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Molecular detection of phytoplasmas in oats, barley, and *Triticosecale* and their classification based on 16S rRNA gene polymorphisms

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Diseased plants of oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and *Triticosecale* Wittm. ex A. Camus (*Triticum* L. × *Secale* L.) were observed in fields in the Vilnius region of Lithuania. Disease symptoms exhibited by the plants included yellowing of leaves, general stunting, sterility and deformation of spikes, dwarfed spikes, and twisted awns, indicating possible infection by phytoplasmas. A phytoplasma-characteristic fragment of 16S rDNA was amplified in nested polymerase chain reactions which were primed by phytoplasma universal primers, confirming phytoplasma infection in the symptomatic plants. RFLP analysis of amplified 16S rDNA indicated that diseased oats were infected by the group 16SrI (aster yellows, AY, group) phytoplasma strain belonging to the subgroup 16SrI-L, and that barley and *Triticosecale* were infected by strains belonging to the subgroup 16SrI-B. The phytoplasma strains found in oat, barley and *Triticosecale* were named oat yellows (OatY), barley deformation (BaDef), and *Triticosecale* stunt (TrSt) phytoplasmas, respectively. This is a first reported characterization of AY phytoplasmas in cereal crops of barley and *Triticosecale.*

Key words: cereal crops, phytoplasma, detection, PCR, RFLP

INTRODUCTION

Oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and *Triticosecale* Wittm. ex A. Camus (*Triticum* L. × × *Secale* L.) are economically important grain crops in Lithuania. These and other cereal grains are the most widely grown crops in the country. While oats are used as animal feed, human consumption of oats is increasing, especially in North America, because of their health-related, neutraceutical value. In human health, consumption of oats and other whole grain cereal foods can provide significant benefits, including reduced risk of coronary heart disease [15].

Barley is one of the primary food crops produced in Lithuania, where it is used for direct human consumption, for the production of beer, and as livestock feed. Globally, barley is mainly a livestock feed, and in some countries is the major feed grain. However, health benefits have been associated with human consumption of barley, including benefits for regulating blood sugar levels in diabetics, and for lowering cholesterol and heart disease. Barley kernel components are also providing neutraceuticals for the pharmaceutical market [15].

Triticosecale (triticale) is a polyploid that resulted from a cross between wheat (*Triticum* L.) and rye (*Secale* L.). Triticales are sown increasingly in Lithuania. They are valuable plants and their crops will get a due place among winter cereals in the future. Triticale is mainly a livestock feed. It is also used for making whole-grain breads and other foods for human consumption. Triticale grains contain more proteins than wheat and rye [8].

Considering the importance of grain crops in the agricultural economy of Lithuania, we began an investigation of diseases in grains, with the focus on diseases caused by insect-transmitted pathogens termed phytoplasmas [7]. Phytoplasmas are minute bacteria that lack a cell wall and have very small genomes. Taxonomically, they are classified with mycoplasmas that are responsible for diseases in man and animals, but in contrast to most mycoplasmas, phytoplasmas cannot be isolated in culture [13]. For this reason, molecular methods have been adopted to achieve sensitive detection of phytoplasmas and to classify them [9].

The present study was prompted by the recent discovery of a phytoplasma in diseased oat plants in

Lithuania [7], raising the possibility that phytoplasmas might also infect other grain crops in the country. Here we report that phytoplasmas are present in diseased plants of barley and *Triticosecale* growing in Lithuania, and we classify the phytoplasmas based on polymorphisms in the 16S ribosomal (r) RNA gene.

MATERIALS AND METHODS

Plant samples, PCR (polymerase chain reaction)

Samples of diseased plants exhibiting yellowing, stunting, sterile deformed spikes, shorter spikes and twisted awns were observed in the field in Vilnius region in Lithuania. Template DNA was extracted from the tissues using Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania) and was used in nested polymerase chain reaction (PCR) for amplification of phytoplasmal 16S rDNA. In nested PCR, the first reaction was primed by the phytoplasma-universal primer pair P1/P7 [4, 14]. Products obtained in the first PCR were diluted 1:50 with sterile water and used in the second (nested) PCR primed by the primer pair R16F2n/R16R2 (F2n/R2) [6, 9]. Both amplifications were conducted under the same conditions (94° for 1 min, 55° for 2 min, 72° for 3 min) for 35 cycles in a Perkin Elmer PCR buffer, 0.25 mM dNTP, 0.4 μM of each primer, and 1 unit of recombinant Taq polymerase per 50 μl of reaction mixture. PCR products were analyzed by electrophoresis through 1% agarose gel, stained with ethidium bromide, and DNA bands visualized using a UV transilluminator.

