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SHORT COMMUNICATION

Identification and amplification of candidate genes for virus defence in common ash (*Fraxinus excelsior* L.)

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Abstract

Across various European countries, *Fraxinus excelsior* L. is facing a decline caused by *Hymenoscyphus fraxineus*. The underlying mechanisms of the disease are still insufficiently understood in detail. Observations suggest that, in addition to the main pathogen, viral infections may also contribute to the decline of ash trees. We described homologues in the *F. excelsior* genome of formerly annotated virus resistance genes in model species and tested the applicability of primers on these gene fragments. Further, a prediction of cis-acting regulatory elements and transcription factor binding sites was accomplished, to determine the exact location of the promoters of these genes in the ash genome. After annotation and filtering the low-complexity regions, primers for use with genomic DNA were designed on sequences that showed a high degree of similarity and equality in length to translated regions and promoters of these regions. From the newly designed primers, those with the most favourable primer selection parameters were selected and tested on eight virus infected—four symptomatic and four asymptomatic—samples. The amplified products were analysed by agarose gel electrophoresis. From all producing one-band amplicons, 36 primer pairs amplifying translated, and 36 primers amplifying promoter regions were selected. Our study is the first step of a SNP discovery and marker development test. The further marker development on these sequences could be useful for the selection of ash individuals with various resistance to viral infections, providing a route for a more thorough understanding of the ash dieback–virus resistance interaction.

Keywords *Fraxinus excelsior* · Virus defence genes · SNP markers · Marker-assisted selection · FraxVir

Introduction

Across various European countries, the *Fraxinus excelsior* L. is facing a decline caused by the fungal disease ash dieback (Przybył 2002), the underlying mechanisms of which remain poorly comprehended. However, first steps have been taken towards the molecular basis of healthier ash trees compared to diseased ones (Doonan et al. 2023). The limited knowledge concerning ash dieback and its interactions with other stress factors gives rise to uncertainties regarding the most effective approaches (Dobrowolska et al. 2011). Consequently, there is a pressing need to thoroughly comprehend all influencing factors.

The observations suggest the existence of factors like air pollution (Reiner et al. 1996), other fungi (Peters et al. 2023), mycoplasma (Olivier et al. 2009), or virus infections (Hibben and Silverborg 1978; Sinclair and Griffiths 1994; Rumbou et al. 2021; Marçais et al. 2022; Shamsi et al. 2022; Büttner et al. 2023), that have not yet been fully understood but should be considered alongside the known causes of ash

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dieback. To generate comprehensive findings on individual stress factors and their interaction, FraxVir, a supplementary study to the FraxForFuture demonstration project, started in 2021 in Germany with aim of detection, characterisation, and analyses of the occurrence of viruses and ash dieback in special stands of *F. excelsior*.

F. excelsior is subject to attack by various viruses (Cooper and Sweet 1975; Nienhaus and Castello 1989; Gaskin et al. 2021; Svanella-Dumas et al. 2023). To evade plant immunity and establish infection, viruses encode various effector proteins that manipulate host cellular processes. In response, plants have evolved various mechanisms to recognize and combat viral infections (Ray and Casteel 2022). Although resistance to viruses is conferred by several mechanisms, perhaps the best-characterized is mediated by resistance (R) genes (Soosaar et al. 2005; Pandolfi et al. 2017). Another mechanism of defence is by small interfering RNAs (siRNAs) (Carbonell et al. 2019), and in addition to these innate immune mechanisms, plants can also mount adaptive immune responses to viral infections by receptor-like kinases which can trigger downstream signalling pathways leading to the activation of transcriptional reprogramming and the production of phytohormones and defence-related molecules (Zorzatto et al. 2015; Alazem and Lin 2015). To date, many R genes have been identified in various plant species (Hammond-Kosack and Kanyuka 2007). The R gene-mediated resistance usually confers a specific but reliable dominant resistance against a virus species or group of related viruses (Sett et al. 2022). In recent years, several public databases have been developed to store and disseminate information on genes involved in antiviral defence (Plant Resistance Gene Database; Plant R Gene Database, Amigo 2) (Sanseverino et al. 2012; Gene Ontology Consortium 2015; Calle García et al. 2022; Saxena et al. 2023). The databases provide information from a variety of plant species, including their sequences, annotations, and functional characteristics. By comparative and structural search of these sequences, it is possible to predict loci that may be associated with virus resistance in ash. Once candidate genes have been identified, SNP markers can be developed and used to analyse genetic variation between virus infected or not, symptomatic, or asymptomatic ash trees.

