Species specific marker genes for systemic defence and stress responses to leaf wounding and flagellin stimuli in hybrid aspen and silver birch

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Abstract:

In Northern Europe, climate warming is driving the northward expansion of deciduous tree species such as aspen and silver birch. At the same time, it is intensifying biotic stresses from pests and pathogens, necessitating the development of innovative biotechnological approaches to enhance forest resistance and resilience. However, progress in understanding the genetic basis of stress responses in temperate deciduous trees remains limited, largely due to the absence of reproducible laboratory model systems and well-characterized molecular stress markers. In this study, we aimed to identify and validate known plant defense gene markers to support future functional research on stress responses in hybrid aspen and silver birch. We used sequence mining and phylogenetic analyses to identify homologues of biotic stress-response genes in the genomes of both tree species. We then employed *in vitro* propagated tree clones to test defense gene activation in distal leaves following systemic signal induction via leaf wounding and bacterial flagellin application, assessed at 4- and 24-

hours post-treatment. We present a short communication of a novel *in vitro* methodological approach for the preliminary evaluation of molecular responses to systemic signaling in birch and aspen. The findings represent a methodological advancement for studies of systemic signalling and molecular defense responses in ecologically and economically important tree species and offer a pipeline for gene homologue discovery for the future research on tree-microbe interactions and resilience.

Keywords: tree stress responses, systemic signals, defence markers, *Populus tremuloides* × *P. tremula* L., *Betula pendula* Roth, clonal plantations, *in vitro* assays, forest productivity

1. Introduction

Accelerating climate change, intensifying weather extremes and their legacy effects, such as outbreaks of pests and diseases (Meier *et al*., 2022; Allen *et al*., 2015), are subjecting trees to increasingly intense stresses (Seidl *et al*., 2017). These stresses can exceed the adaptive capacity (i.e., phenotypic plasticity) of local tree populations (Aitken and Bemmels, 2016), emphasizing the urgent necessity for adaptive management strategies (Sousa-Silva *et al*., 2018) to cope with growing environmental challenges (Isaac-Renton *et al*., 2018). Given the long life cycle of trees and the stresses associated with large-scale environmental changes (Moran *et al*., 2017; Seidl *et al*., 2017), effective adaptive measures should be self-sustaining and grounded in a thorough understanding of ecological, ecophysiological, and biochemical processes (Jansson *et al*., 2017). Examples of such measures include locally finetuned management (Murray *et al*., 2025; Forrester and Bauhus, 2016), as well as the inoculation of plants with beneficial microbes, such as mycorrhizal fungi, which have been suggested as promising strategies to enhance forest resilience against increasing environmental stresses (Lutter *et al*., 2023; Ciadamidaro *et al*., 2017; Bois *et al*., 2005).

In the eastern Baltic region, climate-driven shifts in forest species distribution are anticipated, with deciduous species expected to push conifers northward (Buras and Menzel, 2019). Under these conditions, the prevalence of silver birch (*Betula pendula*) and aspen (*Populus tremula*) — both currently of high commercial importance — is projected to increase (Dubois *et al*., 2020; Tullus *et al*., 2012). However, both species are sensitive to climate (Matisons *et al*., 2022; Šēnhofa *et al*., 2015), and thus, an intensification of biotic legacy effects, such as insect pest outbreaks following climatic disturbances, is also expected (Gely *et al*., 2020; Seidl *et al*., 2017). Additionally, the use of specific, highly productive genotypes — such as the most economically valuable birch clones or superior hybrid aspens

— is recommended for commercial forestry (Bāders *et al*., 2024; Tullus *et al*., 2012). This underscores the need to identify innovative biotechnological approaches for improving stress tolerance in selected high yielding genotypes and to enhance understanding of tree stress responses at a molecular level (Estravis-Barcala *et al*., 2020; Bradshaw *et al*., 2019; Lelu-Walter *et al*., 2013).

Molecular stress responses have been extensively studied in herbaceous crop plants and model species such as *Arabidopsis thaliana*, however, trees with their large and complex genomes have been comparatively understudied and often limited to genus *Populus* (Harfouche, 2014). This has hitherto limited functional studies on the genetic mechanisms involved in biotic stress responses in other economically important tree species. Nevertheless, recent advances in sequencing the genomes of important forestry species such as silver birch (Salojarvi *et al*., 2017) or several aspen species (Lin *et al*., 2018) have catalysed recent progress in elucidating the molecular basis of tree responses to abiotic stresses (Estravis-Barcala *et al*. 2020). Similar to *Arabidopsis*, tree responses to chilling (frost), heat and drought, are likely mediated by abscisic acid (ABA), ROS and Ca^{2+} signalling, mitogen-activating protein kinases (MAPK), heat-shock proteins (HSP), MYB and bZIP family transcription factors (among others), resulting in antioxidant production, changes in membrane fluidity and repair (reviewed by Estravis-Barcala *et al*., 2020; Harfouche *et al*., 2014). Still, markers for tree responses to herbivore attack or microbial infections remain to be disclosed.

The study aimed to identify plant defence gene homologues in hybrid aspen and silver birch and to use *in vitro* propagated tree clones for characterisation of tree leaf responses to systemic wounding and immunogenic peptide flagellin that mimic the mechanical damage by chewing herbivores and bacterial elicitor recognition by plant PTI receptor FLS2 (Chinchila, 2006), respectively. To this end we mined hybrid aspen and silver birch genomes for plant wound response genes in the jasmonic acid pathway, including *LOX, JAZ* (Fürstenberg-Hägg, 2013), as well as response genes associated with salicylic acid defence responses to bacterial elicitors, including *FRK1, NHL10, MPK3, PAD4 and MYB51* (Boudsockq *et al.*, 2010; Cui *et al.*, 2017). For the first time we employed *in vitro* propagated tree clones to identify tree defence marker genes and characterise their activation in distal leaves after the induction of systemic signals by leaf wounding and treatment of bacterial flagellin at different time-points. Here we present a novel *in vitro* methodological approach for the initial testing of molecular responses to systemic signals in birch and aspen for future downstream application in intra- and inter-tree signalling research.

2. Materials and Methods

2.1. In-vitro propagation of tree clones

Silver birch and hybrid aspen clone cultures were obtained from the clonal collection of the LSFRI "Silava" plant physiology laboratory. For hybrid aspen *Populus tremuloides* × *tremula*, clone "44" was selected, as it shows superior field performance (Šēnhofa *et al*., 2016). For silver birch, clone "54-146-143", which shows above average field performance and exceeds wild populations by 7–30 % in volume growth (Gailis *et al*., 2020; Jansson *et al*., 2017), was used. The clones were cultivated *in vitro* on 1X Murashige and Skoog (MS) media, supplemented with MS vitamins, MS micronutrients, 20 g/L sucrose, 6 g/L of Agar and 0.1 mg/L indole-3-butyric acid (IBA) at pH=5.8 (Kondratovičs *et al*. 2022).

