





RESEARCH COMMUNICATION

In vitro screening reveals high-level resistance to a virulent Erwinia amylovora isolate in native Malus sieversii

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Abstract

Fire blight, caused by the bacterium *Erwinia amylovora*, is a destructive disease that threatens global apple production and the native forests of *Malus sieversii* in Kazakhstan, the primary progenitor of domesticated apples. To identify sources of genetic resistance, we screened ten native *M. sieversii* genotypes using an *in vitro* shoot inoculation assay with a virulent local isolate of *E. amylovora*. Disease severity was measured as the percentage of lesion length (PLL). Genotype 6.1 exhibited complete resistance (0 % PLL), whereas three other genotypes (1.2, 5.3 and 6.2) were highly resistant. In contrast, genotypes 1.1 and 5.1 were highly susceptible to infection. Molecular screening for sequence-characterised amplified region (SCAR) markers linked to the major fire blight resistance locus, *FBF7*, revealed that the highly resistant genotype 5.3 was positive for the AE10-375 marker. However, the completely resistant genotype 6.1 and the other highly resistant individuals lacked both *FBF7*-linked markers. These results demonstrate the presence of the known *FBF7* locus and suggest the existence of potentially novel genetic resistance sources in this wild population.

Keywords: Erwinia amylovora; fire blight; in vitro screening; Malus sieversii; molecular markers; plant pathology

Introduction

Fire blight, caused by the necrotising bacterium *Erwinia amylovora*, is one of the most destructive diseases of apples (*Malus domestica*), pears (*Pyrus communis*) and other pome fruits worldwide (1). As one of the ten most important plant-pathogenic bacteria, this pathogen can quickly kill entire trees and orchards, resulting in significant economic losses (2). *E. amylovora* is originally from North America, where it was first recorded in 1780 (3). It has become a successful invasive species, now found in New Zealand, Europe, the Mediterranean basin and more recently, parts of Asia (3, 4).

The current way of dealing with fire blight is to use a mix of cultural, chemical and biological controls. However, each of these has serious limitations, making the whole system a costly and sometimes ineffective defence (1, 5). Cultural practices, such as the pruning of cankers to reduce primary inoculum, are labour-intensive and often ineffective to prevent outbreaks under weather conditions favourable for the pathogen. Chemical management, including pre-bloom copper sprays and antibiotic applications, is also limited. The efficacy of streptomycin has been severely compromised by the emergence and spread of antibiotic-resistant strains of *E. amylovora* (6). Moreover, the precise timing required for effective antibiotic application is often difficult to achieve during extended periods of rain. These constraints can be overcome

by the development of cultivars with durable, host-plant genetic resistance.

Fire blight was first reported in Kazakhstan in 2008 (7). Now it poses a significant threat to native M. sieversii forests, the wild ancestor of domesticated apple and a key global source of apple genetic diversity (7, 8). The genetic diversity within M. sieversii is vital for breeding programs, providing novel alleles for disease resistance and other important horticultural traits absent in modern cultivars (9, 10). As a result, researchers have screened M. sieversii for fire blight resistance, mainly using exsitu germplasm collections in North America and Europe (11). These evaluations, which used North American pathogen isolates in greenhouse and field trials, identified many resistance sources and confirmed a high frequency of resistance alleles in the species. However, with the pathogen's arrival in Kazakhstan, a critical research gap has emerged. Resistance to E. amylovora can be strain-specific, so resistance to foreign isolates may not be effective against local pathogen populations in Central Asia (12). Additionally, because E. amylovora is a quarantine pathogen in Kazakhstan, open-field screening is not possible and alternative evaluation methods are required (13, 14). This study addresses this urgent gap. To our knowledge, this is the first report of in vitro resistance screening of native Kazakh M. sieversii genotypes against a virulent, locally sourced E. amylovora isolate.