Genetic variation of gene candidates is a structural characteristic of these genes. However, to unravel the mechanisms that regulate their expression, one way is to identify cis-regulatory elements represented by non-coding DNA sequences. These are short sections of DNA located in the upstream region of the genes and serve as binding sites for transcription factors, being frequently summarized as sequence motifs (Santana-Garcia et al. 2022). For now, the DNA-binding motifs of hundreds of plant transcription factors have been determined (O'Malley et al 2016); tools for transcriptional regulation prediction and analyses were developed, inferring regulatory interactions between transcription factors and non-coding DNA sequences, and searches for the enriched upstream regulators in the input gene set (Jin et al. 2017). Discovering regulatory elements within natural genomic sequences is an important part of the prediction and validation of promoters (Contreras-Moreira et al. 2016).

Our study was performed to (1) describe homologues in the *F. excelsior* genome to formerly annotated genes involved in antiviral response in *Arabidopsis thaliana* L., (2) to predict and computationally validate these candidates' promoters by common regulatory motif prediction, (3) to design and test the applicability of primers on these genes' coding and promoter fragments in virus infected symptomatic and asymptomatic ash samples.

Material and methods

Plant material

Within the FraxVir project five ash tree study sites in different regions of Germany, including two intensive monitoring plots (IBF) from FraxForFuture (BB 1 Stegelitz/Melzower Forst and BY 1 Monheim/Kaisheim), two seed orchards (Emmendingen and Schorndorf) and a clone archive (Grabenstätt) were visually assessed and sampled (Table S1). For a preliminary PCR test of the newly designed primers, four DNA samples randomly selected from the Emmendingen seed orchard were used. Following the preliminary test, PCR amplification on DNA from eight leaf samples previously investigated in terms of virus infection (all infected, four with and four without symptoms, respectively) (Table S2) was performed. For virus detection, leaves from ash trees displaying virus-suspected symptoms, unspecific symptoms as well as from trees showing no symptoms were sampled between 2021 and 2023. Detailed information on the wet-laboratory procedures accomplished for virus detection (RNA isolation and cDNA synthesis, detection of viruses), sequencing, and RNA Seq analyses can be found in Rehanek et al. (2024).

Data sources

From AmiGO, the official web-based set of tools for searching and browsing the Gene Ontology database (https://amigo.geneontology.org/), 94 candidate genes were identified and annotated for GO term 0051607 (defence response to virus) in *Arabidopsis thaliana* L. (Table S3). Based on this list, a multiFASTA EST library containing exonic parts of these genes was constructed with publicly available FASTA sequences from *A. thaliana*, accessed, and retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/).

The latest *F. excelsior* and *Olea europaea* var. *sylvestris* L. reference genome assembly (RefSeq assembly accession: GCA_019097785.1 FRAX_001, GCF_002742605.1, respectively) was downloaded from the National Center for Biotechnology Information database (Sollars et al. 2017; Besnard et al. 2011; Unver et al. 2017). A protein BLASTX database comprising 22,913,321 sequences from all plant species was also generated with protein sequences accessed and retrieved from the NCBI database. The transcription factors and their binding sites prediction were accomplished by using data from PlantTFDB v5.0 (Jin et al. 2017), a portal for the functional and evolutionary study of plant transcription factors.

Bioinformatics data mining

The complete workflow is shown as a flowchart, presented in Fig. S1.