Each clone (plantlet) was cultivated individually in a 150 mL jar filled with 15 mL of the media. During the cultivation, the jars were covered with an aluminium foil cap to prevent microbial contamination whilst enabling air flow and easy access to the plantlet for manipulation and stress treatments. To ensure controlled environmental conditions, all the clone plantlets were grown in a climate chamber maintained at 30-40% relative humidity and 25 □ on four multi-layer shelf systems equipped with luminaries. All clones were grown under the same illumination with a photon flux density of 110 ± 10 µmol m^{-2} s^{-1}, for the wavelengths ranging from 400 to 750 nm with 16 h light and 8 h dark photoperiod to imitate long-day conditions of growing period. For each tree species, a total of 32 plantlets were cultivated for the assessment of stress responses in orthogonal time-course experimental design, allowing four biological replicates per treatment and observation combination.

2.2. Stimulation of local leaves and sample collection

To assess the defence-related gene expression, the plantlets were subjected to three different treatments: a chemical stimulus with 22 amino acid fragments of bacterial flagellin (*flg22*), mechanical injury to leaves and the combination of both. These stimuli were chosen to imitate a herbivore attack (mechanical injury) or the attack of a bacterial pathogen (*flg22*) to activate defence responses related to jasmonic and salicylic acid pathways, respectively (Felix *et al*., 1999; Vlot *et al*., 2009; Fürstenberg-Hägg, 2013). The mechanical injury was applied by squeezing the leaf with tweezers in three parallel lines, while the flagellin stimulus was applied by pipetting 5 µL of 1 µM *flg22* solution (in water), on the abaxial surface of the leaf. For the combined treatment, *flg22* was applied on the wounded abaxial surface of the leaf. As the control, 5 µl of distilled nuclease free water was used.

The stimuli, including the control, were applied to the third fully unfolded leaf from the apex, marking the stimulated leaf on the outside of the jar. Plantlets were treated in a sterile environment of laminar flow before returning to the climate chamber. For the gene expression analysis, a non-stimulated (naïve) leaf - second fully unfolded leaf from the apex - distal from the treated leaf was collected 4 and 24 h after the treatment to characterise the short-term dynamics of systemic plant responses. To preserve RNA, the samples were flash-frozen in liquid nitrogen and stored at -80 °C.

2.3. RNA extraction and cDNA synthesis

To extract total RNA from the leaf samples, a modified protocol from Rubio-Piña and Zapata-Pérez (2011) was used. Prior to the extraction, all labware was treated with RNaseZap™ tissues to eliminate any traces of contaminants. The 1.5- and 2-mL test tubes were submerged in 0.1% DEPC solution for 24 h and autoclaved three times at 121 °C for 40 min, using saturated steam with pressure of at least 103 kPa (15 psi), to ensure the degradation of DEPC. Leaf samples were homogenized in liquid nitrogen using a pestle and mortar before transferring the material into 2 mL test tubes with 900 µl CTAB extraction buffer (2-cetyltrimethylammonium bromide; 0.1M Tris-HCl (pH 8); 1.4M NaCl; 20mM Ethylenediaminetetraacetic acid (EDTA) (pH 8); 2% polyvinylpolypyrrolidone (PVPP)) and 100 µl of β-mercaptoethanol). The mixture was shaken for 30 s and incubated in a thermoblock for 10 min at 65 □. After incubation, 800 µl of chloroform were added to the mixture, which was followed by repeated shaking for 30 s. The mixture was then centrifuged for 10 min at 4 □ and 10’000 rpm.

The supernatant was transferred to a new 2 mL test tube together with 800 µl of a phenol/chloroform/isoamyl alcohol mixture (50:49:1) and shaken for 30 s. Samples were then centrifuged for 10 min at 10’000 rpm and 4 □ and the supernatant was transferred to a new 2 mL test tube together with an equal volume of a chloroform and isoamyl alcohol mix (24:1). The samples were then shaken for 30 s and centrifuged at 10’000 rpm at 4 □. The supernatant was transferred to a new 1.5 mL test tube together with a third of the supernatant volume of 8M LiCl. Subsequently the samples were incubated for 24 h at -20□, after which they were centrifuged for 20 min at 4 □ and 10000 rpm.

The sediment pellets were then washed with 1 mL 96% and 70% ethanol and centrifuged for 5 min at 10000 rpm. After centrifugation, ethanol was discarded, and the samples were dried at room temperature. The sediment pellets were then dissolved in 15 µl of 1x TE buffer with 1 µl of 1u/µl RNasin® Plus ribonuclease inhibitor solution (Promega). The

mix was then treated with RQ1 Rnase free Dnase kit (Promega) according to manufacturer instructions. This process was followed by RNA extraction using a phenol/chloroform/isoamyl alcohol mixture (50:49:1). For convenience, 230 µl of 1x TE buffer were added to 10 µl of the sample. The phenol/chloroform/isoamyl alcohol mixture was added at a ratio of 1:1 to the sample solution.

After the extraction, RNA was sedimented by adding 0.1 volume of 3M sodium acetate and 2 volumes of 96% ethanol, which was followed by 24 hours of incubation at -20 □. Following the incubation period, the mixture was centrifuged for 15 min at 13'000 rpm and 4 □ and subsequently washed with 70% ethanol. The RNA sediment pellets were dried at room temperature and dissolved in 20 µl of TE buffer together with 1 µl of RNase inhibitor. The mRNA samples were stored at -80 □. Total mRNA sample concentration and purity were measured using a "NanoDrop 2000" spectrophotometer (Thermo Scientific).

2.4. rt-qPCR analysis

To carry out qPCR the extracted total mRNA was used as a template for cDNA synthesis. Reverse transcription reaction mix was prepared using the Thermo Scientific Maxima First Strand cDNA synthesis kit according to manufacturer instructions using 500 ng of total RNA. The reaction was carried out using a BIO-RAD T100™ thermal cycler and the reaction mix was subsequently diluted to 800 µl. The qPCR reactions were carried out using the Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) kit, with each reaction containing 2 µl of 0.3 µM primer solution (containing both forward and reverse primer), 5.5 µl of sample cDNA solution and 7.5 µl of the Maxima SYBR Green/ROX qPCR Master Mix (2X). For each sample, three qPCR reactions (technical replicates) were carried out with each primer pair. The resulting average Ct value was then used in further calculations.