LAURA ET AL 2

Materials and Methods

Bacterial isolate and pathogenicity verification

Infected apple tissues exhibiting characteristic fire blight symptoms were collected from two orchards in the Almaty region of Kazakhstan from a total of six symptomatic trees during the 2024 growing season. Small sections of tissue from the margin between the healthy and necrotic areas were excised, surface-sterilised and homogenised in sterile phosphate-buffered saline (PBS). The resulting macerate was streaked onto selective media MM2Cu and Luria-Bertani (LB) agar supplemented with 5 % sucrose and incubated at 28 °C for 48-72 hr. On MM2Cu, the virulent isolate, designated EaAla_1, formed yellow, mucoid colonies typical of E. amylovora (15). The identity of the isolate was confirmed using a loop-mediated isothermal amplification (LAMP) assay (16). Bacterial DNA was extracted as described previously (17). LAMP primers (Table S1) were designed using Primer Explorer V5 (http:// primerexplorer.jp/lampv5e/index.html (accessed 12 September 2025); Eiken Chemical Co. Ltd., Tokyo, Japan). The 25 μL LAMP reaction contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1 % Tween 20, 400 μM dNTP mix, 600 mM betaine, 0.4 μ M each of forward inner primer (FIP) and backward inner primer (BIP) primers, 0.1 µM each of F3 and B3 primers, 20-30 ng of DNA and 8 units of Bsm DNA polymerase (Thermo Scientific, Waltham, MA, USA, Cat. num. EP0691). The reaction was incubated for 1 hr at 56 °C, followed by a 10-minute enzyme deactivation at 80 °C. The reaction products were analysed by electrophoresis on a 1.8 % agarose gel (Fig. S1).

The pathogenicity of the EaAla_1 isolate was confirmed using an immature pear fruit assay (18). For whole-fruit assays, 20 μL of a bacterial suspension (1 × 10 7 cells/mL) was injected into surface-sterilised unripe pear fruits. For segment assays, 5 μL of the suspension was applied to the surface of 5 mm thick fruit slices. The controls were treated with sterile PBS. After incubation at 28 $^{\circ}$ C for 5 days, the inoculated fruits and segments were visually observed for tissue necrosis and bacterial ooze production.

In vitro plant culture and resistance assay

Seeds of M. sieversii were taken from ten healthy trees in the Tau Turgen Mountains, Kazakhstan (43°21' 31.6" N; 77°40' 16.6 "E). Following a 1.5-month cold stratification at 4 °C to break dormancy, seeds were surface-sterilised and germinated in vitro on a Murashige and Skoog (MS) medium (PhytoTech Labs, Inc., Lenexa, KS, USA, Cat. Num. M5501). Shoots were proliferated on MS-based medium supplemented with 1 6-benzylaminopurine (BAP), 1 mg/L 1-naphthaleneacetic acid (NAA) and 0.1 mg/L indole-3-butyric acid (IBA) to generate clonal replicates for each of the ten genetically distinct, seed-derived genotypes and maintained at 24 ± 2 °C under a 16-hr photo-period. For the resistance assay, ten micropropagated plantlets were used for each genotype. A suspension of the EaAla_1 strain was prepared in phosphate buffer with a final concentration of 1×109 CFU/mL. The apical 3 mm of actively growing micropropagated shoots was excised and a 3 μL droplet of the inoculum was applied to the cut surface (14). Control shoots were treated with sterile buffer. Disease severity was quantified by measuring the length of the necrotic lesion and

total shoot length at 6 and 12 days post-inoculation (DPI). The extent of disease was expressed as PLL, calculated using the formula (19).

PLL=(lesion length/total shoot length) × 100 (Eqn. 1)

PLL and standard deviation were calculated using Microsoft Excel 2021. Statistical analyses were performed using the Excel Data Analysis ToolPak. Differences in PLL between 6 and 12 DPI for each genotype were compared using a two-sample Student's t-test assuming unequal variances. To assess differences between genotypes, a one-way Analysis of Variance (ANOVA) test was performed on the 12 DPI data.

Molecular marker analysis

Genomic DNA was extracted from the ten genotypes used in the inoculation assay, as well as from an additional five genotypes from the collection, using a CTAB protocol (20). PCR analysis was performed using two SCAR markers, AE10-375 and GE-8019, which flank the major fire blight resistance quantitative trait locus (QTL) FBF7 (21). DNA from the Summer Red and Discovery cultivars was used as positive and negative controls, respectively (22). PCR was performed with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 1.5 min at 72 °C, with a final elongation step of 15 min at 72 °C. Amplification products were separated by electrophoresis on a 2.0 % agarose gel and visualised under ultraviolet (UV) light.