Searching for sequence similarities in the *F. excelsior* genome

A search of the *A. thaliana* sequences against the *F. excelsior* genome was performed by the Galaxy/Europe NCBI BLAST + BLASTN package (Camacho et al. 2009; Cock et al. 2015); the *A. thaliana* multiFASTA file was searched as nucleotide query against the genome as a nucleotide database. For this purpose, with the *F. excelsior* contigs (FASTA sequences served as inputs) used as the first step, a BLAST database was generated using makeblastdb, applied with no additional masking data. The BLASTN application was performed by setting shorter word sizes and using the best-hit-filtering algorithm, searching for only the best matches for each query reporting a match. The BLASTN search results were displayed in tabular format, with the –outfmt option. Alignments were visualised by Multiple Sequence Alignment Viewer 1.21.0 (https://www.ncbi.nlm.nih.gov/projects/msaviewer/). The second phase constituted the validation of the BLASTN results, by a subsequent BLASTX search of coding sites for proteins involved in the molecular control of defence against viruses.

Prediction of proteins encoded by the previously identified genes

As previously mentioned, the putative proteins involved in the molecular control of virus defence were identified and selected by mining the reference protein library of all plant species using the basic local alignment search tool BLASTX algorithm. For this purpose, with all plant protein libraries (FASTA sequences as inputs) compiled serving as the first step, a BLAST database was generated using makeblastdb, performed with no additional masking data. The BLASTX activity was performed by the Galaxy/Europe NCBI BLAST + s BLASTX tool (Camacho et al. 2009; Cock et al. 2015), with the following parameters: standard query genetic code, E-value of 1e-3, filtering of low-complexity regions, 0 maximum hits to consider, 1 maximum HSP to retain for any single query-subject pair, and 99–100 minimum query coverage per HSP. The BLASTX search results were displayed in tabular format with the –outfmt option.

Gene Ontology annotation of the predicted proteins

GenBank identifiers of the protein sequences were converted into UniProt identifiers using g:Profiler (Kolberg et al. 2020). To understand the biological meanings of the output of the BLASTX search result list, GO annotation was performed using the String web server (Snel et al. 2000) web application. The enriched biological themes, particularly GO terms, the enriched functional-related gene groups, the redundant cluster annotation terms pointing out the interactive proteins and the highlighted protein functional domains and motifs were also identified.

In-silico *determination of the candidate genes' promoters by transcription factor binding site prediction*

As a first step towards identifying regulatory signatures, each gene previously identified in *F. excelsior* with successful primer laboratory test result was searched against the well-annotated *Olea europaea* L. genome and the − 1500–0 bp upstream sequence relative to the transcription starting site of each was defined. In case of each candidate this 1500-bp-long sequence was searched again in the *F. excelsior* genome and extracted from the reference genome assembly. Then each candidate was scanned for transcription factor binding sites with help of PlantTFDB v5.0 (Jin et al. 2017) by checking the best hit with threshold p-value $\leq 1e-4$, the best hit to retain for any single query. Following this the transcription factor binding motif, the location of the binding site in the 1.5 kbp of the 5′ upstream region of each gene from the *F. excelsior* genome and the transcription factor that binds to each binding site was defined.

Primer design and PCR amplification

The design of the primers on the coding and promoter region of R gene candidates was performed with the primer design toolkit of CLC Genomic Workbench. The primer pairs were tested in silico using the Primer-BLAST toolkit (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) to search for paralogous sequences, in order to avoid simultaneous amplification of these paralogous loci.

