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Rapid Identification of Potato Scab Causing *Streptomyces* spp. Using Pathogenicity Specific Primers

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The potato scab is caused by several species of *Streptomyces*. Among these species, only pathogenic strains were found to produce thaxtomin A characterized by necrotic bioassay and HPLC. In this study, identification of the pathogenic strains of *Streptomyces* was performed through the polymerase chain reaction (PCR) by using specific pathogenicity primer sets derived from the *nec*1 gene sequences of *Streptomyces scabies*. The expected PCR products were obtained approximately 580 bp and confirmed by sequencing. This PCR technique can be used effectively to identify the pathogenic *Streptomyces* species, that cause scab on potato tubers.

Keywords **:** *nec*1 gene, pathogenicity, potato scab, *Streptomyces scabies*.

Potato scab is the most economically important tuber disease in potato-growing regions of the world. It reduces the marketability of table stock, processing, and seed potato tubers. Scab symptoms caused by *Streptomyces* species have diversified on tuber surface to have rough circular, raised, tan to brown, and corky lesions. Similar diseases on potato tuber due to *Rhizoctonia* and *Spongospor*a are not readily differentiated from the scab by the naked eye (King et al., 1989; Kuske et al., 1998; Loria et al., 1995; GreenShare, html).

Since several species of *Streptomyces* cause the same diseases on potato tuber, there must be a common mechanism regarding pathogenicity involved in the disease development. The mechanism is known to be caused by the production of phytotoxic secondary metabolite thaxtomin A (4-nitroindol-3-yl-containing 2,5-dioxopiperazine) by the pathogenic strains of *Streptomyces*. The metabolite has been purified from potato tuber tissues infected by *S. scabies* and oatmeal-based media (Babcock et al., 1993; King et al., 1991; Kutchma et al., 1998; Leiner et al., 1996).

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It has also been reported that thaxtomin A production is positively correlated with pathogenicity. Bukhalid and Loria (1997) cloned 2.4 Kb gene from *S. scabies*, of which necrogenic activity and the proximity to transposase gene had been horizontally transferred from another genus. Detection of necrogenic activity through a bioassay and determination of pathogenicity by specific primers encode *nec*1 gene from *Streptomyces* species were reported in this study.

Material and Methods

Bacterial strains and culture conditions. The pathogenic strains isolated from a common scab lesion on potato (*Solanum tuberosum* L. cv. Dejima) in Jeju area were identified as *Streptomyces scabies* and *S. acidoscabies* by characteristics of morphological features (Kim et al., 1999). *S. scabies* ATCC49173 and *S. acidoscabies* ATCC49003 were purchased from the American Type Culture Collection (ATCC). The strains were grown on both oatmeal (OMA) and malt extract agars (MEA).

Isolation and bioassay of thaxtomin. Isolation of thaxtomin A was described by King et al. (1989). *Streptomyces scabies* was incubated for 5 days at 28 on OMA and extracted twice by using equal volume of chloroform. Crude extracts were loaded into a pre-coated thin layer chromatography (TLC: silica-gel 60F254). A yellow band was scraped off and eluted by methanol. The yellow band was characterized by high performance liquid chromatography (HPLC), Hewlett Packard System with reverse C18 column. Thaxtomin A was eluted with a 50% methanol for over 20 minutes and monitored at 254 nm. A potato tuber slice assay was used to assess the necrosis inducing abilities of thaxtomin A that was isolated from *S. scabies*. Tuber slices of scab-susceptible cultivar Dejima were treated with the yellow band compound on a wet Whatman filter paper and incubated for 3-7 days. Necrosis and collapse of tuber cells were carefully observed.

Polymerase chain reaction (PCR). Genomic DNA was isolated from frozen *Streptomyces* mycelia according to a method described by Kutchma et al. (1998). Pathogenicity-specific primers were designed on the basis of published sequence data for the 1.5 Kb DNA (GenBank Accession No. AF073320, Table 1). For the PCR amplification, 20 μl of the mixture was reacted with

Table 1. List of primers used in this experiments

Names	Sequences (5'→3':bp)	Positions*
Nec15	cgaagatctgccggtggccgtt (22-mer)	725-746
Nec13	atccggagctcacccgagacaac (23-mer)	1724-1746
Nec35	cctgcagagaggacgtgtagtg (22-mer)	1045-1066
Nec33	ccagtcagctactttc6tcgtta (22-mer)	1605-1626

*Sequence data was enrolled Genbank Acession no. AF073320 (Bukhalid & Loria, 1997).

