REAL-TIME PCR (qPCR) ASSAY FOR RHIZOCOTONIA SOLANI ANASTOMOSES GROUP AG2-2 IIIB

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Abstract

Rhizoctonia solani anastomosis group AG2-2 IIIB is a severe sugar beet and maize pathogen. It causes crown and root rot disease which leads to yield losses world-wide. The soil-borne pathogen is difficult to detect and quantify by conventional methods. We developed a real-time PCR (qPCR) assay for the quantification of genomic DNA of Rhizoctonia solani AG2-2 IIIB based on the ITS region of rDNA genes. The limit of quantification of the assay is 1.8 pg genomic DNA. The amplification efficiency was 96.4%. The assay will be helpful in the diagnoses of Rhizoctonia solani infection of sugar beet and maize roots and in the quantification of R. solani AG2-2 IIIB inoculum in plant debris and soil.

Introduction

Genus Rhizoctonia comprises a complex mixture of filamentous fungi with an imperfect state designated Rhizoctonia anamorph (Rhizoctonia solani Kühn). The anamorphic state does not produce conidia. The taxonomical classification of the Rhizoctonia anamorphs is based on morphological features, dividing the complex into anastomosis groups (AG’s) and subgroups (Sharom et al., 2006). Disease symptoms caused by R. solani on host plants are also used as diagnostic features (Gonzalez & Rubio, 2006). It is sometimes difficult to accurately assign an isolate to an AG group because certain isolates do not anastomose with known representative of AG group or lose the ability of anastomoses (Hyakumachi & Ui, 1987), while some isolates anastomose with more than one anastomosis group (Sharom et al., 2006).

Rhizoctonia solani is a soil-borne pathogen with a broad host range, causing diseases in a variety of crops, ornamentals and trees (Naz et al., 2008, Gonzalez & Rubio, 2006, Anderson 1982). In seedlings, R. solani causes damping off disease, producing black lesions in seeds and root of plant parts which were in contact with soil during plant growth. Germination of basidiospores on the leaves surface results in foliar lesions. Fourteen anastomoses (AG’s) and subgroups (Sharon et al., 2006). Rhizoctonia solani is an anastomosis group AG2-2 IIIB is a severe sugar beet and maize pathogen. It causes crown and root rot disease which leads to yield losses world-wide. The soil-borne pathogen is difficult to detect and quantify by conventional methods. We developed a real-time PCR (qPCR) assay for the quantification of genomic DNA of Rhizoctonia solani AG2-2 IIIB based on the ITS region of rDNA genes. The limit of quantification of the assay is 1.8 pg genomic DNA. The amplification efficiency was 96.4%. The assay will be helpful in the diagnoses of Rhizoctonia solani infection of sugar beet and maize roots and in the quantification of R. solani AG2-2 IIIB inoculum in plant debris and soil.

Material and Methods

Fungal isolates and DNA extraction: Rhizoctonia solani isolates used are listed in Table 1. Fungal DNA was extracted according to a CTAB protocol (Brandfass & Karlovsky, 2006).

Primer selection and specificity test: A number of forward and reverse primer combinations were tested. Forward primers selected from ITS1 18S region were Rhs1 (AACAAGGTTTCCGTAGGTG), AG2sp (ATTATGGAATTTAACAAG), and AG22sp2 (TAGCTGGATCCATTAGTTT) (Salazar et al., 2000). These primers were combined with the reverse primer 5.8SKhotR (GTTCAGAYTCGATGATTCAC) (Fredricks et al., 2010), amplifying a product of 32 2 to 330 bp. The qPCR system with SYBR Green detection was optimized for 25 µL reactions containing PCR buffer (Bioline, Germany), 3mM MgCl₂, 200µM dNTPs (Bioline, Germany), 0.3µM forward primer, 0.3µM reverse primer, 10mM fluorescence in, 0.1x of SYBR Green 1:1000 (Molecular Probes, USA), 0.25U of Taq DNA polymerase (Bioline, Germany) and 1µL of template DNA. This qPCR system was used with the following temperature profile: initial denaturation 94 °C/3 min was followed by 40 cycles of 94°C/30s, 50-60°C gradient/20 s and 72°C/30s and by a final extension
72°C/5 min.
Table 1. Fungal strains.