The pre-test and final test of the applicability of the primers in the laboratory were carried out as follows: in

a 20 µl volume containing 1 µl of genomic DNA (about 20 ng), 0.12 µl Platinum TM Taq DNA Polymerase (5 U/µl) (Thermo Fischer Scientific, 2 µl 10 × PCR Rxn Buffer, 1 µl $MgCl_2$ (50 µM)); 0.6 µl dNTPs (2,5 mM each), 1.6 µl primers (10 µM each), by the following PCR (HOT PCR) programme: denaturation at 94 °C for 3 min (1); 94 °C for 50 s (2), 70 °C for 1 min (3), 10 cycles for step 2–3; 94 °C for 30 s (4); low stringency annealing amplification 55–60 °C for 50 s (5); 70 °C for 2 min (6), 35 cycles for steps 4–6; and final 5 min at 4 °C (7). The PCR products were analysed visually concerning their presence or absence, number, and size, by 1.75% agarose gel electrophoresis with Biozym LE Agarose and 1 × TBE as electrophoresis buffer.

Results

Sequence similarities in the *F. excelsior* genome

The NCBI BLAST + BLASTN search of all *A. thaliana* ESTs in the *F. excelsior* genome revealed 60 genomic sequences with high similarity. The identified *F. excelsior* DNA sequences in a next step were translated into proteins which were used to mine the "all plant" protein library. By this search with one exception (Nr. 23, identified in *Fraxinus americana* L.), all best hits were in *Olea europaea* L. (Table S4).

Annotation and functional association

As a result of the ontology association by STRING software, molecular functions, biological processes, and cellular component in case of 46 proteins were determined (Table S5). To summarize the correlations among significant pathways, the encoded proteins were clustered to a specified MCL inflation parameter set on three into five clusters, presented in Fig. 1 and summarized in Table S6, associated with RNA-mediated post-transcriptional gene silencing (cluster 1), cap binding complex (cluster 2), defence response (cluster 3), regulation of defence response to virus (cluster 4), and inositol phosphate metabolism (cluster 5).

The transcription factor binding site for each candidate gene was inferred to predict the promoter position. Altogether, 54 transcription factors were identified. Upstream regions of genes with successfully tested primer pairs matched known core DNA-binding elements. These elements and their position in the 1500-bp-long upstream region from the transcription starting site of each gene are presented in Table S7.

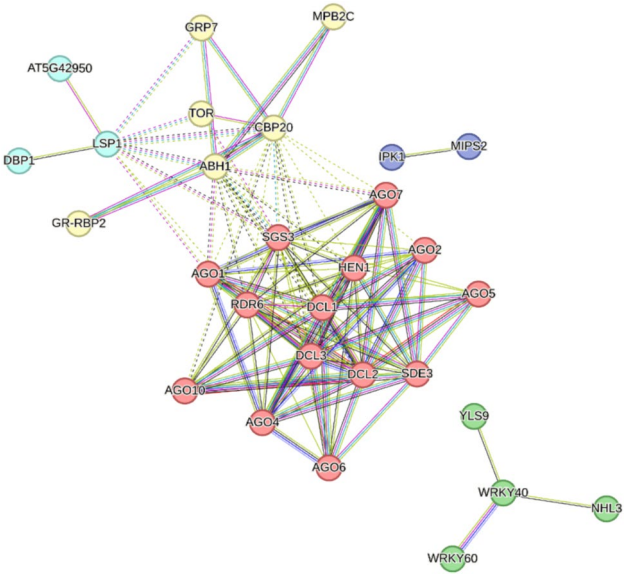

Fig. 1 MCL clusters based on the stochastic flow of the searched proteins generated by STRING. Cluster 1 (red)—RNA-mediated post-transcriptional gene silencing; Cluster 2 (yellow)—cap binding complex; Cluster 3 (green)—defence response; Cluster 4 (turquoise)—regulation of defence response to virus; Cluster 5 (blue)—inositol phosphate metabolism. Disconnected nodes in the network are not shown

Primer design and laboratory test of the primers

Initially, 60 primer pairs were designed and tested in the laboratory for coding regions. Primer sequences and the expected PCR products' length are presented in Table S8. Out of these 60 primers, 54 provided one-band amplicons in expected length (Fig. S2). To be able to analyse the cis regulation of these candidates, in a second step, new primers on these 54 genes' upstream promoter regions were designed (Table S9). From all promoter primers, 36 provided one-band amplicons (Fig. S3). Finally, of the total of 60 designed coding and 54 promoter primer pairs, 72 (36 + 36) were selected. With the selected primers, PCR amplification and sequencing of DNA from a larger number of—more easily symptom-accessible—seedlings will be performed. The selected primer pairs included only those that successfully generated single-band amplicons for both the coding region and promoter of each candidate gene.