Accupower PCR Premix Kit (Bioneer Co.). Amplification of DNA was carried out in a Perkin Elmer Model Genamp PCR system 9700 Thermocycler (Norwalk, Conn.) as follows: 1 cycle at 95°C for 2 minutes, 35 cycles at 94°C for 1 minute, 52°C for 1 minute, and 1 cycle at 70°C for 10 minutes. Following amplification, 20 µl of the reaction mixture was added to 5 µl of the mixture containing 35 sucrose, 0.125 bromophenol blue, and xylene cyanol. The entire sample was subjected to electrophoresis with 2 SeaKem GTG agarose gel (FMC Bioproducts, Rockland). Electrophoresis buffer was composed of 40 mM Tris, 20 mM of sodium acetate, and 2 mM of EDTA at pH 8.0 containing 50 ng of ethidium bromide per ml. DNA was visualized by transillumination with UV light and photographed with a type 55 polaroid film (Polaroid Co.). PCR marker was used as the standard size. The nucleotide sequence of the amplified *nec*1 fragment was determined by using an ABI automatic DNA sequencer (Perkin Almer, USA). The PCR fragment was excised from the gel and purified with a QIAquick gel extraction kit (QIAGEN, Germany). The analysis of DNA sequence was performed by using the BLAST program through NCBI database.

Results and Discussion

Several species of *Streptomyces* namely, *S. scabies, S. acidiscabies,* and *S. turgidiscabies,* are known to be the causal pathogens of the potato scab throughout the world. Symptoms of potato scab caused by three pathogens are difficult to distinguish from another scab-like symptoms caused by *Rhizoctonia, Spongospora*, and others. However, similarities in host range and causing symptoms on potato tuber caused by three *Streptomyces* species were investigated by using a possible common mechanism of their pathogenicity (Loria et al., 1997). The pathogenicity was primarily caused by a phytotoxic secondary metabolite, thaxtomin A. The tuber slice assay was reported to be an efficient technique for assessing pathogenicity of *Streptomyces* species. Crude extracts of pathogenic *S. scabies* grown on OMA produced a necrotic reaction on potato slices. Necrosis was observed when crude extracts of *S. scabies* contained phytotoxic compounds and thaxtomin A (yellow component peak) estimated by TLC. The thaxtomin A that was detected by HPLC analysis revealed pathogenicity (Fig. 1).

Several *Streptomyces* species causing the same diseases

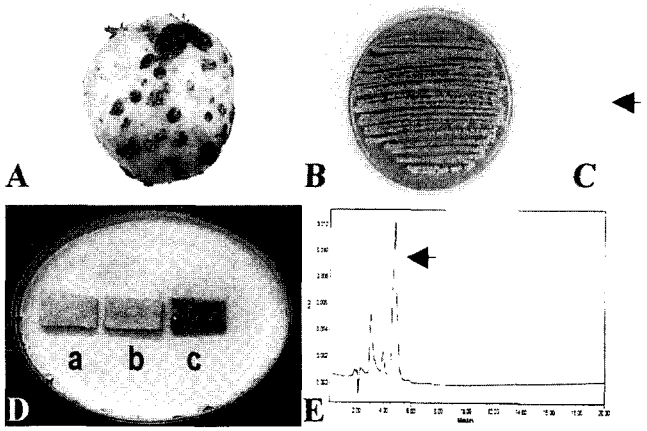

Fig. 1. Purification of thaxtomin A isolated from *Streptomyces scabies* and its necrogenic activities. (A) A field sample of potato showing scab symptom. (B) Colony pattern of *Streptomyces scabies* on oat meal agar. (C) The yellow component (arrow) with authentic thaxtomin A from *S. scabies* on TLC. (D) Bioassay on slice tuber: a, control; b, chloroform; c, yellow component. (E) Identification of thaxtomin A from yellow component by HPLC. Arrow indicates thaxtomin A peak.

