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Studies on Phylogenetic Analyses and Detection Method of Fire Blight Pathogen and its Closely Related Bacteria

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Summary

1. Introduction

Fire blight, caused by *Erwinia amylovora*, is the world's most important bacterial disease of pear and apple. It was first reported in the eastern area of the United States of America (Hudson Valley, New York State) in the 1780s. Thereafter the disease spread south and westward in the USA. In early 1900s, the disease was confirmed all over the USA. Furthermore, the first occurrence of fire blight was mentioned as early as the 1840s in Canada. Since 1924, the disease has spread to all pear and apple growing areas of Canada. On the other hand, the reports of fire blight came from New Zealand in 1919, from England in 1958, and from Egypt in 1964. Fire blight has been spreading in European and Middle Easetern countries and occasionally causes severe damage. There are about 40 countries known with fire blight worldwide at present.

Erwinia amylovora has not been isolated in Japan. However, bacterial shoot blight of pear with symptoms resembling fire blight was reported and the causal pathogen closely related to *E. amylovora* was assigned to *Erwinia* sp. This disease was eradicated by Japanese governmental emergency control measures and has not been reported since eradication. *Erwinia amylovora* also has not been isolated in South Korea. In 1999, a new disease with symptoms resembling fire blight was reported on Asian pears in South Korea. The causal agent was named *E. pyrifoliae*.

In this study, the phylogenetic relationships among *E. amylovora*, *Erwinia* sp. (the pathogen of bacterial shoot blight of pear), and *E. pyrifoliae* were revealed on the basis of their common nucleotide sequences. For the detection and identification of each bacterium, PCR primers were designed using the nucleotide sequences. Epiphytic bacteria on Japanese pear flowers, which may cause misidentification in the diagnosis of fire blight using direct PCR, were investigated. The sensitivity and suitable sites for direct PCR were also investigated.

2. Phylogenetic relationships among *E. amylovora*, *Erwinia* sp. (the pathogen of bacterial shoot blight of pear), and *E. pyrifoliae*

Phylogenetic trees were made based on the sequences of 16S rRNA, *gyrB*, and *rpoD* genes of *E. amylovora* (28 isolates), *Erwinia* sp. (the pathogen of bacterial shoot blight of pear 9 isolates), and *E. pyrifoliae* (2 isolates). The *gyrB* genes encode the subunit B protein of DNA gyrase which is the enzyme responsible for introducing negative supercoils into bacterial chromosomes and plays a crucial role in the replication of chromosomes. The *rpoD* genes encode the σ^{70} factor which is one of the sigma factors that confers promoter-specific transcription initiation on RNA polymerase. The tree based on 16S rRNA

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gene sequences demonstrated low reliability. On the other hand, the trees based on *gyrB* and *rpoD* demonstrated very high reliability. In each tree, the 39 isolates formed two groups: "group 1" containing fire blight pathogen isolates and "group 2" containing bacterial shoot blight pathogen and Asian pear pathogen isolates. Furthermore, the tree based on combined 16S rRNA, *gyrB*, and *rpoD* sequences was similar to the *gyrB*-based and *rpoD*-based trees. Thus, phylogenetic analyses suggested that isolates from the three diseases could be divided into two groups.

The *gyrB* and *rpoD* sequences of the 25 *E. amylovora* isolates, except for isolates from *Rubus* sp., were almost identical. Meanwhile, the *gyrB* and *rpoD* sequences of the 9 isolates of bacterial shoot blight of pear pathogen were slightly different from one another. The 25 *E. amylovora* isolates had been isolated from different geographical areas, different host plants, and at different times. The 9 isolates of bacterial shoot blight of pear had been isolated only from the Hokkaido region in Japan, only from pear plants, and only during a period of several years. These results suggested that *E. amylovora*, except for *Rubus* strains, was a homogeneous group which had been spread worldwide, and that the pathogen of bacterial shoot blight of pear which was a heterogeneous group, existed in a limited region.

3. Design of primers for the detection and identification of bacteria belonging to group 1 and 2

The primers which can distinguish bacteria belonging to group 1 and 2 based on the *rpoD* gene sequences were designed. The specificity of these primers was investigated using 71 isolates of *E. amylovora*, 14 isolates of bacterial shoot blight of pear pathogen, 3 isolates of *E. pyrifoliae*, and 11 isolates of closely related bacteria. The group 1-specific primer set amplified 375 bp of DNA from all of the 71 *E. amylovora* isolates, but not from other isolates. The group 2-specific primer set amplified 375 bp of DNA from all of the 14 isolates of bacterial shoot blight of pear pathogen and *E. pyrifoliae*, but not from any of the 71 *E. amylovora* isolates, the only other size of amplification was recognized in 4 isolates of closely related bacteria. The detection limits of these primers were $10^{4\sim5}$cfu/ml.

