNA can be performed by plotting the CP value of a sample on a standard curve. Recently, a dual-labelled probe was used to enhance the specificity of real-time PCR and is now widely accepted as the most reliable quantitative technique for the evaluation of fungal biomass within the host tissue (Kulik, 2011; Pavón et al., 2012; Su’udi et al., 2012; Zhao et al., 2012).

Initially, short sequences of rDNA or internal transcribed spacers (ITS) were the preferred target regions for primer design owing to limited fungal genome data (Borneman & Hartin, 2000; Gil-Serna et al., 2009; Martin & Rygiewicz, 2005). However, the copy number of both elements is not fixed (Sone et al., 2000). Moreover, designing a primer pair specific to the target organism is difficult because these sequences are highly conserved and ubiquitous across all filamentous fungi. Recently, the fungal genome project and accumulated fungal sequences in the public database have made an enormous amount of sequence information available.

The objective of this study was to reveal the relationship between symptom progression and *Alternaria brassicicola* proliferation in two pathosystems: *Ara. thaliana*, the representative model plant, and Korean cabbage (*Brassica rapa* subsp. *pekinesis*), one of the most important vegetables in Korea. We have developed a convenient and reliable protocol to produce objective and quantitative evaluation of *Alternaria brassicicola* within infected tissue DNA by employing a primer pair and a dual-labelled probe specific to the unigene *AbSCD1*, which encodes SCYTALONE DEHYDRATASE1. An orthologue of *AbSCD1* in *Chocliobolus miyabeanaeus*, *CmSCD1*, has been used for quantification of brown leaf spot disease in rice owing to its presence in the fungal genome as a single copy (Su’udi et al., 2012).

**METHODS**

**Fungal strains and pathogenicity assay.** The fungal isolates used in this study (Table 1) were obtained from the Korean Agricultural Culture Collection (KACC), Rural Development Administration (Korea). Fungal inocula were prepared as follows: *Alternaria brassicicola* and *Magnaporthe oryzae* were grown on oatmeal agar (50 g oatmeal flakes and 20 g agar per litre of distilled water); *C. miyabeanaeus* was grown on sucrose proline agar (SPA) (Ahn et al., 2005); and *Botrytis cinerea*, *Fusarium* and *Rhizoctonia solani* were cultivated on potato dextrose agar (PDA, Difco). All strains were grown at 22 °C under constant fluorescent light. Subsequently, conidial suspension or actively growing mycelial block was transferred into complete liquid medium and grown for 3 days at 25 °C, 150 r.p.m., in the dark. Prior to sampling, the mycelial mass was filtered with Miracloth (Calbiochem), subsequently frozen in liquid nitrogen and stored at −80 °C.

Korean cabbage (*Bra. rapa* subsp. *pekinesis*) cv. ‘Seoul’ and *Ara. thaliana* Col-0 ecotype were used for the inoculation assay. *Arabidopsis* were germinated in pots containing a soil–vermiculite (10:1) mixture and grown in a growth chamber at 22 °C, 70 % relative humidity, with a light intensity of 250 μmol photons m⁻² s⁻¹ and a 16 h photoperiod. Inoculation was performed by spraying conidial suspension of each *Alternaria brassicicola* strain at a concentration of 2 × 10⁶ conidia ml⁻¹ and 250 μg ml⁻¹ Tween 20 onto five Korean cabbages or ten *Arabidopsis* plants until all the leaves were covered with fine droplets. The infected plants were kept at 25 °C in a dark chamber at almost 100 % humidity for 20 h and then transferred into normal growth conditions (22 °C, 70 % relative humidity). The leaf samples for DNA preparation were collected at the indicated times. The images of symptom progression were taken at 4 days post-inoculation (days p.i.). The pathogenicity assay was performed at least three times and similar trends were obtained.