Primer efficiency (E%) was calculated using the formula $\left(10^{\frac{-1}{Slope}}-1\right) \times 100$, with slope being obtained by plotting the $\log_{10}$ values of dilution factors (0-7) in a serial dilution of the gene specific qPCR product on the x axis and average Ct value of three technical replicates on the y axis. The expression of each gene was normalized to the expression of the chosen reference gene *ACT2* using the formula $\frac{E_r^{Ct_r}}{E_g^{Ct_g}}$ where E_r - the amplification factor of the reference gene, E_g - the amplification factor of the gene of interest, Ct - the average Ct value of the three qPCR technical replicates for the reference gene and the gene of interest

respectively. In total, four expression values were obtained for each gene, for all stimuli groups and at each time point for both birch and hybrid aspen.

2.5. Primer design

In order to observe potential differences in defence related gene expression in naïve systemic plant tissue (distal leaf), 8 different genes with a well described role in *Arabidopsis thaliana* defense mechanisms were chosen as the qPCR targets (**Supplementary material, Table S1**); *EIN2, FRK1, PAD4, LOX2, MPK3, NHL10, JAZ10, MYB51*, with *ACT2* chosen as a constitutively expressed reference gene for both taxa. Using gene specific protein sequences obtained from The Arabidopsis Information Resource (TAIR), BLAST was used to find potential defense gene homologues in the genomes of both tree taxa. To increase the chances of finding optimal gene homologues, BLAST search was performed in two databases for each taxon: the National Centre for Biotechnology Information (NCBI) database was used for both taxa (*Populus tremula x tremuloides taxid:47664; Betula pendula taxid:3505)*, while Comparative Genomics (CoGe) and PlantGenIE were used for birch (*Betula pendula scaffold assembly id35079, id35080*) and hybrid aspen (*Populus tremula x tremuloides v10*), respectively. The BLAST search results with the highest homology identifiers (query coverage, percent identity and e-value) were chosen as the potential homologues of each gene and subjected to reciprocal BLAST against *A. thaliana* and other taxa included in the NCBI database.

A complementary phylogenetic analysis of the selected tree homologues was performed using the MEGA (v11.0.13) Maximum likelihood algorithm (bootstrap=500). In addition to the potential tree homologues, additional *A. thaliana*, *Medicago truncatula* and *Marchantia polymorpha* sequences from NCBI and MarpolBase were included in the phylogenetic trees. Based on the *in silico* gathered data, a single potential homologue was selected for each gene. Using NCBI Primer-BLAST, a primer pair was designed for each selected gene sequence. All primer pairs were designed with T_m of around 60 □ (with ±1 □ as the maximal deviation) and PCR product size of 100-300 nt.

2.6. Statistical analysis

To assess differences in gene expression between treatment groups, a two-way, single factor analysis of variance (ANOVA) was performed along with Tukey HSD post-hoc tests in cases of significant differences, using base R. In cases where ANOVA assumptions were not met, non-parametric Kruskal-Wallis tests and subsequent post-hoc Dunn tests were

performed. Kruskal-Wallis tests were done using base R, while the "dunn.test" package (Dinno A, 2024, R package version 1.3.6.) was used for the Dunn post-hoc tests. A significance level α of 0.05 was used for all hypothesis significance tests. Heatmaps were generated using the *pheatmap* function from the "pheatmap" package (Kolde R., 2019, R package version 1.0.12) in R with centroid linkage clustering based on Euclidean distance. To assess the interaction between wound or *flg22* treatments and the different time-points of gene-expression across the 6 gene panel, a multivariate linear model was used where gene Fold Change ~ Treatment * GeneID * Time-point. The statistical models were checked for compliance with assumptions via diagnostic plots. Data analysis was conducted in R version 4.5.0. (R Core Team).

3. Results

3.1. Identification of plant stress response gene homologs and primer design

To identify plant defence gene homologs in silver birch and hybrid aspen, the BLAST hits from *Arabidopsis thaliana* query protein sequence were used in an additional reciprocal BLAST against all NCBI plant taxa. Candidate tree sequences with 99% coverage to any plant protein with a matching gene annotation were shortlisted by the employed BLAST algorithms. To confirm the homology, a phylogenetic analysis using all candidate homologues for a gene of interest (GOI) from hybrid aspen (**Supplementary material, Figure S1**) and silver birch (**Supplementary material, Figure S2**) were compared alongside well annotated herbaceous plant (*A. thaliana* or *M. truncatula)* homologues and liverwort *M. polymorpha* homologue as an outgroup. For each GOI, tree sequences with highest coverage and identity to *A. thaliana* and *M. truncatula* and different from *M. polymorpha* were selected for primer design (**Supplementary material, Table S1**). Primer pairs displaying a single-peak melting curve, as well as yielding the desired amplicon (**Supplementary material, Figure S3 A, B**) were further tested for their PCR efficiency (E%) which typically ranged between 90-110% (**Supplementary material, Table S1**). Tree *ACT2* homologue was selected as a reference gene as it displayed earlier amplification (Ct=18) and more uniform expression across different treatments compared to *TUB*, *SAND* or *GAPDH* (**Supplementary material, Figure S3 C**).

3.2. Characterisation of systemic response marker expression

The tested wounding and *flg22* stimuli and their combination demonstrated significantly different effects (relative to the control) on gene expression patterns in silver

birch ($p<0.01$), as well as hybrid aspen ($p=0.03$), indicating clear inducible systemic responses in the distal leaves (**Supplementary material, Table S2**). The gene expression patterns showed strong dependency on time after stimulation of the local leaf, highlighting species specific early and late response markers of biotic stress (**Figure 1**). Birch showed significant early induction of *LOX2* ($p=0.03$), *MPK3* ($p<0.01$) and *EIN2* ($p=0.03$) 4h after wounding. The effect, however, was clearly transitory as it disappeared after 24h. In contrast, *flg22* treatment did not display any significant gene induction in systemic tissues neither 4 h nor 24 h after stimulation of the local leaves. 4h after the application of the combined wounding and *flg22* treatment only *MPK3* expression was significantly elevated ($p=0.03$), indicating potential antagonistic responses to wounding and *flg22* treatment in silver birch.

