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Enhanced lignin synthesis and ecotypic variation in defense-related gene expression in response to shade in Norway spruce

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Abstract

During the growth season, northern forests in Sweden daily receive more hours of far-red (FR)-enriched light or twilight (shade) as compared to southern forests. Norway spruce (shade-tolerant) are adapted to latitudinal variation in twilight characterized by a northward increase in FR requirement to maintain growth. Shade is a stressful condition that affects plant growth and increases plant's susceptibility to pathogen attack. Lignin plays a central role in plant defense and its metabolism is regulated by light wavelength composition (light quality). In the current work, we studied regulation of lignin synthesis and defense-related genes (growth-defense trade-offs) in response to shade in Norway spruce. In most angiosperms, light promotes lignin synthesis, whereas shade decreases lignin production leading to weaker stem, which may make plants more disease susceptible. In contrast, enhanced lignin synthesis was detected in response to shade in Norway spruce. We detected a higher number of immunity/defense-related genes up-regulated in northern populations as compared to south ones in response to shade. Enhanced lignin synthesis coupled with higher defense-related gene expression can be interpreted as an adaptive strategy for better survival in northern populations. Findings will contribute to ensuring deployment of well-adapted genetic material and identifying tree families with enhanced disease resistance.

KEYWORDS

conifer, immunity, light quality, local adaptation, R:FR ratio, RNA sequencing, latitudinal cline, disease resistance, red light, far-red light

Summary statement

Enhanced lignin synthesis was detected in Norway spruce under shade in contrast to angiosperms. Shade also revealed ecotypic variation in defense-related gene expression in Norway spruce.

Abbreviations: R:FR, red:far-red ratio; RNA-seq, RNA-sequencing.

1 | INTRODUCTION

Light is one of the major factors that govern forest productivity, while biotic stress reduces tree growth and its survival rate (Bayat et al., 2021; Teshome et al., 2020). One of the parameters that define light is its wavelength composition, so-called light quality. Vegetative shade, characterized by a low red:far-red (R:FR) ratio, is the result of the absorbance of R light by the pigments of the neighbouring plants, while FR light is reflected (Ballare et al., 1987). Astronomic shade, twilight, is also characterized by a low R:FR ratio, which is caused by sunlight scattering in the upper atmosphere, and the duration of twilight depends on the latitude and the time of the year (Nilsen, 1985). Sweden's geographic location results in a pronounced latitudinal difference in the duration of twilight. This difference is more pronounced during the growing season when the northern latitudes receive longer daily exposure to FR-enriched light (twilight) as compared to the southern latitudes (Supporting Information: data 2, Figure S1 and references therein represents how the local light conditions differ in Sweden throughout the year).

Shade tolerance is the ability of a tree to continue to become established, survive and thrive in shade; tree species that can compete well in fully shaded conditions are shade-tolerant, while those that require full sunlight and limited competition are shade-intolerant (Grebner et al., 2021). Norway spruce (*Picea abies* [L.] H. Harst) is shade-tolerant and it is one of the most economically important conifers in Boreal forests. Norway spruce shows ecotypic variation in response to twilight, which is characterized by a northward increase in the requirement for additional FR light to maintain growth (Clapham et al., 1998; Ranade & García-Gil, 2021). This character could be interpreted as an adaptive response that allows trees to leave dormancy, grow and survive during the growing season when the northern latitudes are daily exposed to longer hours of twilight. In other words, the northern trees interpret FR enriched or shade-like conditions as a signal for growth and development, unlike the southernmost populations.