**Mycolological characteristics.** For morphological characterization, fungal conidia from each strain were retrieved from the medium with sterilized distilled water, mounted on glass slides and observed under a light microscope (Zeiss Axioplan 2). In each strain, the cell number was estimated in more than 1000 conidia.

For the observation of conidial chain formation, one of the important keys in *Alternaria brassicicola* diagnosis, the surface of the colony was lightly touched with transparent vinyl tape. Then, the tape was mounted on a glass slide with a drop of sterilized distilled water, and the slide was observed under a microscope. After taking pictures, the width and length of each conidiurn were measured using the Java image processing software ImageJ (http://rsweb.nih.gov/ij/download.html).

**DNA preparation and Southern blot analysis.** Genomic DNA from the fungal mass or infected plant materials was prepared using the hexadecyl trimethyl ammonium bromide (CTAB) method (Stewart & Via, 1993). DNA was precipitated using 2-propanol and the concentration was quantified using a NanoDrop (Thermo Scientific) and further confirmed by band brightness comparison with the HindIII-digested lambda DNA marker (Invitrogen) using the ImageJ software. The DNA concentrations from fungal and plant samples were adjusted to 50 ng μl⁻¹ and fungal DNA was serially diluted as indicated for standard curve construction by Taqman real-time PCR.

For Southern blot analysis, fungal genomic DNA (5 μg) from four *Alternaria brassicicola* strains (Ab44877, Ab44415, Ab43923 and Ab44414) was digested with EcoRI or Sphi, incubated at 37 °C for 4 h and loaded onto 0.7 % agarose gel. The gel was blotted onto a Hybond membrane (Amersham Pharmacia Biotech), and the membrane was further processed according to standard DNA blot procedure (Southern, 1975). A 268 bp fragment of *AbSCD1* was used to make a radiolabelled probe. The primers used for probe amplification were *AbSCD1-7F* (5’-GAG AAG AAC AAG TTA TAT TAC-3’) and *AbSCD1-275R* (5’-ATT GCC TCC CAG ATC TTG TC-3’) (Fig. 1). The probe was labelled with [³²P]dCTP by random priming (Feinberg & Vogelstein, 1983) using the Rediprime II DNA
### Table 1. Fungal strains used in the evaluation of primer pair specificity

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Strain/collection no.</th>
<th>PCR result*</th>
<th>Host†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria brassicicola</td>
<td>KACC42464</td>
<td>+</td>
<td>Armoracia rusticana</td>
</tr>
<tr>
<td>Alternaria brassicicola</td>
<td>KACC44877</td>
<td>+</td>
<td>Brassica rapa</td>
</tr>
<tr>
<td>Alternaria brassicicola</td>
<td>KACC44415</td>
<td>+</td>
<td>Raphanus sativus</td>
</tr>
<tr>
<td>Alternaria brassicicola</td>
<td>KACC43923</td>
<td>+</td>
<td>Armoracia rusticana</td>
</tr>
<tr>
<td>Alternaria brassicicola</td>
<td>KACC44414</td>
<td>+</td>
<td>Brassica oleracea</td>
</tr>
<tr>
<td>Magnaporthe oryzae</td>
<td>K197</td>
<td>–</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>Cochliobolus miyabeanus</td>
<td>Cm85</td>
<td>–</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>KACC40574</td>
<td>–</td>
<td>Cucumis sativus</td>
</tr>
<tr>
<td>Fusarium moniliforme</td>
<td>KACC41032</td>
<td>–</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
<td>KACC46434</td>
<td>–</td>
<td>Hordeum vulgare</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>KACC40101</td>
<td>–</td>
<td>Solanum tuberosum</td>
</tr>
</tbody>
</table>

*+, Strain detected; –, strain not detected.
†Original host harbouring each strain.