In contrast to silver birch, hybrid aspen showed significant interaction between treatment type, identity of the activated genes and timepoints after stimulation ($p<0.001$) (**Supplementary material, Table S2**), indicating complex control. Only the combined wounding and *flg22* treatment induced significant aspen marker gene activation at 4 h and 24 h (**Figure 1**). *JAZ10* was significantly ($p=0.01$) induced 4h after the combined wounding and *flg22* treatment and displayed increased expression relative to the water control in the wounded plants as well, albeit not significantly. Surprisingly, the wound-responsive JA pathway marker *LOX2* showed no significant induction in hybrid aspen at neither 4 h nor 24 h after the stimulation. The ethylene signalling gene *EIN2* displayed significant suppression ($p=0.01$) 24 h after the combined wounding and *flg22* treatment. Still, it demonstrated a tendency to be induced after wounding, suggesting potential opposing effects of *flg22* and wounding in the systemic responses of hybrid aspen leaves (**Figure 1**).

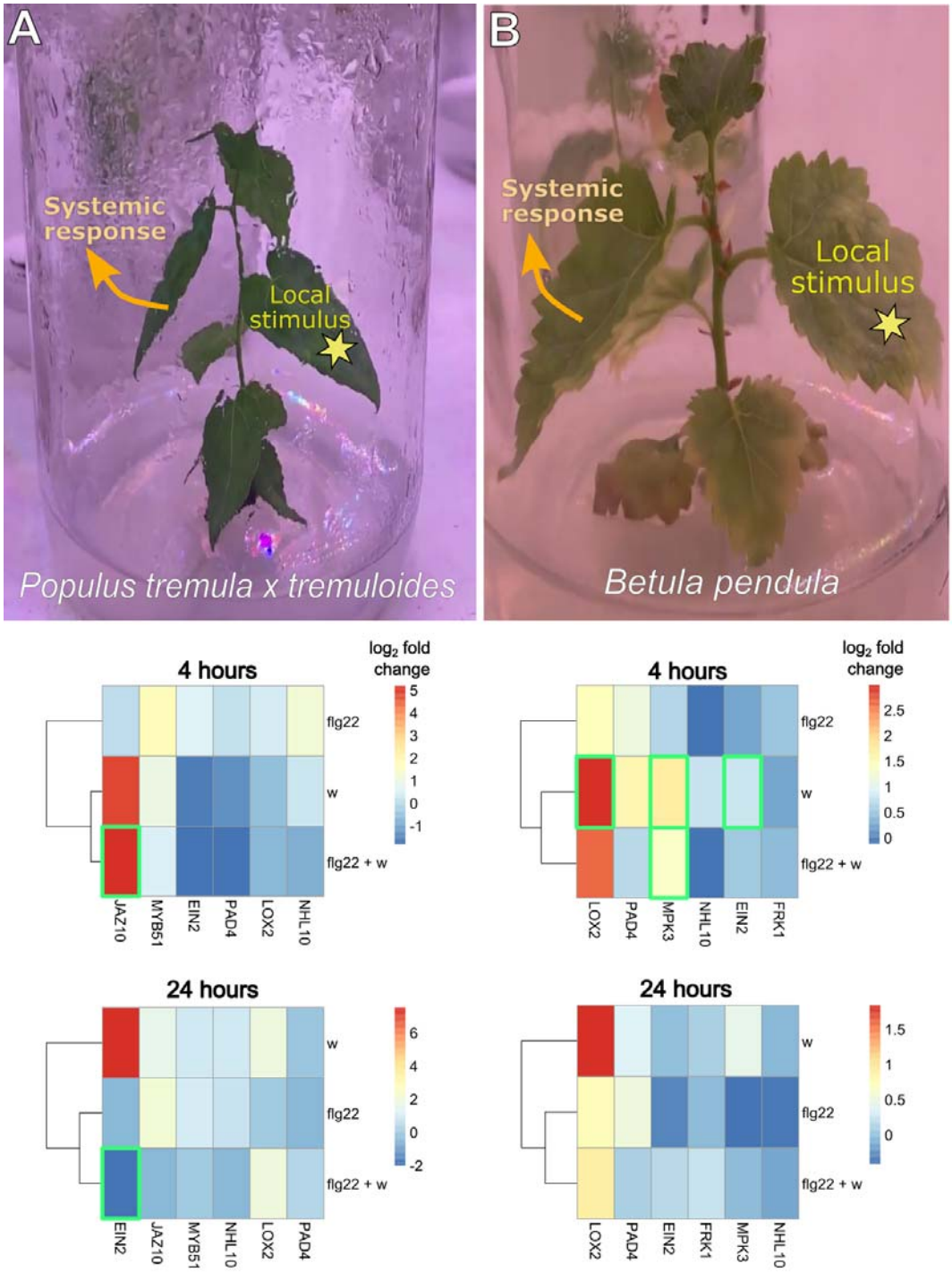

Figure 1. Experimental setup and relative marker gene expression in systemic leaves of hybrid aspen (column A) and silver birch (column B) clones in response to wounding and *flg22* treatment of local leaves. The heatmaps demonstrate $\log_2$ fold changes in gene expression (x-axis) for each treatment group (y-axis) relative to the water control at 4 hours and 24 hours post stimulation. The green rectangles indicate statistically significant ($p<0.05$) differences in gene expression in systemic leaves based on ANOVA post-hoc tests. Local leaf stimuli are *flg22* - flagellin; *w* - wounding; *flg22 + w* - combination of flagellin with wounding. Response similarity to treatments based on centroid linkage clustering by Euclidean distance. Full relative expression data along with baseline expression in control treatments are displayed in **Figure S4 (Supplemental material).**

4. Discussion

4.1. In vitro tree clones as models for plant systemic signalling research

The present study is the first to our knowledge to employ *in vitro* propagated tree clones as a model for studying intra-tree responses to stress signals and offers a scalable screening platform for design and discovery of tree stress response marker genes to diverse biotic stressors and their combinations. The applied methods provide certain potential advantages, including rapid propagation time, genetic uniformity due to clonal propagation, controlled growth and media conditions, sterile environment absent from uncontrolled pest or pathogen infections and the ability to precisely subject trees to selected defence elicitors or individual stress elements, potentially overcoming many challenges in future elucidation of molecular mechanisms for tree biotic and abiotic stress tolerance associated with heterogeneity and interaction of multiple environmental and stress factors in greenhouse and field studies (Estravis-Barcala, 2020; Harfouche *et al*., 2014). The early identification of stress markers via the *in vitro* system would accelerate follow-up functional studies in saplings grown on soil in growth chambers, greenhouse or field conditions for a range of different biological questions.