| Fungal species          | Isolate | Anast. group | Source | Fungal species       | Isolate | Source |
|-------------------------|---------|--------------|--------|----------------------|---------|--------|
| Rhizoctonia solani      | MP93    | AG2-2 IIIB   | Source | Fusarium equiseti    | FE 5     | Source |
| Rhizoctonia solani      | Tester  | AG1-IB       | 1      | Fusarium cerealis    | FCKW 3   | 5      |
| Rhizoctonia solani      | Tester  | AG2-1        | 1      | Fusarium cerealis    | FCKW 4   | 5      |
| Rhizoctonia solani      | Tester  | AG3-PT       | 1      | Fusarium sacchari    | FSAC 1   | 3      |
| Rhizoctonia solani      | Tester  | AG4-HGII     | 1      | Fusarium sacchari    | FSAC 2   | 3      |
| Rhizoctonia solani      | GC      | AG5          | 1      | Fusarium culmorum    | FC 15SS  | 5      |
| Rhizoctonia solani      | PL      | AG5          | 1      | Fusarium culmorum    | FC 22SS  | 5      |
| Rhizoctonia solani      | PL-1047 | AG5          | 1      | Fusarium culmorum    | FC 2     | 5      |
| Rhizoctonia solani      | GER     | AG5          | 1      | Fusarium culmorum    | FC H15   | 5      |
| Rhizoctonia solani      | GER 1042 | AG5        | 1      | Fusarium culmorum    | FC H69   | 5      |
| Rhizoctonia solani      | Tester  | AG8          | 1      | Fusarium culmorum    | FC H71   | 5      |
| Rhizoctonia solani      | 5P      | AG8          | 1      | Fusarium culmorum    | FC H73   | 5      |
| Rhizoctonia solani      | GB      | AG11         | 1      | Fusarium verticillioides | FV 3   | 3 |
| Rhizoctonia solani      | Tester  | AG11         | 1      | Fusarium verticillioides | FVER 420 | 5 |
| Rhizoctonia solani      | 1079    | AGD          | 1      | Fusarium verticillioides | FVER 429 | 5 |
| Rhizoctonia solani      | Tester  | AGE          | 1      | Fusarium proliferatum | FPROL 12 | 5 |
| Fusarium oxysporum      | FO 2    | 2            | 2      | Fusarium proliferatum | FPROL 1  | 3      |
| Fusarium oxysporum      | FO      | 5            | 5      | Fusarium proliferatum | FPROL 2  | 3      |
| Fusarium oxysporum      | FO 125  | 5            | 5      | Fusarium proliferatum | FPROL 4  | 3      |
| Fusarium oxysporum      | FO 436  | 5            | 5      | Fusarium proliferatum | FPROL 5  | 3      |
| Fusarium oxysporum      | FO 121SS | 5        | 5      | Fusarium proliferatum | FPROL 6  | 5      |
| Fusarium avenaceum      | FA 3    | 5            | 5      | Fusarium proliferatum | FPROL 7  | 5      |
| Fusarium avenaceum      | FA 9    | 5            | 5      | Fusarium proliferatum | FPROL 8  | 5      |
| Fusarium avenaceum      | FA 1.2  | 3            | 3      | Fusarium proliferatum | FPROL 9  | 5      |
| Fusarium avenaceum      | FA 5.2  | 5            | 5      | Fusarium proliferatum | FPROL 11 | 5 |
| Fusarium acuminatum     | FACU 1  | 4            | 4      | Fusarium graminearum | FGR 62048 | 3 |  |
| Fusarium acuminatum     | FACU 3  | 5            | 5      | Fusarium graminearum | FGR 62722 | 3 |
| Fusarium acuminatum     | FACU 5  | 5            | 5      | Fusarium graminearum | FGR 67638 | 3 |
| Fusarium equiseti       | FE 2    | 4            | 4      | Fusarium graminearum | FGR 83649 | 3 |

1- Strain collection of Division of general plant pathology and crop protection, Georg-August-University, Göttingen, Germany  
2- Mykothek FAP (Dr. M. Winter)  
3- Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany  
4- International Center for Agricultural Research in the Dry Areas, Aleppo, Syria  
5- Strain collection of the Molecular phytopathology and mycotoxin research division, Georg-August-University, Göttingen, Germany

Sequence analysis: PCR product obtained from R. solani AG2-2 IIIB (strain MP93) was directly sequenced by using primers used for the amplification (MWG Biotech, Munich, Germany).