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**Fig. 1.** Genomic DNA sequence of AbSCD1 and Southern blot analysis. (a) Three exon regions are indicated with capital letters and the translated amino acid sequences are shown. Primer pairs (AbSCD1_123F and AbSCD1_219R) and probe sequence for Taqman real-time PCR are designated with dotted boxes and an arrow. The primer pair AbSCD1_7F and AbSCD1_275R is indicated with dashed boxes and the probe was amplified using this pair. The single SphI restriction site is indicated with a solid box. (b) Southern blot analysis of AbSCD1. After digestion of genomic DNA with EcoRI and SphI, the membrane was hybridized with the 269 bp amplicon as the probe. (c) Restriction map of the ATCC 96866 genomic DNA spanning 8,000 bp around AbSCD1 (arrow). Asterisks indicate the three SphI sites that might be responsible for the difference in Southern blot patterns between Ab43923 and Ab44877, Ab44415 and Ab44414. There were no EcoRI sites and only a single SphI site within the probe region of AbSCD1.
Plasmid construction and PCR amplification. A 97 bp fragment was amplified from *Alt. brassicicola* genomic DNA using *Pfu* polymerase (Nanohelix). The primers used in the PCR were AbSCD1_123F (5’-GCA GAC AGC TAC GAT AGC AA-3’) and AbSCD1_219R (5’-GAT GCA TTT GCG GAG AC-3’) (Fig. 1). The amplicon was purified, A-tailed and ligated into the pGEM-T Easy vector (Promega) at 4°C for 16 h. The ligation product was transformed into *Escherichia coli* strain DH5α, plated on Luria–Bertani (LB) agar medium supplemented with ampicillin (Amp) and incubated at 37°C for 16 h. Several colonies were picked and grown in LBamp broth for plasmid preparation. The resulting plasmid construct (pGEM-T Easy::AbSCD1) was confirmed by EcoRI digestion and DNA sequencing.

For the primer specificity test, PCR was performed with primer sets AbSCD1_123F and AbSCD1_219R using fungal genomic DNA (83.3 ng) as a template. PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were loaded onto a 2% (w/v) agarose gel containing ethidium bromide. The pictures were taken using a gel documentation system (Bio-Rad).

Cloning of the ITS regions. For the molecular identification of the fungal strain, the 582 bp ITS regions were amplified from the genomic DNAs of five *Alt. brassicicola* strains using the primers AbITS1 (5’-TCC GTA GGT GAA CCT GCG-3’) and AbITS4 (5’-TCCT TCC GCT TAT TGA TAT GC-3’). After cloning the product into the pGEM-T Easy vector, the nucleotide sequences of the cloned ITS regions were identified using M13F and M13R primers.

**Taqman real-time PCR.** The fluorescent dyes 6-carboxyfluorescein (FAM) and matching Black Hole quencher 1 (BHQ1) were added to the 5’ and 3’ ends to generate dual-labelled probe. The probe sequence for Taqman real-time PCR was 5’-tca cac atg gca gGA CTG GGA CC-3’. A 10-fold dilution series (1010 to 10-6 gene copies) of pGEM-T Easy::AbSCD1 plasmid was used as a template to generate a standard for gene copy number. Similarly, *Alt. brassicicola* genomic DNA was diluted threefold, ranging from 83.3 ng to 1 pg, to generate a standard for fungal DNA concentration. Real-time PCR was performed in triplicate on the LightCycler 480 II (Roche) with triplicates for 40 cycles (15 s at 95°C and 15 s at 60°C), starting with an initial incubation at 95°C for 4.5 min. The CP values obtained from this reaction were set automatically by the system. For the evaluation of pathogen ramification in planta, the leaves from five Korean cabbages or ten whole plants of infected *Arabidopsis* were combined and roughly ground. DNA preparation, quantification and Taqman real-time PCR were performed as described above. The amount of pathogen DNA (in nanograms) within 1 μg of plant DNA was determined by plotting CP values on a standard curve.