The experimental system herein allowed for optimal selection of tree stress inducing treatments and their response time estimation. For example, we discovered that only the combined treatment of wounding and *flg22* application to local leaves triggered significant systemic activation of JA-response gene *JAZ10* after 4h in hybrid aspen (**Figure 1**), while the individual treatments failed to induce consistent early responses. In contrast, leaf wounding displayed the strongest early response in silver birch compared to the other treatments. Selection of reliable and reproducible stress inducing treatments and their marker genes could be a key primer for several applications such as mechanistic studies on inter-tree signals and responses (Orlovskis *et al*., 2024) in the future.

4.2. Species specificity of SA or JA marker responses upon wounding or flg22 application

While certain markers of early responses in pattern triggered immunity (PTI) have been well described in *Arabidopsis* such as early response genes *NHL10 (NDR1/HIN1-LIKE 10*) or MAPK-specific target gene *FRK1 (FLG22-INDUCED RECEPTOR KINASE1)* (Boudsocq *et al*., 2010), no significant gene expression differences compared to the water control treatment were observed for these markers in *flg22* treatment in birch or aspen, potentially indicating different timing or species specific differences in the mechanisms of

early FLS2-mediated PTI signalling and their responses. These can be attributed to species-specific differences in the recognition of shortened *flg22* ligands by FLS2 receptor- co-receptor complexes (Mueller *et al.,* 2012) or, alternatively, heterogeneity of flagellin fragments (Colaianni *et al.,* 2021) produced by leaf endophyte communities that may interfere with responses to exogenously applied pathogenic flagellin.

PAD4 (PHYTOALEXIN DEFICIENT4) and *EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1)* constitute a well-established module in PTI (Pruitt *et al*., 2021), as well as abiotic stress (Szechynska-Hebda *et al*., 2016) signalling and a positive regulator of salicylic acid (SA) pathway in *Arabidopsis* (Wiermer *et al*., 2005). However, *PAD4* did not display significant response to *flg22* or combined wound and *flg22* treatments of the tree species, suggesting a potentially different *flg22* perception and signalling mechanisms than *Arabidopsis*. Both *PAD4* and plant transcription factor *MYB51* are also involved in glucosinolate and callose metabolism in innate immune responses to *flg22* (Bednarek *et al*., 2009; Clay *et al*., 2009) and mediate plant responses to phloem feeding insects (Kim & Jander, 2007; Luis & Shah, 2015). When tested in hybrid aspen, *MYB51* did not show any significant responses to wounding or *flg22* treatments.

LOX2 (LIPOXIGENASE2) encodes a key enzyme in jasmonic acid (JA) biosynthesis (Wasternack *et al*., 2013) while *JAZ10 (JASMONATE ZIM-Domain10)* is an important component in downstream JA signalling (Chini *et al*., 2007) in wound responses and control of glucosinolate production in *Arabidopsis* (Fürstenberg-Hägg *et al*., 2013). Interestingly, *LOX2* was a good early wounding marker in birch but not in aspen (**Figure 1**), indicating species specific responses or response timing to wounding. However, *JAZ10* appeared to be a good marker for the combined wounding and *flg22* treatment in aspen and displayed upregulation upon wounding as well. Since the JA and SA pathways are considered antagonistic (Gimenez-Ibanez & Solano, 2013), this may potentially illustrate a more complex interaction between SA and JA pathways in hybrid aspen compared to *Arabidopsis*.

MAPK (MITOGEN ACTIVATED PROTEIN KINASES) are important regulators of herbivory associated local wounding as well as plant systemic responses (Hettenhausen *et al*., 2015), and appear to be a useful marker for the systemic combined wounding and *flg22* responses in silver birch (**Figure 1**). *MPK3* and *MPK6* are known activators of ethylene (ET) biosynthesis in *Arabidopsis* (Liu & Zhang, 2006; Broekgaarden *et al*. 2015). As reviewed in Broekgaarden *et al*. (2015), ET signalling also regulates *flg22*–triggered PTI and hormonal crosstalk between SA-mediated responses to biotrophic pathogens and JA-mediated responses to wounding and chewing herbivores. *ETHYLENE INSENSITIVE2 (EIN2)* is an

important ET signalling component (Yoo *et al*., 2009) and was induced in silver birch upon wounding, consistent with the observed *LOX2* and *MPK3* responses and involvement in wound signalling (Broekgaarden *et al*. 2015). Interestingly, the tendency for the opposite regulation in *EIN2* during wounding and the combined wounding and *flg22* treatment in hybrid aspen may be indicative of the potential crosstalk between *flg22*-dependent SA pathway and wound-inducible JA pathway in hybrid aspen.

Conclusion

We have identified *BpLOX2*, *BpMPK3* and *BpEIN2* as suitable early response markers for wounding responses in silver birch, while *PttJAZ10* and *PttEIN2* as strong markers for hybrid aspen response to combined wounding and flagellin application. Selection of biotic stress treatments and response marker identification will aid future studies on intra- and inter-tree systemic stress responses using *in vitro* and soil propagated tree clones.

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Supplementary materials

Table S1. List of silver birch and hybrid aspen marker genes used in the study. Primer sequences are provided alongside the corresponding gene ID from TAIR, GOGE, PlantGene databases. Amplification factor was calculated based on 10-fold template DNA dilution series ranging from 100ag to 1ng and displayed for all genes used in **Figure 1**. Genes that did not display a specific single band in the electrophoresis and did not produce consistent melting curves (**Figure S3**), the amplification factor was not calculated.