4. Epiphytic bacteria inhabiting Japanese pear flowers

Epiphytic bacteria were isolated from Japanese pear flowers in Japan, and were investigated for the relationship to *E. amylovora*, the pathogen of bacterial shoot blight of pear, or *E. pyrifoliae*. A total of 278 bacteria were isolated from Japanese pear flowers in three areas (Ibaraki, Nagano, and Tottori prefectures) using LB medium. A total of 60 bacteria were also isolated using a semi-selective medium for *E. amylovora*. Partial 16S rRNA gene sequence similarity and colony count revealed that *Pseudomonas* spp. and *Enterobacteriaceae* dominated on Japanese pear flowers. These bacteria were not closely related to *E. amylovora*, the pathogen of bacterial shoot blight of pear, or *E. pyrifoliae*. All of the 338 bacterial isolates were checked for the false positive reaction using the present and newly designed primers. Thus, the newly designed primers were considered to be practical for the detection and identification of *E. amylovora* even from Japanese pear flowers.

5. PCR detection of *E. amylovora* in infected plant material

It was necessary to identify suitable sites on diseased plants for PCR and to remove inhibitors from them for PCR. Therefore, an *E. amylovora* isolate tagged with bioluminescence genes was used to examine the behavior and movement of the pathogen in inoculated apple shoots. The sections of shoots with or without symptoms and luminescence were used for isolation and PCR detection. DNA was extracted from the sections using a simple grinding tube and glass fiber filter paper. From symptomatic

sections, $10^{5\sim7}$cfu of *E. amylovora* tagged with bioluminescence genes were isolated, and the 375bp DNA fragment was mostly detected by PCR. However, from one symptomatic section, neither *E. amylovora* nor the fragment was detected. On the other hand, from asymptomatic sections, $10^{2\sim4}$cfu of the pathogen were isolated, but the fragment was not detected by PCR. The border of healthy and diseased tissue was a suitable site for detecting the pathogen and the fragment by PCR. Apple shoots inoculated with a wild *E. amylovora* isolate were also investigated. Eleven out of all 12 inoculated shoots showed the typical symptoms. From the symptomatic shoots, $10^{5\sim6}$cfu of wild *E. amylovora* were isolated, and the fragment was detected by PCR. On the other hand, from the one symptomless shoot, *E. amylovora* was isolated, but the fragment was not detected.

6. Conclusion

In this study, the phylogenetic relationships among *E. amylovora* (the pathogen of fire blight), *Erwinia* sp. (the pathogen of bacterial shoot blight of pear), and *E. pyrifoliae* (the pathogen of Asian pear blight) were investigated using the sequences of 16S rRNA, *gyrB*, and *rpoD* genes. Thirty-nine isolates belonging to the species formed two distinct monophyletic groups in the phylogenetic trees constructed based on the *gyrB* gene, *rpoD* gene, or a combination of the three genes, "group 1" containing the pathogen of fire blight and "group 2" containing the pathogen of bacterial shoot blight of pear and the pathogen of Asian pear blight. The pathogen of fire blight, except for isolates from *Rubus* sp., was homogeneous for sequences of *gyrB* and *rpoD*. The primers which could amplify the *rpoD* gene sequences specific to group 1 and 2 were designed. These primers were demonstrated only for each group containing isolates amplifying a unique DNA fragment. The suitable site for direct PCR detection of fire blight pathogen from the infected plant was revealed. The border of healthy and diseased tissue was very suitable for PCR.

These results will contribute to the detection and identification of the pathogen of fire blight from infected plants if the pathogen enters into Japan. Furthermore, these methods should also be applicable to other plant pathogenic bacteria.

する試料部位の調査から，直接的PCRに供する最も適した部位は発病部と健全部の境界部位であることが明らかとなった．また，野生火傷病菌を用いた試験でも同様の結果が得られた．さらに，直接的PCRと同時に行った選択培地による火傷病菌の分離結果から，直接的PCRの検出限界は供試部位あたり10^5cfuであることが示された．Llopら[29]は，火傷病菌が持つ特異的なプラスミドであるpEA29を標的として，nested-PCRにより7×10^{-1}cfu/mlもの高感度な手法を開発し，植物体から火傷病菌を検出している．しかし，pEA29を持たない火傷病菌の存在が報告されていることから，火傷病菌の植物体からの検出においては総合的にいくつかの技術を組み合わせる必要があるとしている（Llopら[30]，Llopら[31]）．EPPOの火傷病診断マニュアルにおいても，病徴を示している植物体からの初期の迅速な同定は3種類の異なる試験（2種の血清学的試験，1種の特異的PCR）を行い，3種の試験とも陽性とならない場合は，同時に行う火傷病菌の分離結果に基づいて同定を行っている．今回，試料の磨砕に利用した温州萎縮ウイルス(SDV)検定用簡易磨砕容器は，試料磨砕時の試料間の相互汚染を起こしにくく，また，この磨砕液からガラス繊維ろ紙を利用したDNA抽出法を用いることにより，火傷病菌のPCRによる検出ができた．さらに磨砕液からの分離も可能であることが示された．このことから，今回行った手法は，火傷病類似症状が発見された場合，同じ試料から同時に火傷病菌の検出，分離を行うことで，初期の迅速な同定に十分利用できると考えられる．