Fungal ramification in planta. To observe fungal growth within the plants, the leaves were recovered at 3 days p.i. The leaves were stained with 2.5 mg ml-1 aniline blue staining solution and resuspended in lactophenol (equal volumes of glycerol, lactic acid and phenol). After boiling gently for 1 min, the samples were further stained in the same solution for 1 day. Excess aniline blue was removed with several lactophenol washes. Then, the mycelial growth within the infected tissue was observed under a light microscope (Peng et al., 1986).

**RESULTS**

Validation of *AbSCD1* for evaluation of *Alt. brassicicola* proliferation

To establish a Taqman real-time PCR-based *Alt. brassicicola* quantification system, a primer pair and dual-labelled probe specific to the *AbSCD1* gene encoding SCYTALONE DEHYDRATASE1 were designed. In addition to its presence as single copy within the fungal genome, this gene might be involved in melanin biosynthesis. In fungi, melanin is indispensable for the survival and longevity of fungal hyphae and spores (Wheeler, 1983). Melanin is also important for appressorium formation and for prepenetration development. Initially, the genomic sequence of *AbSCD1* was retrieved from the *Alt. brassicicola* strain ATCC 96866 genome database (http://genome.jgi.doe.gov/Altbr1/Altbr1.home.html). This strain was isolated from cabbage (*Brassica oleracea*) seeds with *Alternaria* black spot symptoms. This gene spans three exons which are separated by two short intronic sequences (Fig. 1a). The open reading frame (ORF) consists of 558 nt encoding 185 amino acids. From the whole genomic sequence, a 97 bp region was selected for primer and probe design (Fig. 1a). Nucleotide sequencing also indicated that *AbSCD1* in our strains is identical with that in ATCC 96866 (data not shown).

One of the most important requirements of a gene as a detection target for Taqman real-time PCR is that the gene should exist in a fixed copy number in the genome (Salvioli et al., 2008; Baumgartner et al., 2010). A Southern blot was performed to determine the copy number of *AbSCD1* within the *Alt. brassicicola* genome. A 269 bp fragment of this gene was used as a probe (Fig. 1a, c). In the probe region and the whole genomic DNA sequence of *AbSCD1*, no EcoRI site and only a single *SphI* site were present. As shown in Fig. 1b, single- and double-bands were evident in the genomic DNA digested with EcoRI and *SphI*, respectively. These data indicated that *AbSCD1* exists as a single copy within the *Alt. brassicicola* genome. Therefore, *AbSCD1* is an appropriate target in the establishment of a Taqman real-time PCR. *AbSCD1* was also present as a single copy in *Ab43923*, and its Southern blot pattern was different from those of other strains (Fig. 1b). The 2675 697 bp contig 2 of ATCC 96866 contains *AbSCD1* (Fig. 1c).

Specificity and sensitivity are important for most quantification strategies (Francis et al., 2006; Pfaffl, 2004). In our work, the specificity of the primer pair was tested by comparing the amplification patterns of five *Alt. brassicicola* and several other fungal species (Table 1). PCR was performed using the same primer set used for cloning as described in the Methods section. Fig. 2 shows that the primers used in the PCR specifically and successfully amplified the 97 bp *AbSCD1* fragments. The primer pair did not react with DNA of other fungal species. Owing to its specificity, the primer pair can be used in the evaluation of *Alt. brassicicola* in biological materials.
relationships among CP value, copy number, and amount of genomic DNA

With respect to sensitivity, Taqman probe real-time PCR promises more accurate assessment than real-time PCR using SYBR Green (Gunel et al., 2011; Matsenko et al., 2008; Yin et al., 2001). We prepared two standard curves dealing with the relationships among CP value, copy number, and amount of fungal genomic DNA (Fig. 3). Taqman probe and primers were designed as shown in Fig. 1(a). The standard curve for the relationship between CP values and gene copy number was generated by using pGEM-T Easy : : AbSCD1 plasmid DNA as the template in a Taqman probe real-time PCR. The templates were serially diluted 10-fold from 1010 to 10−3. The slope obtained from this standard curve was −3.5072, with a high linear correlation (R2=1) (Fig. 3a). The standard curve for the relationship between CP values and the amount of fungal DNA was generated using a similar strategy; known concentrations of fungal genomic DNA were serially diluted threefold from 83.3 ng to 1 pg. The linear correlation between DNA concentrations and CP values was high (R2 >0.998), with a regression slope of −3.0491 and −3.1494 for Ab44877 and Ab44415, respectively (Fig. 3b). Hence, we estimated the genome copy number and fungal DNA concentration by plotting the CP values from the infected samples on the above linear regressions.