Gene name	TAIR reference number	Species	Reference number	Forward primer	Reverse primer	Amplification factor E
EIN2	at5g03280	*Betula pendula*	Bpev01.c0890.g0001.m0001	CTGTTGGGAGCGAAGGAGTT	AATCTCTCCCTGCCAGCCAC	2.0509[illegible]
		Populus tremuloides x tremula	Potri.04[illegible]g[illegible]25.4	CCTAATTGTTGGGGAGGGCTG	CTGGTGGGAGCAGGGATTT	2.2[illegible]
LOX2	at3g45140	*Betula pendula*	Bpev01.c0328.g0011.m0001	TTAGCGTTGGTGGCTTGCTC	CCCCGAAGTCTCTGGGACT	1.9[illegible]
		Populus tremuloides x tremula	Potri.0[illegible]3g[illegible].5	AGAGGGCACATTAAGGCCAC	GATGCATAACGCTGAGCTGC	2.0[illegible]
PAD4	at3g52430	*Betula pendula*	Bpev01.c0134.g0001.m0001	GGACACCGAACCTGAACAGT	TTGACCCATTTAGCACGCCT	1.9[illegible]
		Populus tremuloides x tremula	Potri.05[illegible]g[illegible].1	GGATTGCTTGCTGTGCCAACG	AATGCTACACCTAGGCGACC	2.0[illegible]
RNL28	At3g[illegible]	*Betula pendula*	Bpev01.c0157.g0001.m0001	AGAGCTTTGGCACGCGCGTG	TCGCAGTTGATCTTTGGCCTT	1.9[illegible]
		Populus tremuloides x tremula	Potri.047[illegible]g[illegible]77.1	CGTGACCCTCCTCCTCAAAC	TAGCGGGCCATGTTGTTGGT	1.9[illegible]
RLP2	at3g[illegible]	*Betula pendula*	Bpev01.c1235.g0009.m0001	AGCCCAATCCAAGAGGCGTA	ACCACTGGCATTGCGGGAGA	1.9[illegible]
		Populus tremuloides x tremula	Potri.0[illegible]g[illegible].3	ACCACTGCTGAACGGGAAAT	CTGGGCAACGGAATCTCTCA	2.0[illegible]
MPK3	At3g45640	*Betula pendula*	Bpev01.c1085.g0001.m0001	CGACTCACAGAGGGCAGTTT	TCTTCATCGCGAGCATCTCG	2.0[illegible]
		Populus tremuloides x tremula	Potri.0[illegible]g[illegible].1	CTCGGCCAACTTCGGAGAAT	CATGGGTTTGCCAAGAAGC	ND
PAL1	At2g19990	*Betula pendula*	Bpev01.c0[illegible].g0001.m0001	TTTCATTTCCGGTTGGGCTC	GCCCACCCTGAGTGTATGTC	2.0[illegible]
		Populus tremuloides x tremula	AC[illegible].1	GTGGCACAGGGGATTTGCTA	ACCATACTCTGGGCCATTGT	ND
JAZ10	at5g13220	*Betula pendula*	Bpev01.c0[illegible].g0001.m0001	GCAAAGCGCCATTTCCAAGA	ATGTTCTCGGCTTGTTCTGG	ND
		Populus tremuloides x tremula	Potri.00[illegible]g[illegible].1	GGAACCGTTGCGGTTTTCAA	ATGGGGACACCGAAGTCAAC	1.9[illegible]
MYB51	AT1G18570	*Betula pendula*	Bpev01.c0421.g0001.m0001	AGTTGCAGGCTGAGGTGGAC	ACGTCCTGGAAGATGGCTTG	ND
		Populus tremuloides x tremula	Potri.0[illegible]g[illegible].1	TCTTCCCAACCCACAAGTCG	TGGAGCCCATTCCATTGCTT	1.7[illegible]

Table S2. Generalized linear model results for testing the differences in gene expression between treatments across the panel of genes used in Figure 1. The model tests for the interaction of wound or *flg22* treatments with 4h and 24h timepoints and individual expression patterns of each gene (Fold Change ~ Treatment * GeneID * Time-point).

Silver birch	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**Significance**
Treatment1	**2**	**8,428**	**4,2139**	**5,4797**	**0,00545**	******
Gene	**5**	**9,33**	**1,866**	**2,4266**	**0,04002**	*****
Timepoint	**1**	**26,904**	**26,9036**	**34,9851**	**4,20E-08**	*******
Treatment1:Gene	**10**	**6,048**	**0,6048**	**0,7865**	**0,64162**	
Treatment1:Timepoint	**2**	**0,677**	**0,3385**	**0,4401**	**0,64513**	
Gene:Timepoint	**5**	**9,334**	**1,8668**	**2,4275**	**0,03995**	*****
Treatment1:Gene:Timepoint	**10**	**2,668**	**0,2668**	**0,347**	**0,96565**	
Residuals	**105**	**80,745**	**0,769**			
Adj. R2	**0,2531**					
Hybrid aspen	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**	**Significance**
Treatment1	**2**	**15,368**	**7,6839**	**3,7558**	**0,02661**	*****
Gene	**5**	**78,781**	**15,7561**	**7,7013**	**3,41E-06**	*******
Timepoint	**1**	**0,037**	**0,0367**	**0,0179**	**0,89371**	

Treatment1:Gene	**10**	**32,845**	**3,2845**	**1,6054**	**0,11515**	
Treatment1:Timepoint	**2**	**6,501**	**3,2503**	**1,5887**	**0,20911**	
Gene:Timepoint	**5**	**68,361**	**13,6722**	**6,6827**	**1,95E-05**	*******
Treatment1:Gene:Timepoint	**10**	**112,282**	**11,2282**	**5,4881**	**1,63E-06**	*******
Residuals	**104**	**212,774**	**2,0459**			
Adj. R2	**0,4603**					