RFLP analysis and phytoplasma classification

Products (1,2 kbp) of the nested PCR, primed by the primer pair F2n/R2, were subjected to enzymatic restriction fragment polymorphism (RFLP) analysis using the restriction endonucleases *Alu*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*II, *Mse*I, *Rsa*I, *Tag*I, *Kpn*I and *Sau*3AI (MBI Fermentas, Vilnius, Lithuania) and electrophoresis through 5% acrylamide gel. DNA bands were stained with ethidium bromide and visualized using UV transilluminator. Phytoplasmas were classified in groups and subgroups, through comparisons of RFLP patterns previously published, in accordance with the classification scheme of Lee et al. (1998).

RESULTS AND DISCUSSION

Subgroup 16SrI-L in *A. sativa*

Symptoms exhibited by diseased oats included stunting, development of numerous short tillers at the plant's base, and sterile deformed spikes (Fig. 1). A phytoplasma-characteristic 1.2 kbp rDNA fragment was amplified in nested PCR primed by phytoplasma universal primers and containing DNA template extracted from diseased oats, confirming infection by phytoplasma (data not shown). The collective RFLP patterns of amplified rDNA were indistinguishable from characteristic RFLP patterns previously published for rDNA from group 16SrI subgroup B phytoplasmas [9] except for the *Hinf*I RFLP pattern (Fig. 2). The sum of the sizes of rDNA fragments in the *Hinf*I RFLP pattern exceeded the size of 1.2 kbp. We interpreted this pattern to indicate the presence of two sequence heterogeneous 16S r RNA genes in the genome of the phytoplasma. A similar *Hinf*I RFLP pattern had been previously published for rDNA of a phytoplasma classified in the subgroup 16SrI-L and found in primrose and aster plants in Germany [10]. On this basis the phytoplasma present in oats was classified in group 16SrI, subgroup L and named OatY (oat yellows) phytoplasma. Previously, subgroup 16SrI-L phytoplasma strains had been detected in hyacinth, rape, and gladiolus plants in Lithuania [1, 16]. Our discovery of subgroup I-L in *A. sativa* adds yet another monocotyledonous plant to the known host range of subgroup I-L and underscores the need to learn more about the potential of phytoplasmas to damage oat crops.

The literature contains only few reports of phytoplasmas possibly infecting oats. In some early reports, only phytoplasma-characteristic disease symptoms were reported.

For example, naturally symptomatic *A. sativa* was reported in 1969 in Canada by Gill et al. [11]. In Siberia, mycoplasma-like organisms (phytoplasmas) were observed in diseased oat by electron microscopy [5], but the identity of phytoplasma

Fig. 1. *Avena sativa* L. plant infected by phytoplasma (Photo L. Urbanavičienė)

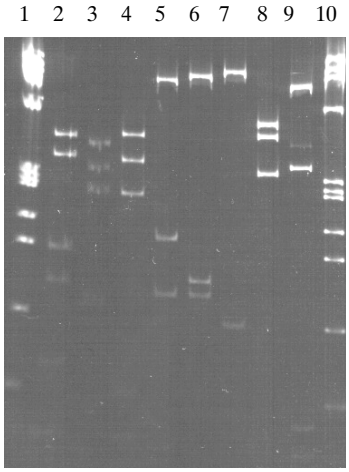

Fig. 2. RFLP patterns of 16S rDNA (F2n/R2 PCR product) from the subgroup 16SrI-L phytoplasma from infected oat. Lanes: 1, 10 – ØX174/*Hae*III digest, size standard, 2 – *Alu*I, 3 – *Mse*I, 4 – *Rsa*I, 5 – *Hpa*II, 6 – *Hha*I, 7 – *Hae*III, 8 – *Kpn*I, 9 – *Hinf*I

Fig. 4. *Triticosecale* Witt. ex A. Camus plant infected by phytoplasma.
Healthy spike on the left (Photo L. Urbanavičienė)

remains unknown. To date, the only phytoplasmas definitely identified in plants of oats are the subgroup 16SrI-A phytoplasma discovered in diseased oats in a field in Raseiniai region of Lithuania [7] and the subgroup 16SrI-L detected in symptomatic oats in Vilnius region (this paper).