Mycological and molecular identification of Alt. brassicicola

The colony morphology on oatmeal agar was typical of growth of Alt. brassicicola. Approximately 5 to 6 days p.i., the colony colour changed to dark black, and active sporulation was evident. To confirm the species diagnosis, we analysed other mycological characteristics and molecular evidence, such as the shape/size of the conidia, the formation of conidial chains during sporulation, and the nucleotide sequences of the ITS regions (Kirk et al., 2008).
Pathogenicity assay of *Alt. brassicicola* in the two pathosystems and microscopic observation

Among the six strains of *Alt. brassicicola* tested, Ab44877 and Ab44414 showed the highest virulence on *Bra. rapa* subsp. *pekinensis* and the remaining three strains also induced some degree of disease symptoms on the same host species (Fig. 5a). No visible alteration was evident on the plants at 1 day p.i. At 2 days p.i., almost all the infected leaves showed abundant small, water-soaked spots, and some part of the leaves had turned yellow. The spots developed into big lesions at 3 days p.i. At 4 days p.i., almost all the infected leaves had turned yellow, and some leaves were severely withered. All tested strains induced severe chlorosis and withering on the outer leaves; however, Ab44877 and Ab44414 provoked severe yellowing and black spot symptoms on the inner leaves. In the other host, *Ara. thaliana* ecotype Col-0, strain Ab44877 was also highly virulent and the remaining strains also induced disease symptoms (Fig. 4a). Massive water-soaked lesions were evident on the infected leaves at 1 day p.i., and the yellowing and withering occurred at 4 days p.i. In contrast with the results from *Bra. rapa* subsp. *pekinensis*, Ab44414 did not cause severe symptoms; the symptoms were almost identical to those induced by Ab43923 and Ab44415.

To observe the pathogen’s mycelial growth, we harvested infected *Arabidopsis* leaves at 4 days p.i. and analysed them under a microscope after aniline blue staining (Fig. 4a). Active conidial germination, appressorium formation, and massive invasive/mycelial growth were evident on the *Arabidopsis* leaves inoculated with strain Ab44877; however, invasive mycelial growth within the leaves challenged with Ab43923, Ab44415 and Ab44414 was relatively restricted. In addition, some degrees of epiphytic growth were observed on the *Arabidopsis* leaves infected with *Alt. brassicicola*.

**Quantification of *Alt. brassicicola* in *Arabidopsis* and Korean cabbage**

Two host species, *Ara. thaliana* Col-0 and Korean cabbage, *Bra. brassica rapa* subsp. *pekinensis*, were spray-inoculated
with conidial suspensions from the five strains of *Alt. brassicicola* and the pathogens’ growth within infected tissues harvested at 4 and 3 days p.i. was evaluated by Taqman real-time PCR. By plotting the CP values on the standard curve, the amount of fungal DNA and the number of genomes within 1 µg of infected tissue DNA were calculated (Figs 4b and 5b). As expected, the amount of Ab44877 DNA within and on the Arabidopsis tissues was 40,950 pg or 3256,033 genomes per microgram of infected tissue DNA. In contrast, the amount of Ab44414 DNA was only 5870 pg or 658,516 genomes (Fig. 4b). The amount of *Alt. brassicicola* within the infected Korean cabbage tissues was estimated chronologically (Fig. 5b). At 1 day p.i., there were no distinct differences among the strains and the mean amount of fungal DNA within 1 µg of infected Korean cabbage DNA was 236 pg. Ab44415, Ab4393 and Ab42464 DNA did not increase noticeably by the next day; however, Ab44877 and Ab44414 fungal DNA increased and reached 2376 and 1241 pg by 2 days p.i. The amounts of DNA of the remaining three strains were less than 1 ng at 3 days p.i., but those of Ab44877 and Ab44414 sharply increased to 15 ng and 4 ng, respectively.