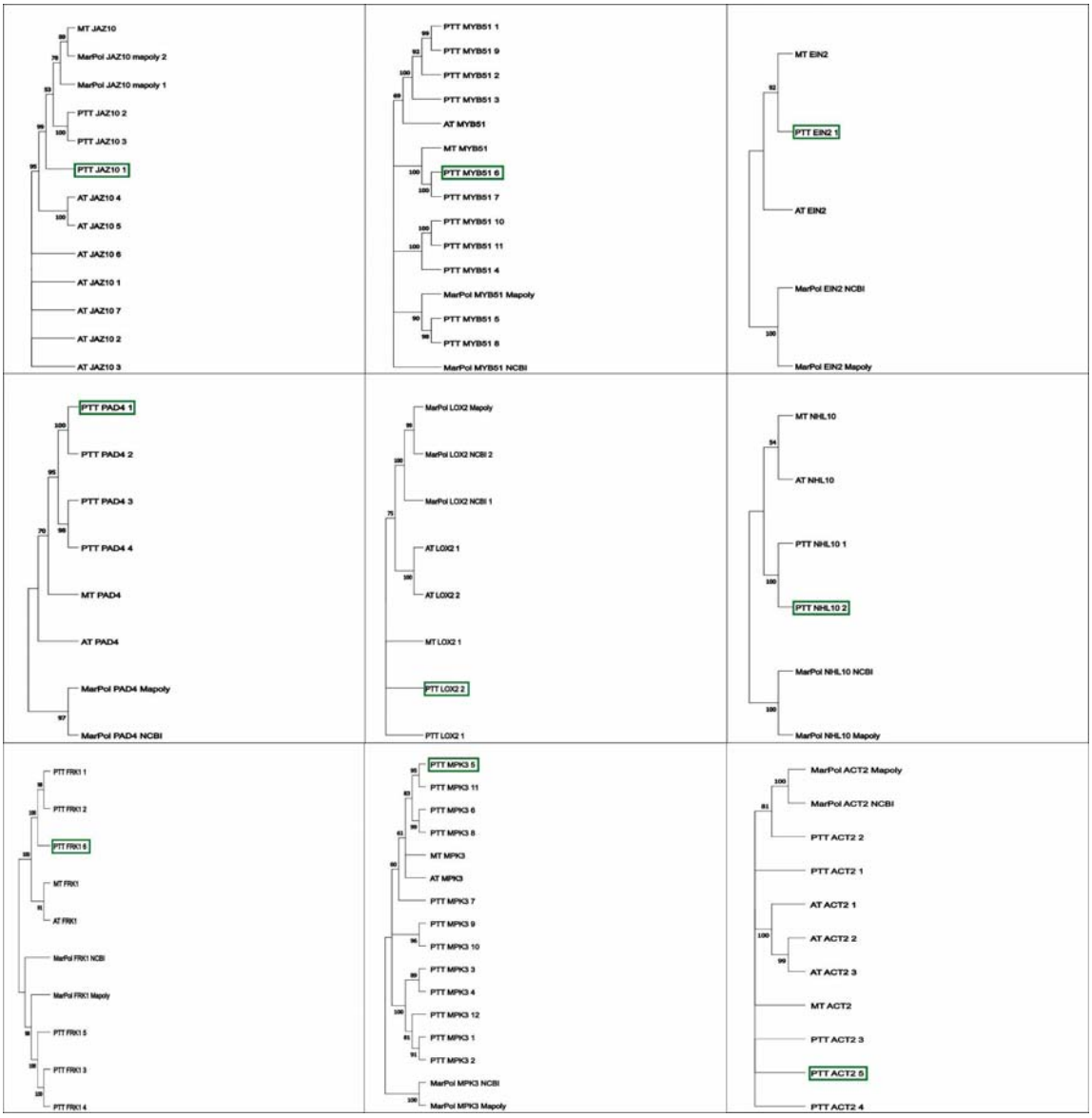


Figure S1. Cladograms of putative *Populus tremula x tremuloides* marker gene homologs. Phylogeny was based on translated protein sequences of *JAZ10*, *MYB51*, *EIN2*, *PAD4*, *LOC2*, *NHL10*, *FRK1*, *MPK3*, and reference gene *ACT2* from *Populus tremula x tremuloides* (PTT), *Arabidopsis thaliana* (AT), *Medicago truncatula* (MT), *Marchantia polymorpha* (MarPol). In case of multiple spliced variants, all sequences were included for tree construction using the Maximum likelihood method. Green box denotes the homolog used for primer design in the study.

Figure S2. Cladograms of putative *Betula pendula* marker gene homologs. Phylogeny was based on translated protein sequences of *JAZ10*, *MYB51*, *EIN2*, *PAD4*, *LOC2*, *NHL10*, *FRK1*, *MPK3*, and reference gene *ACT2* from *Betula pendula* (BP), *Arabidopsis thaliana* (AT), *Medicago truncatula* (MT), *Marchantia polymorpha* (MarPol). In case of multiple spliced variants, all sequences were included for tree construction using the Maximum likelihood method. Green box denotes the homolog used for primer design in the study.

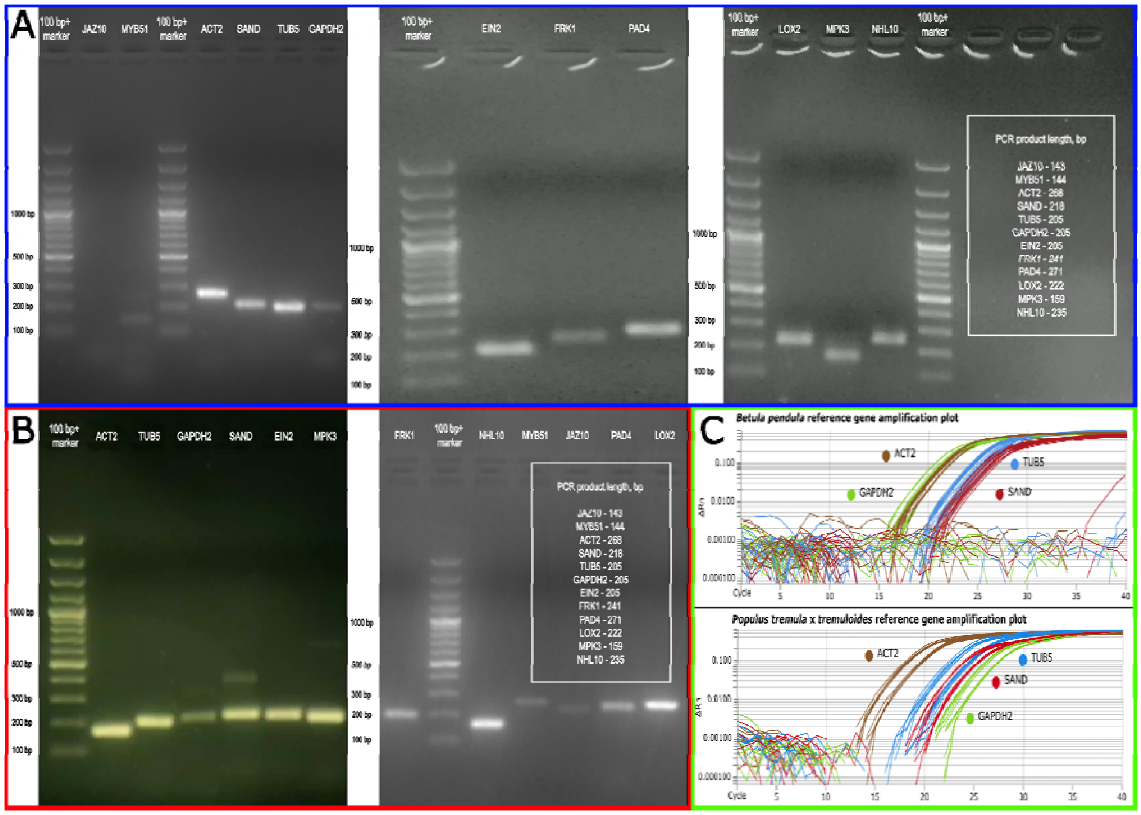


Figure S3. Electrophoresis gels of stress marker gene amplicons in *Betula pendula* (A) and *Populus tremula x tremuloides* (B) as well as marker gene amplification curves for tree reference gene selection (C). 35-cycle PCR amplicons were used to check for primer specificity, target size and absence of double bands. rt-qPCR analysis was performed for all primers with a visible single band to check for a single uniform melting-curve. Reference gene *ACT2* was selected based on earlier amplification and uniform expression across the different stress treatments in the qPCR amplification curve **(C).** deltaRn indicates the intensity of SYBR signal corresponding to target amplification during 40 qPCR cycles.