Results and Discussion

A virulent E. amylovora isolate, designated EaAla_1, was successfully recovered from an infected apple tree in an orchard in the Almaty region of Kazakhstan. From the six symptomatic trees sampled, a total of three isolates were recovered. All three isolates were confirmed as E. amylovora and displayed identical colony morphology on selective media. As these isolates were phenotypically identical, one representative isolate (EaAla_1) was selected for further work. The pathogenicity of the isolate was confirmed through bioassays on both whole, immature pear fruits and fruit slices, which developed characteristic symptoms of extensive necrosis and bacterial ooze. The identity of the isolate as E. amylovora was further verified using the LAMP assay (Fig. S1). While the pear bioassay provided clear qualitative confirmation of pathogenicity, a quantitative comparison with a known reference strain was not performed. However, the high PPL in susceptible genotypes during the in vitro assay (PLL > 75 %) confirms the high virulence of the EaAla_1 isolate.

In vitro screening using this confirmed that pathogenic isolate reveals a wide spectrum of responses among the ten tested *M. sieversii* genotypes (Fig. 1). A one-way ANOVA confirmed that these differences between genotypes at 12 DPI were highly significant (F(9, 90) = 41.57, p < 0.0001). The most significant result was observed in genotype 6.1, which showed no disease symptoms at either time point (0.00 % PLL), demonstrating complete resistance to high infection pressure (Table S2). Three additional genotypes - 1.2, 5.3 and 6.2-were identified as highly resistant, with mean PLL values at 12 DPI of 2.05 %, 2.58 % and 4.82 %, respectively. In stark contrast, genotypes 1.1 and 5.1 were highly susceptible, with lesions progressing to 77.53 and 79.79 % PLL by 12 DPI, respectively. These values were statistically confirmed to be significantly different (p < 0.001, Student's t-test)

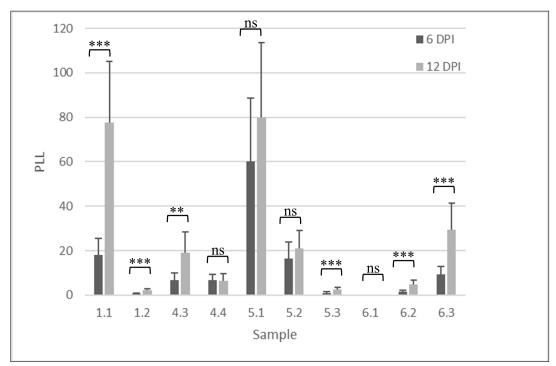


Fig. 1. Fire blight resistance of ten *Malus sieversii* genotypes at 6 and 12 DPI with *Erwinia amylovora*. Data are presented as the mean PLL. Error bars represent standard deviation. Asterisks indicate a statistically significant difference between 6 DPI and 12 DPI measurements for a given genotype, as determined by a Student's t-test (ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001).

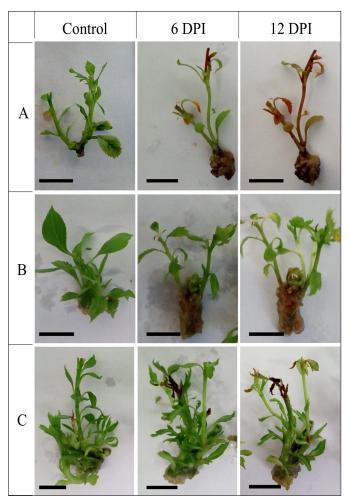


Fig. 2. Phenotypic response of *Malus sieversii* genotypes to *in vitro* inoculation with *Erwinia amylovora*. **A.** Representative images of a highly susceptible genotype (sample 1.1), **B.** a completely resistant genotype (sample 6.1), **C.** a moderately susceptible genotype (sample 4.4) are shown. Scale bars = 1 cm.

from the completely resistant genotype 6.1. The phenotypic

differences between the resistant, moderately susceptible and highly susceptible genotypes were visually distinct (Fig. 2).

Molecular analysis using two SCAR markers flanking the FBF7 locus revealed that the highly resistant genotype 5.3 tested positive for the AE10-375 marker but was negative for the GE-8019 marker (23). The presence of only the AE10-375 marker, but not GE-8019, could suggest a recombination event within the FBF7 region or the presence of a different resistance allele at this locus. Alternatively, it may indicate that this genotype possesses a different allele of the FBF7 locus that is not recognised by the GE-8019 marker. Further fine-mapping would be required to confirm the integrity of the FBF7 QTL in this genotype. In contrast, the completely immune genotype 6.1, along with the other highly resistant genotypes (1.2 and 6.2), tested negative for both the AE10-375 and GE-8019 markers (Fig. S2). This result suggests that their resistance is conferred by a genetic mechanism independent of the FBF7 locus and calls for further investigation into a potentially novel source of resistance.