**DISCUSSION**

Real-time PCR-based strategy is a powerful tool for molecular detection and quantification. This method is widely used not only in human disease-related clinical detection (Rodu *et al.*, 1991; Taylor *et al.*, 2010), but also in the evaluation of microbial population (Fierer *et al.*, 2005; Kolb *et al.*, 2003; Mühleng, *et al.*, 2008). PCR-based detection and quantification are also convenient, fast and reliable methods for many other biological applications (Mandal *et al.*, 2011; van Brouwershav et al., 2010).

Previously, the quantification of *Alt. brassicicola* was performed using SYBR Green I in combination with 5.8S rDNA-specific primers (Brouwer *et al.*, 2003). Subsequently, another SYBR Green fluorescence-dependent evaluation was presented (Gachon & Saindrenan, 2004). The significant development in the latter experiment was the introduction of a primer pair specific to CUTINASE A, a gene that exists within the *Alt. brassicicola* genome in a fixed copy number. Although we have attempted the same experiment several times employing the same primer pair, the standard curves obtained were not reliable and the $R^2$ values were less than 0.96 (data not shown). In this investigation, we present relationships among the CP value, copy number, and amount of fungal genomic DNA with high reliability (Fig. 3b). To further confirm the reliability of our evaluation system, we constructed two standard curves using two series of CP values from two serial dilutions of genomic DNA from two different strains, Ab44877 and Ab44415. Southern blot analysis revealed that AbSCD1 exists as a fixed, single copy within their genome. If all of the experimental conditions, such as template DNA quantification, are ideal, then the above two standard curves should be identical regardless of the strain differences. As expected, the two standard curves almost completely merged with a high degree of reliability. These results indicate that our DNA evaluation is preferable and reliable for fungal quantification of *Alt. brassicicola* biomass. Our method was able to meet all of the requirements with respect to specificity (Francis *et al.*, 2006; Pfaffl, 2004) and sensitivity (Guner *et al.*, 2011; Matsenko *et al.*, 2008; Yin *et al.*, 2001). In addition, this method is relatively free from rDNA contamination during the PCR because the target of this system is fungal AbSCD1, which exists as a single copy within the fungal...
genome. Fixed gene copy number is one of the most important requirements for the proportional relationship between the amount of fungal DNA and fungal biomass within infected tissue (Baumgartner et al., 2010; Salvioli et al., 2008). Therefore, this method should be preferred over previously reported methods (Brouwer et al., 2003; Gachon & Saindrenan, 2004).

Based on the above system, we have evaluated the in planta growth of each strain in the two different pathosystems Ara. thaliana ecotype Col-0 and Korean cabbage, Bra. rapa subsp. pekinesis. Strain Ab44877 caused the most severe black spot symptoms. The quantification of growth of Ab44877 based on CP values and the microscopic observation of the infected tissues indicated the active proliferation of this strain in both host species. Although Ab44414 also induced intensive symptoms on Korean cabbage, the development of symptoms on Arabidopsis by this strain was low and similar to those on Arabidopsis caused by Ab43923 and Ab44415. A comparison of CP values revealed 3.8-fold lower mycelial growth of Ab44414 than of Ab44877 in Korean cabbage. Similar to symptom development, strains Ab44414, Ab43923 and Ab44415 did not show significant differences with respect to fungal growth within and on the Arabidopsis plants. Uncoupling strong symptom development from relatively lower in planta mycelial growth of Ab44414 on Korean cabbage indicated the additional contribution of other unknown virulence factor(s) such as enzymes and/or toxic secondary metabolites that specifically affect symptom progression by Ab44414 in Korean cabbage. A similar disagreement between symptom development and fungal growth within the host tissue has been described in the interactions between necrotrophic Septoria glycines and soybean and between necrotrophic Bot. cinerea and Arabidopsis (Hoffman et al., 1999; Thomma et al., 1999). Alt. brassicicola is also a representative necrotrophic pathogen and several studies have focused on the effects of molecules synthesized and secreted during prepenetration morphogenesis and symptom development (Fan & Köller, 1998; Wight et al., 2009). The results for symptom development and mycelial growth of the strains Ab44877 and Ab44414 indicate the importance of fungal strains in the evaluation of resistant germplasms or genetic resources. Furthermore, black spot evaluation methods should be chosen carefully and appropriately according to the aims of the investigation.