Discussion

F. excelsior is relevant in the forest ecosystem throughout much of Europe (Pautasso et al. 2013), occurring as an admixed tree species in various forest communities (Ellenberg 2010). Moreover, ash has an important role in primary and secondary succession (Peterken 1993; Tapper

1996; Emborg et al. 2000; Marigo and Peltier 2000), in urban settings for its aesthetic appeal (Boshier et al. 2005; Beck et al. 2016) and is widely cultivated for its timber (Fraxigen 2005; Dobrowolska et al. 2011). Virus infections can have severe impacts on its health and survival, leading to significant losses in both forest and urban populations (Gaskin et al. 2021; Büttner et al. 2023; Vainio et al. 2024). Therefore, the understanding of the complex plant defence mechanism against viral infections, especially in the ash dieback context, is crucial for the development of effective control strategies. Identification of genes involved in defence response after pathogen attack could greatly help the development of gene-targeted molecular markers for breeding that can significantly contribute to the sustainable control of diseases (including virus-induced) in forest tree species (Younessi-Hamzekhanlu and Gailing 2022). Our study was aimed at describing homologues in the *F. excelsior* genome of formerly annotated virus defence-related genes and to test the applicability of primers designed on these gene fragments. By similarity search of *A. thaliana* ESTs in the *F. excelsior* genome, sequences with high similarity were found. Same type of comparative genomic approaches and their benefit towards understanding other non-model plant genomes were reported in several studies like in Hall et al. (2002), Wang et al. (2015), Ingvarssson et al. (2016), Batalova et al. (2022), or Vuruputoor et al. (2023). The translation of the sequences found and search against a protein database revealed that all encoded proteins can be found in the closely related species *Olea europaea* L. presumably for the reason that these sequences are in interspecies conserved regions. Some of the encoded proteins participate in post-transcriptional gene silencing. Such proteins include members of the AGO protein family (DCL1, SDE3, SGS3), and some are involved in disease-resistance (NHL3, RPP8, YLS9, CRH1) and mRNA degradation inhibition (TOR). They play a role in RNA transcription (GRP7, GR-RBP2, NTL9), antibody recognition (MIPS2), salicylic acid, and jasmone acid-mediated defence signalling (SSI2), in processing natural small interfering RNAs (DCL2), and they regulate bacterial and viral pathogen responses (CRY2) or are pathogen-induced transcription factors (WRKY40, WRKY60). Despite being a relevant tree species, relatively limited work in biotic resistance-related marker development has been performed on common ash and mainly in relation to ash dieback (Harper et al. 2016) or emerald ash borer (Hale et al. 2021). As variation in the virus resistance genes and their cis-regulatory elements is essential for the defence process against viruses, in our consideration, the future search in terms of polymorphism of the PCR products amplified by our primers may result in several markers that could be used in prospective studies to unveil divergence between virus-tolerant and susceptible ashes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41348-024-01018-5.

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Author contributions BF and ZAK planned the experiments. SvB, MR, and ZAK collected material on sampling tours. MR performed virus detection; ZAK performed the experiments. ZAK analysed the data and results. ZAK wrote the first draft of the manuscript. BF, SvB, MR, and CB revised and proofread the manuscript. BF supervised the research work. All authors have read and approved the final version of the manuscript.

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Data availability All data generated or analysed in this work are available in the published manuscript.

Declarations

Competing interests We have no conflicts of interest and all authors have agreed to submit this manuscript to Journal of Plant Diseases and Protection.

Ethical statement We confirm this work is not under consideration for publication elsewhere, and all authors and institutions have approved the manuscript for submission.

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