Shade is a stressful condition for any plant leading to a weaker stem, making the plant more susceptible to diseases and pathogen attacks (Hussain et al., 2019), explained by changes in the response pathways to biotic and abiotic factors (Courbier & Pierik, 2019). Lignin, which is the second most abundant heterogeneous polymer after cellulose, provides structural support and protection to the plants against pathogen invasion by acting as a physical (Lee et al., 2019; Malinovsky et al., 2014) and chemical (Xie et al., 2018) barrier. This secondary metabolite, which is mainly present in secondary cell walls of vascular plants, forms one of the most important components involved in the plant defense mechanism. Lignin biosynthesis is a highly energy-consuming and irreversible process that responds to many developmental and environmental cues including light (Dixon & Barros, 2019; Zhao & Dixon, 2011), and is regulated by a complex network of transcription factors (Ohtani & Demura, 2019; Zhao, 2016; Zhong & Ye, 2009). Lignin deposition generally occurs when the cell stops growing and is committed to programmed cell death leading to secondary thickening of the cell wall (Rogers & Campbell, 2004). This means that when a plant is responding to a pathogen through lignification, the plant stops growing. This is referred to as the growth-defense trade-off. Secondary metabolites including lignin are involved in the defense response in any plant species (Isah, 2019), and therefore are important candidates to understand the defense mechanism. Light quality affects lignin synthesis—in most angiosperms, there is a sharp decrease in the lignin production in response to low light or shade, leading to a weaker stem, making the plant more susceptible to diseases/pathogens (Hussain et al., 2019; Wu et al., 2017). However, the lignin biosynthetic process and its molecular regulation remain under-explored in conifers, particularly in response to light quality and with reference to the growth-defense trade-offs, as compared to the well-studied model plants like *Arabidopsis thaliana* (*Arabidopsis*).

Quantitative Trait Loci for plant defense response have been previously reported in Norway spruce (Elfstrand, Baison, Lunden et al., 2020; Elfstrand, Zhou, Baison et al., 2020). However, the defense mechanism at the gene regulation level and the metabolites involved in the process remain unclear in coniferous trees. Even less understood is the effect of changes in natural light conditions on the regulation of the defense mechanism. We previously reported that conifers respond to twilight (low light intensity and low R:FR) in a different way than angiosperms, although some aspects of shade response appear to be conserved (Ranade et al., 2019). Further, transcriptomic and exome capture analysis revealed the presence of an adaptive latitudinal cline in allele frequencies of the differentially expressed genes (DEGs) under twilight in Norway spruce (Ranade & García-Gil, 2021). Those DEGs belong to pathways involved in cell wall synthesis, lignin biosynthesis and immunity, suggesting a potential role of these genes in local adaptation to variation in exposure to twilight (Ranade & García-Gil, 2021). For example—MYB DOMAIN PROTEIN 3 (*MYB3*), a transcription factor that is involved in the repression of the lignin biosynthetic pathway, was found to be down-regulated under a low R:FR ratio in the northern Norway spruce populations as compared to the southern populations. A missense single nucleotide polymorphism (SNP) in the coding region of *MYB3* showed a steep latitudinal cline in allelic and genotypic variation. Likewise, RESISTANT TO P. SYRINGAE 2 (*RPS2*), a gene that is involved in plant defense, was found up-regulated under twilight in the northern Norway spruce populations and two missense SNPs from its coding region displayed a latitudinal cline. These polymorphisms in the coding regions of the gene may result in alteration of the protein folding and conformation, consequently influencing their binding-capacity/interaction, thus leading to an alteration in their mode of action.

A comparative transcriptomic study of shade avoidance and shade tolerance in Scots pine and Norway spruce respectively discussed the shade response in conifers in general and highlighted the differential regulation in light signalling pathway genes in both conifer species (Ranade et al., 2019). The same study also revealed differential responses in the defense-related genes under shade in the two conifer species. However, whether the defense-related expression under shade in Norway spruce follows a latitudinal cline is

unknown. In addition, an adaptive cline for shade tolerance was detected in Norway spruce that was associated with latitudinal cline of allele frequencies of SNPs in the DEGs (including genes involved in lignin synthesis and defense) in response to shade (Ranade & García-Gil, 2021). In the current work, we are interested in investigating:

(a) Whether there is any ecotypic variation regarding defense-related gene expression under shade in Norway spruce, and (b) Whether shade affects lignin synthesis in Norway spruce, lignin being one of the major components involved in plant defense.