摘　要

火傷病は，火傷病菌(*Erwinia amylovora*)によって引き起こされるナシ，リンゴの最も重要な細菌性病害の一つで，1780年代に，アメリカ合衆国の東部で初めて確認された．これまでに，北米大陸，ヨーロッパ地域，中東地域等40ヶ国以上で発生し，時に甚大な被害を及ぼしている．本研究では，バラ科植物（特にリンゴ・ナシ）に火傷病及び火傷病類似の病害を引き起こす3種類の細菌（火傷病菌，ナシ枝枯細菌病菌，*Erwinia pyrifoliae*）について，分子系統解析を試みた．さらに，それら細菌を検出・識別するための特異的なPCRプライマーセットを設計した．また，火傷病罹病植物からの直接的PCRによる診断時に，擬陽性反応を起こす可能性のあるニホンナシ花器に生息する常在細菌の調査を行うとともに，既存の簡便な手法を利用し，実際の利用場面において検出に適した試料の採取部位及びその検出感度について調査を行った．

火傷病菌，ナシ枝枯細菌病菌，*E. pyrifoliae* について，16S rRNA遺伝子，*gyrB*遺伝子，*rpoD*遺伝子の部分的塩基配列を決定し，分子系統解析を行った．その結果，3種類の細菌は2つのグループに分かれた．グループ1は火傷病菌，グループ2はナシ枝枯細菌病菌ならびに*E. pyrifoliae*を含んでいた．火傷病菌の*gyrB*，*rpoD*遺伝子の配列は，ほとんど同一であった．一方でナシ枝枯細菌病菌は互いにわずかに配列が異なっていた．このことは，火傷病菌が分子系統学的にはほぼ単一の系統が広く世界に伝搬したのに対して，ナシ枝枯細菌病菌は限られた地域に多様な系統が存在していたことを示唆した．

*rpoD*遺伝子の塩基配列からグループ1，グループ2それぞれを検出・識別するための特異的プライマーセットを設計し，実際に各菌株を用いてその特異性を調査した．グループ1特異的プライマーセットは火傷病菌のみで，単一の375bpのDNA断片の増幅が確認された．また，グループ2特異的プライマーセットはナシ枝枯細菌病菌，*E. pyrifoliae* のみで，単一の375bpのDNA断片の増幅が確認され，その検出限界は$10^{4\sim5}$cfu/mlであった．

国内のニホンナシの花器から網羅的に細菌を分離し，火傷病菌ならびにAsian pear blight pathogenに対する類縁性，及び特異的プライマーセットを用いたPCR診断法で擬陽性反応を起こす細菌の存在について検討した．茨城，長野，鳥取各県から採取した花器より表生細菌の分離を行い，338株の細菌株を得た．16S rRNA遺伝子の部分的塩基配列から属を推定したところ，大部分が*Pseudomonas*属または*Enterobacteriaceae*であった．得られた細菌株の中には，火傷病菌や*E. pyrifoliae*に類似している細菌は存在しなかった．また，これら細菌株について，既存の火傷病菌特異的プライマーセット2種及び今

回*rpoD*遺伝子の部分的塩基配列から作成したグループ特異的プライマーセットを用いてPCRを行ったところ，今回作成したグループ特異的プライマーセットが最も非特異反応がなかった．

PCRによって病原体を罹病植物体から直接検出するために，PCR反応を阻害する物質の除去及び，罹病植物体のどの部位がPCR用試料として適しているかを調査した．発光遺伝子標識された火傷病菌及び野生の火傷病菌を盆栽ヒメリンゴの新梢に付傷接種し，細菌の再分離と直接的PCRを試みた．簡易磨砕容器で試料を磨砕し，ガラス繊維ろ紙を用いた簡便迅速なDNA抽出法により，PCR用の鋳型DNAを抽出して行った．PCR用試料として最適な部位は発病部と健全部の境界部位であり，その時の検出限界は切り出し部位あたり$10^{5\sim6}$cfuであった．

これらの研究結果は，万が一火傷病菌が日本に侵入した際の同定作業の迅速性，正確性を高めることに寄与するとともに，他の植物病原細菌に対しても応用可能と考えられた．

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