Phytoplasma subgroup 16 SrI-B in *H. vulgare* and *Triticosecale*

Diseased plants of barley (*Hordeum vulgare* L.) and *Triticosecale* Witt. ex A. Camus (triticale) exhibiting symptoms characteristic of phytoplasma infection (yellowing, stunting, sterile deformed spikes, twisted awns, shorten spikes) were collected from naturally infected plants (Figs. 3, 4). Based on symptoms in plant hosts we designated the diseases as barley deformation (BaDef) and *Triticosecale* stunt (TrSt).

Phytoplasmas were detected in barley and *Triticosecale* plants on the basis of amplification of phytoplasmal 16S rDNA in nested PCR primed by a phytoplasma universal primer pair (F2n/R2) (data not shown). BaDef and TrSt phytoplasmas were similar to each other on the basis of collective RFLP patterns of rDNAs using up to ten restriction enzymes (Figs. 5, 6). A comparison of these collective RFLP patterns with the collective patterns presented in a phytoplasma classification scheme (9) indicated that phytoplasmas infecting barley and *Triticosecale* belong to the subgroup 16SrI-B. BaDef and TrSt are the first phytoplasmas identified in barley in Lithuania and in *Triticosecale* worldwide (this paper).

Fig. 3. *Hordeum vulgare* L. plants infected by phytoplasma. Healthy spike on the left (Photo L. Urbanavičienė)

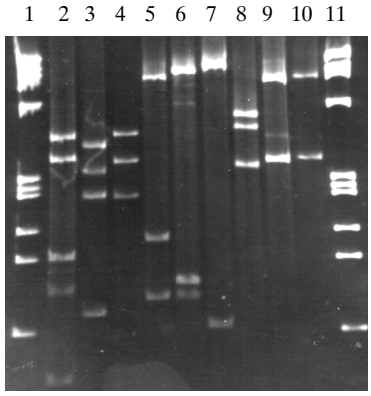

Fig. 5. RFLP patterns of 16S rDNA (F2n/R2 PCR product) from the subgroup 16SrI-B phytoplasma from infected barley. Lanes: 1, 11 – ØX174/*Hae*III digest, size standard, 2 – *Alu*I, 3 – *Mse*I, 4 – *Rsa*I, 5 – *Hpa*II, 6 – *Hha*I, 7 – *Hae*III, 8 – *Kpn*I, 9 – *Hinf*I, 10 – *Tag*I

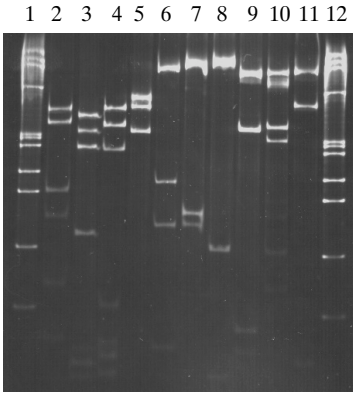

Fig. 6. RFLP patterns of 16S rDNA (F2n/R2 PCR product) from the subgroup 16SrI-B phytoplasma from infected *Triticosecale*. Lanes: 1, 12 – *Ø*X174/*Hae*III digest, size standard, 2 – *Alu*I, 3-*Mse*I, 4 – *Rsa*I, 5 – *Kpn*I, 6 – *Hpa*II, 7 – *Hha*I, 8 – *Hae*III, 9 – *Hinf*I, 10 – *Tag*I, 11 – *Sau*3AI

OatY, BaDef and TrSt are the first examples of phytoplasmas belonging to the subgroups 16SrI-L and 16SrI-B reported in monocotyledonous host plants in Lithuania.

In 1963 it had been reported that the aster yellows infections in barley and wheat resulted in severe losses in Canada and North America [3]. Subsequently, six grasses have been reported belonging to the host range of AY infections in the United States and Canada [2]. In Finland, the disease caused by aster yellows symptoms was readily transmitted by *Macrosteles laevis* (Rib.) to and from graminaceous plants [12].