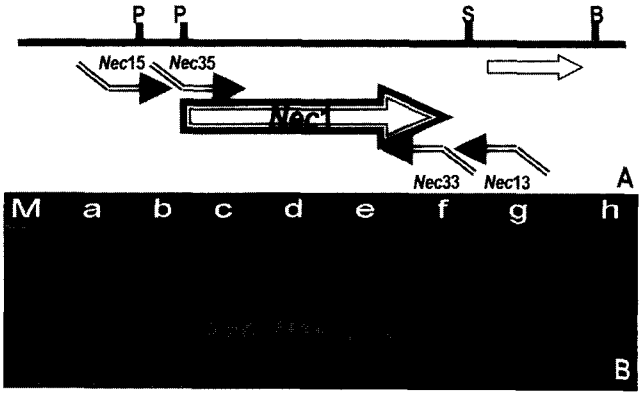

Fig. 2. Detection of pathogenicity from *Streptomyces scabies* and *S. acidiscabies* by specific primers of *Nec*1 gene. (A) Specific primers were designed from *Nec*1 gene (GenBank Accession No. AF073320) and Primer3 program. (B) PCR products of *S. scabies* and *S. acidiscabies* by specific primers. Pathogenic strains used to Lane a, c and f: *S. scabies*, Lane b, d and h: *S. acidiscabies*, Lane e: *S. scabies* ATCC49173, and Lane g: *S. acidiscabies* ATCC49003. Specific primers amplified with Lane a and b: *Nec*15 and *Nec*13, and Lane c-h: *Nec* 35 and *Nec* 33.

of the potato scab imply that common pathogenic genes are required for thaxtomin A production which had been horizontally transferred among these diverse pathogens. The gene responsible for the putative pathogenicity or virulence is known as *nec*1 that is conserved in plant pathogens but absent in non-pathogens. Subsequently, strong correlation existed between thaxtomin production and the presence of *nec*1 in *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* strains (Bukhalid et al., 1998, Bukhalid and

Fig. 3. Alignment of nucleotide sequences of PCR products with the primers designed from *Nec*1 gene. s: *Streptomyces scabies*, a: *S. acidiscabies*, t: *S. turgidiscabies*. Rectangular were primer sequences designed from *Nec*1 gene (*Nec*35 and *Nec*33). M mark encoded start codon of *Nec*1 gene. Stop codon marked with an asterisk.

Loria, 1997). Identification of the unusual *nec*1 gene and the sequences of *nec*1 among three described species of *Sterptomyces* prompted an investigation of the genetic region flanking. The *nec*1 gene was amplified with nested species-specific oligonucleotide primers designated as *Nec*15 & *Nec*13 and *Nec*35 & *Nec*33, based on the *nec*1 sequence (Fig. 2-A). The PCR amplification of genomic DNA purified from the infected potatoes of Jeju by using the *Nec*35 and *Nec*33 primers within the region of *nec*1 gene consistently produced a fragment of 580 bp, but flanking region primers of *Nec*15 and *Nec*13 did not produce any fragment (Fig. 2-B). Based on the results obtained by using Jeju strains, PCR of *S. scabies* ATCC49173 and *S. acidiscabies* ATCC49003 were performed to detect *Streptomyces* pathogens by using the *Nec*35 and *Nec*33 primers. The PCR results are shown in Fig. 2. Bukhalis et al. (1998) demonstrated pathogenic strains of *Streptomyces* by hybridization of *nec*1 with 0.7 Kb PCR products as a probe. However, southern analysis was time consuming and actually revealed variable sizes in *S. scabies*. The nucleotide sequence of the PCR product was determined and aligned with that of the strains. The sequence of PCR product was found to have a perfectly identical sequence to 580 bp within the *nec*1 gene from *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* (Fig. 3). The primer sets designed in this study allowed for the rapid identification of pathogenicity of *Streptomyces* species, which caused potato scab.

This complex method using PCR by specific primer of *nec*1 gene and bioassay of thaxtomin A has two major advantages compared with the conventional identification techniques. First, reliable, rapid identification and monitoring field samples are feasible. Second, an identification process reduces time by using a series of exchange morphologic→bioassay→genetic into a genetic→bioassay →morphological course.

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