These findings support the importance of M. sieversii as a key source of genetic variety for improving apples (11). The identification of four genotypes with high to complete resistance from a small sample suggests a high frequency of strong resistance alleles within the native Kazakh population. For comparison, previous extensive field screening assessed nearly 200 M. sieversii accessions across multiple years and locations, ultimately identifying 12 accessions (6 %) exhibiting consistent resistance comparable to highly resistant controls (11). Our higher frequency possibly indicates the preliminary nature and restricted sample size of our in vitro screening, which aims to identify promising candidates, whereas the lower percentage observed in the field study represents a more rigorously validated figure following multi-year trials. A major advantage of this study was using a local virulent E. amylovora isolate. Using a local isolate provides a more accurate evaluation of the interaction between host germplasm and a pathogen strain relevant to the area. This is particularly important for developing regionally adapted cultivars, as pathogen

LAURA ET AL 4

populations can vary in virulence and some resistance QTLs can be strain-specific (24).

Complete immunity in genotype 6.1 was the most significant finding. This response, observed under high inoculum pressure, is rare in fire blight resistance research. This phenotypic observation may be conferred by a novel genetic mechanism, as 6.1 tested negative for markers flanking the major FBF7 locus. Such complete immunity could be governed by a strong qualitative resistance gene, potentially triggering a rapid hypersensitive response that halts pathogen proliferation at the point of infection. Further transcriptomic and genetic mapping studies are needed to uncover the specific genes and pathways involved. Furthermore, the confirmation of the FBF7-linked marker in the resistant genotype 5.3 demonstrates that the previously characterised locus is also functional within the ancestral M. sieversii population. This indicates that the Kazakh gene pool contains parallel defence strategies, offering exciting possibilities for breeding, such as pyramiding these distinct resistance genes to achieve more durable protection (25).

The in vitro methodology used here offers significant advantages over traditional greenhouse or field assays, particularly for a quarantine pathogen like E. amylovora in Kazakhstan, as it allows for rapid, space-efficient and biosecure screening in a controlled environment. This is critical for initial high-throughput evaluation. It is important to acknowledge, however, that this was a preliminary study conducted with a single virulent local isolate and a limited number of genotypes from one population. While this approach successfully identified a highly promising candidate, field performance remains the ultimate test. Research indicates that there can be a low correlation between greenhouse and multi-year field trials, as quantitative resistance is strongly influenced by environmental interactions (11). Therefore, the exceptional immunity observed in genotype 6.1 requires validation in whole plants under field conditions over multiple years and across diverse environmental conditions. Genotype 6.1 represents an invaluable genetic resource and its novel immunity makes it a prime candidate for genetic mapping and the development of new molecular markers for marker-assisted selection (MAS). Broadening the genetic base of resistance by introgressing novel genes is critical to prevent pathogens from overcoming single resistance sources. The discovery of novel immunity in 6.1 alongside the FBF7 locus in 5.3 within the same gene pool is a significant finding. This presents a clear opportunity for a gene pyramiding strategy, creating a multilayered defence system that would be significantly more difficult for the pathogen to defeat.

Conclusion

This study successfully used an *in vitro* platform under controlled conditions for rapid screening to identify four *M. sieversii* genotypes with strong resistance to a local virulent strain of *E. amylovora*. The presence of the AE10-375 marker in one resistant genotype confirmed the presence of the *FBF7* locus in this population. The absence of *FBF7*-linked markers in the other resistant genotypes, particularly immune 6.1, indicates a potentially novel genetic source of resistance for further genetic and functional characterisation. These genotypes represent an invaluable resource for breeding programs aimed at developing durable fire blight resistance in apple trees. Future efforts will focus on transcriptomic analysis and genetic mapping of

genotype 6.1 to identify the novel gene(s) responsible for its immunity, which will enable the development of new molecular markers for marker-assisted breeding.

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Authors' contributions

LY conceptualised the work and wrote the original draft. AR conducted *in vitro* work and carried out a resistance assay. KA carried out work on sample collection and molecular analysis. ZB conducted primer design and carried out bacterial identification and isolation. RK carried out pathogenicity tests and edited the draft manuscript. NG carried out conceptualisation and finalised the draft manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflicts of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors did not use generative AI or AI-assisted technologies and take full responsibility for the content of the publication.

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