CONCLUSIONS

1. RFLP analysis of amplified 16S rDNA from diseased oats (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and *Triticosecale* Wittm. ex A. Camus plants confirmed that the crops grown in the Vilnius region were infected by similar phytoplasma strains belonging to the group 16SrI (aster yellows group).

2. OatY (oat yellows) phytoplasma classified to the subgroup 16SrI-L and both BaDef (barley deformation) and TrSt (*Triticosecale* stunt) phytoplasmas classified to the subgroup 16SrI-B infected monocotyledonous host plants.

3. An important new finding in Lithuania is that for the first time phytoplasma infections were revealed in barley and *Triticosecale* plants.

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MOLEKULINIS FITOPLAZMŲ APTIKIMAS AVIŽOSE, MIEŽIUOSE BEI KVIETRUGIUOSE IR JŲ KLASIFIKACIJA PAGAL 16S rRNR GENO POLIMORFIZMĄ

S a n t r a u k a

Sergantys avižų (*Avena sativa* L.), miežių (*Hordeum vulgare* L.) ir kvietrugių (*Triticosecale* Wittm. ex A. Camus) augalai buvo aptikti Vilniaus apskrities javų pasėliuose, Lietuvoje. Ligos simptomai – lapų pageltimas, žemaūgė, varpų sterilumas ir deformacija, varpų sumažėjimas ir akuotų susisukimas rodo galimą fitoplazminę infekciją. Fitoplazmoms būdingas 16S rDNR fragmentas, pagausintas lizdinėje polimerazinėje grandininėje reakcijoje, naudojant fitoplazmoms universalius pradmenis, patvirtino fitoplazminę infekciją simptomatiniuose augaluose. Pagausinto 16S rDNR RFLP analizė parodė, kad sergančios avižos buvo užkrėstos 16SrI grupės (Aster yellows, AY) fitoplazmų kamienais, priklausančiais 16SrI-L pogrupiui, o miežiai ir kvietrugiai buvo užkrėsti kamienais, priklausančiais 16SrI-B pogrupiui. Avižose, miežiuose ir kvietrugiuose aptikti fitoplazmų kamienai buvo pavadinti OatY (oat yellows), BaDef (barley deformation) ir TrSt (Triticosecale stunt) fitoplazmomis. Tai yra pirmasis paskelbtas fitoplazmų miežiuose ir kvietrugiuose apibūdinimas.

Raktažodžiai: javai, 16S rRNR genas, PCR, RFLP

Лайма Урбанавичене, Раса Йомантене, Дейвидас Валюнас, Роберт Едвард Давис

МОЛЕКУЛЯРНОЕ ОПРЕДЕЛЕНИЕ ФИТОПЛАЗМ В ОВСЕ, ЯЧМЕНЕ И *TRITICOSECALE* И ИХ КЛАССИФИКАЦИЯ НА ОСНОВЕ ПОЛИМОРФИЗМА 16S рРНК ГЕНА

Р е з ю м е

В окрестностях Вильнюса обнаружены растения овса (*Avena sativa* L.), ячменя (*Hordeum vulgare* L.) и *Triticosecale* Wittm. ex A. Camus с симптомами болезни. Симптомы болезни – пожелтение листа, карликовость, стерильность и деформация колосьев, уменьшение размеров колосьев и скручивание остьи указывают на вероятность фитоплазменной инфекции. Эти данные были подтверждены методом ПЦР (полимеразная цепная реакция) используя для фитоплазм универсальные праймеры. Результаты 16S рДНК RFLP анализа показали, что больные растения овса были инфицированы штаммами, принадлежащими к группе 16SpI и подгруппе 16SpI-L. Ячмень и *Triticosecale* были инфицированы штаммами, принадлежащими той же группе и подгруппе 16SpI-B. Штаммы фитоплазм, обнаруженные на овсе, ячмене и *Triticosecale,* были названы OatY (oat yellows), BaDef (barley deformation) ir TrSt (Triticosecale stunt) фитоплазмами. AY фитоплазмы, обнаруженные на ячмене и *Triticosecale,* публикуются впервые.

Ключевые слова: злаки, фитоплазма, 16S рРНК ген, ПЦР, RFLP