Calibration and determination of PCR efficiency: To determine the sensitivity of the assay, R. solani AG2-2 IIIB (isolate MP93) genomic DNA was quantified by densitometry as described by Nutz et al., 2011 using ImageJ software (http://rsweb.nih.gov/ij/). Calibration curves were constructed by plotting threshold cycle value versus DNA concentration (3-fold dilution series from 444.4 pg to 1.8 pg). After the PCR, melting curves of the products were recorded using the same thermocycler.

Results

On the basis of preliminary experiments with primer combinations described in the Material and Methods section, forward primer AG22sp2 in combination with reverse primer 5.8SKhotR were selected. qPCR conditions for the primer pair were optimized, leading to the temperature profile specified in Material and Methods section.

The sequence of the forward primer AG22sp2 was compared with sequences in NCBI database using BLAST. All hits with 100% sequence identity originated from Rhizoctonia solani isolates belonging to anastomoses groups AG-2, AG2-2 IIIB and AG-2-2 IV. The sequence of the reverse primer was not tested because it was expected to match most fungal species. Amplicon generated with primer pair AG22sp2/5.8SKhotR from genomic DNA of R. solani isolate MR93, which belongs to anastomosis group AG2-2 IIIB, was sequenced (NCBI accession number JX914627). Comparison with the database the sequence was found identical with sequences from Rhizoctonia solani anastomos group AG2-2 IIIB isolates (Acc. Nos. GU811684.1, FJ492151.3, FJ492138.3, FJ492137.3, FJ492136.3, FJ492124.3, FJ492123.3, and FJ492089.3.). To extend the characterization of the specificity of primer AG22sp2 described by Salazar et al., 2000 who designed the primer (Salazar et al., 2000), genomic DNA of R. solani isolates from anastomosis groups AG1-IB, AG2-1, AG3-PT, AG4-HGII, AG5, AG8, AG11, AGD, AGE and DNA of common fungal pathogens of maize and sugar beet Fusarium oxysporum, F. avenaceum, F. acuminatum, F. equiseti, F. cerealis, F. sacchari, F. culmorum, F. verticillioides, F. proliferatum and F. graminearum (Table 1) were tested in the qPCR assay. The results were negative for
R. solani isolates AG1-IB, AG2-1, AG3-PT, AG8, AG11, AG5 (isolate GC), AG5 (isolate PL), AG5 (isolate PL-1047), AG5 (isolate Ger), AG5 (isolate Ger 1042), AG8 (isolate 5P), AG11 (isolate GB), and AGD (isolate 1079). The assay was also negative for DNAs extracted from fungal species other than R. solani except for Fusarium avenaceum FA5-2 and Fusarium graminearum FGR83649, which produced amplicons late in the PCR with threshold cycles larger than 32. DNA of the other isolates of F. avenaceum and F. graminearum (Table 1) tested negative.

Linear relationship between the logarithm of DNA concentrations and the threshold cycle was found in the range of 1.8 pg to 444.4 pg DNA with a coefficient of determination $R^2 = 0.99$. The amplification efficiency with pure template DNA was 96.4% (Fig. 1). Melting temperature of the products was 86°C (Fig. 2).

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Fig. 1. Standard curve for qPCR for genomic DNA of Rhizoctonia solani AG2-2 IIIB. DNA standard series from 444.4 pg to 1.8 pg was used; whiskers show standard deviation.

Fig. 2. Melting curves of amplification products of Rhizoctonia solani AG2-2 IIIB DNA at three concentrations. The melting temperature was 86°C.
Discussion

Detection of *Rhizoctonia solani* anastomoses groups AG2-2 IIIB, AG-2, and AG-2-2 IV by conventional PCR-RFLP has been reported using ITS1 and ITS4 primers (Hyakumachi et al., 1998). These primers produced an amplicon of 740bp, which is too large for qPCR. Detection of *Rhizoctonia solani* AG2-2 with conventional PCR was also reported by Matsumoto (Matsumoto, 2002) but the primers developed in this work amplified isolates of AG5 and AG7, too. The assay reported in this communication is thus the first qPCR assay for *R. solani* anastomosis group AG2-2 IIIB.

Conclusion

We envision that the assay will be useful in the diagnoses of *R. solani* infection of sugar beet and maize and in the quantification of the inoculum of the pathogen in plant residues and soil.

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