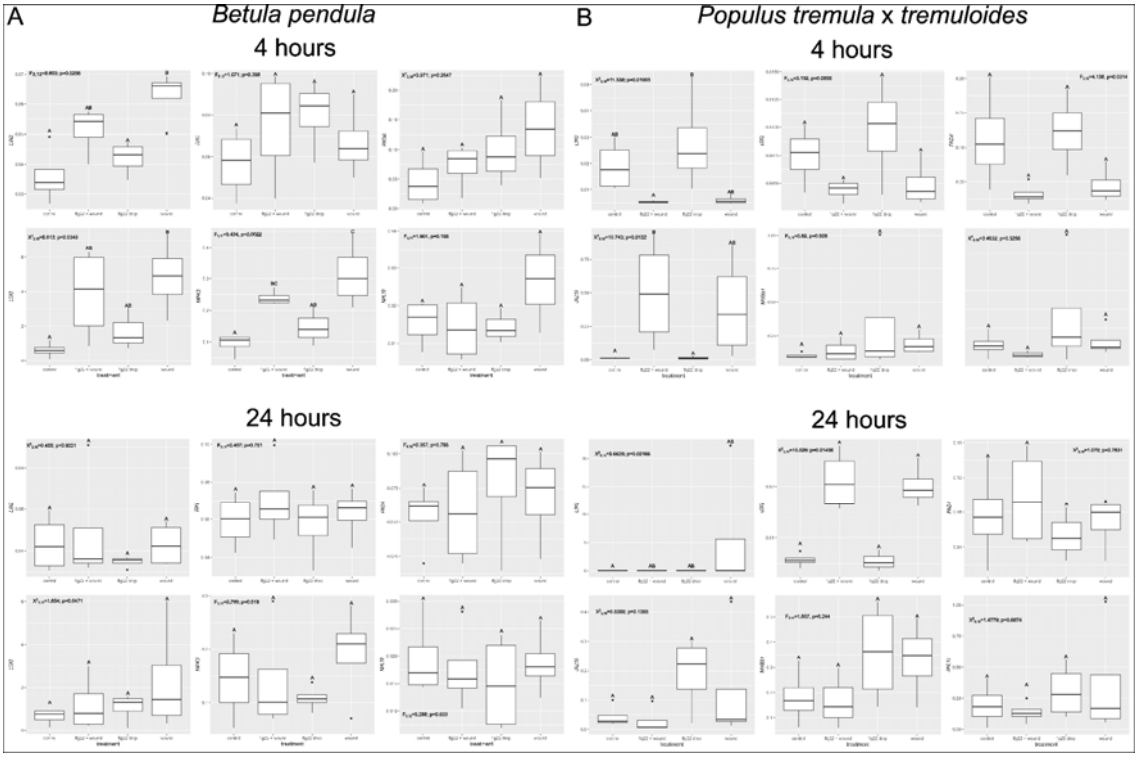
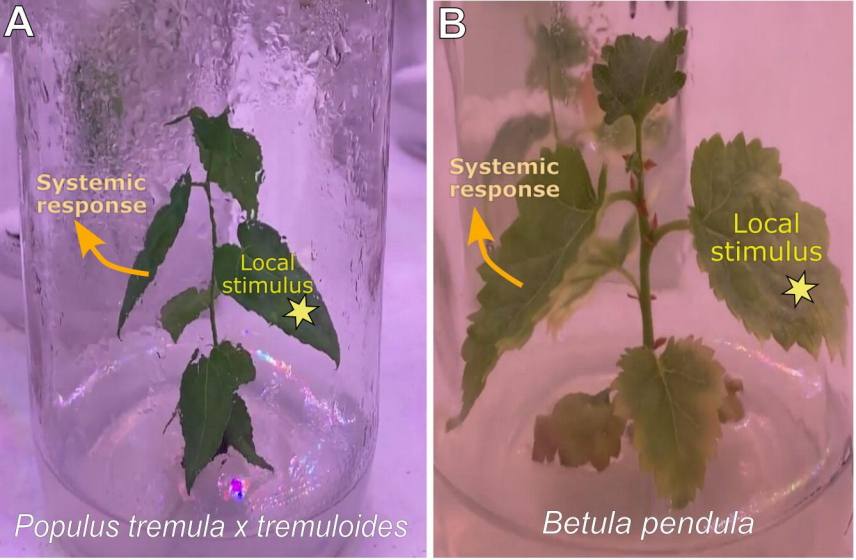
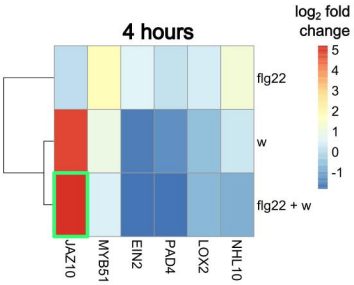


Figure S4. Marker gene expression relative to reference gene in *Betula pendula* (A) and *Populus tremula x tremuloides* (B). Boxplots represent the interquartile range (IQR), the line indicates the median expression, the whiskers - variance within 1.5x IQR. Summary results of ANOVA or Kruskal-Wallis and corresponding post-hoc tests are provided for each marker gene.


A
Systemic response
Local stimulus
Populus tremula x tremuloides
B
Systemic response
Local stimulus
Betula pendula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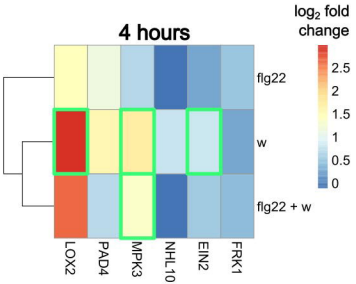
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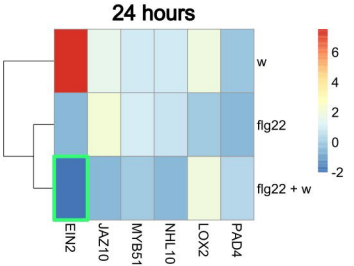
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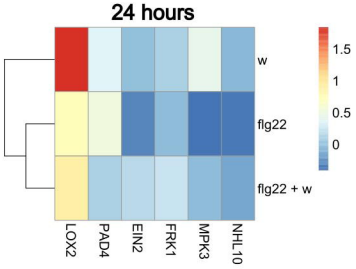
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