Although invasive mycelial growth of Ab44877 was superior to that of the other strains, this strain and the remaining three strains (Ab43923, Ab44414 and Ab44415) also grew epiphytically on Arabidopsis (Fig. 4a). Therefore, some portions of the evaluated fungal DNA originated from these mycelia. Owing to the lack of methods that are able to distinguish real invasive mycelial growth from epiphytic growth, results of in planta Alt. brassicicola growth should be analysed with consideration of epiphytic growth.

Investigation of the 8000 bp region containing AbSCD1 from Alt. brassicicola strain ATCC 96866 revealed the presence of six Sphi sites (Fig. 1c). One of the sites is located within the second exon of the AbSCD1 and the probe regions for Southern blot analysis in this research. The expected Southern blot result is identical to the pattern for Ab43923 which comprises a 3181 bp band containing the 3’ region of AbSCD1 and a 2501 bp band harbouring the 5’ region of AbSCD1 (Fig. 1b). Interestingly, Southern analyses of the remaining Alt. brassicicola strains revealed a shift of the smaller band from 2.5 to 4.3 kbp. These results strongly imply the absence of the three Sphi sites (indicated with asterisks in Fig. 1c) that are located upstream of AbSCD1 in the genomes of Ab44877, Ab44415 and Ab44414. Cloning via PCR and sequencing of these 600 bp regions of Ab44877, Ab44415 and Ab44414 further confirmed the absence of these three Sphi sites (data not shown). Nucleotide sequence analysis indicated that the EcoRI fragment of ATCC 96866 that contains AbSCD1 is 12119 bp long and this result coincided with the Southern blot results from the EcoRI-digested Ab43923. In contrast, other strains’ EcoRI fragments harbouring AbSCD1 are larger by as much as 1.8 kbp.

In sum, we have described a reliable method to evaluate Alt. brassicicola’s mycelial growth based on CP values inversely reflecting the amount of fungal DNA. Our method is dependent on a specific primer pair and an additional dual-labelled probe and guarantees the accurate and objective estimation of fungal biomass within infected tissues. Dependent on the results presented here and several observations not described here, we can make some suggestions for the establishment of a fungal mass estimation system by real-time PCR. Prior to all, the confirmation of the copy number and nucleotide sequence of a target gene is indispensable. After evaluation of the specificity of the primer pair, sequencing of the resulting amplicon and Southern analyses using the amplicon as the probe are recommended. Exact quantification of fungal template DNA and infected tissue DNA is a critical step for the reliable standard curve construction and estimation of fungal DNA among tissue DNA. Several trials based on absorbance at 260 nm did not yield consistent values, but band brightness-based measurements were reproducible. Although the aim of this investigation was to establish a fungal biomass quantification system, our data here also imply the value of symptom development results and the exact characterization of the tested fungal strains. These attributes are especially important in the design of experiments using necrotrophic pathogens and also in the analysis of the results. Finally, the CP values from the infected tissue DNA are composed of two kinds of DNA from actual invasive mycelial growth and epiphytic growth. This fact should not be ignored in the quantitative data analyses.

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