2 | MATERIALS AND METHODS

2.1 | Seed germination and light conditions

Norway spruce seeds were collected from natural populations across Sweden—the northern population from Pellonhuhta (67°2′N) and the southernmost population between latitude 56° and 58° (designated as 56°). These two populations are referred to as the northern and southern spruce populations respectively in this study. Seeds were sampled from unrelated trees at a minimum distance of 50 m from each other to ensure low consanguinity and to capture a representation of the population diversity. The percentage of germination was obtained by germinating soaked seeds on paper discs on a warm bench with controlled humidity and temperature. The percentage of germination was 98% in a batch of 200 seeds (5 seeds per tree). Seventy seeds were germinated under two continuous light treatments (Treatment A, SHADE; Treatment B, SUN) as described previously (Ranade & García-Gil, 2021) in Percival (LED-30 Elite series) growth cabinets, at a constant temperature of 22°C on moist vermiculite. Treatment A (SHADE) represented shade-like conditions containing R and FR light wavelengths only, with a R:FR ratio of 0.2 and total light intensity of 36 µmol m^{-2} s^{-1} (R, 6 µmol m^{-2} s^{-1}; FR, 30 µmol m^{-2} s^{-1}). Treatment B (SUN) was used as control light treatment, which represented sun-like conditions containing R and FR light wavelengths only, with a R:FR ratio equal to 1.2 and a total light intensity of 65 µmol m^{-2} s^{-1} (R, 35 µmol m^{-2} s^{-1}; FR, 30 µmol m^{-2} s^{-1}). We applied only the R and FR light qualities in this experiment, as these are the two main responsible elements that plants use to determine the shade conditions and respond accordingly. Moreover, the light conditions used in the experiments were able to trigger the shade responses in Norway spruce, as described in our earlier work (Ranade et al., 2019).

2.2 | Fourier-transform infrared (FTIR) spectroscopy analysis

Forty seedlings from each light treatment for each species were used for FTIR spectroscopic analyses. Whole seedlings were oven-dried at 40°C for 48 h and ground finely in a bead mill at 30 Hz and for 2 min. Five milligrams of the resulting powder was mixed and manually ground with 395 mg of potassium bromide (KBr; Sigma-Aldrich; FTIR spectroscopy grade) using an agate mortar and pestle. FTIR spectra were recorded in diffuse reflectance mode under vacuum (4 mbar) conditions, using a Bruker IFS 66 v/S spectrometer (Bruker Optik GmbH), at a spectral resolution of 4 cm^{-1} according to the protocol described by Gorzsas & Sundberg, (2014). Pure KBr was used as background. Data in the spectral region 400–1900 cm^{-1} were used in the subsequent multivariate analyses as described previously (Gorzsas et al., 2011). Before multivariate analysis, spectra were baseline corrected and standardized using the built-in 64-point rubberband baseline correction followed by offset- and vector-normalizations of the OPUS software (version 7.0.122; Bruker Optik GmbH) over the 400–1900 cm^{-1} spectral region. Multivariate analysis was performed by the SIMCA-P software package (version 11.0.0.0; Umetrics AB). Individual OPLS-DA (orthogonal projections to latent structures–discriminant analysis) was carried out to highlight specific differences in the chemical composition of the samples. Q2(cum) values were analysed where Q2 is the fraction of the total variation that can be predicted by a component, as estimated by cross-validation. Q2(cum) is the cumulative Q2 for all components, that is, a numerical measure of the predictive ability of the model, with a maximum value of 1.0 corresponding to maximum (100%) predictive ability. Q2(cum) is dependent on the number of components; it cannot reliably be used to compare models with different numbers of components. In addition, models with large differences between R2 and Q2 values (i.e., model fit and predictive ability) should be treated as unreliable. When comparing reliable models with matching numbers of components and a similar number of observations, Q2(cum) values can be used to compare the predictive ability of these models.

2.3 | Transcriptomic analysis

Whole seedlings were used for the extraction of RNA. Three biological replicates were prepared for each of the light treatments by pooling three seedlings per sample to reduce variation between replicates and to increase the statistical power of the analysis. Isolation of total RNA, RNA sequencing (RNA-seq) and pre-processing of RNA-seq data were carried out as described in our previous work (Ranade et al., 2019). In short, total RNA was isolated using Spectrum Plant Total RNA Kit (Sigma) following the manufacturer's instructions. RNA library preparation and subsequent sequencing (HiSeq. 2500; Illumina) were performed at SciLifeLab.

The data pre-processing was performed as described here: https://www.epigenesys.eu/en/protocols/bio-informatics/1283-guidelines-for-rna-seq-data-analysis. Reads were aligned to v1.0 of the *P. abies* genome retrieved from the PlantGenIE resource (Sundell et al., 2015). The RNA-seq data were deposited to the ENA and are accessible under the accession number PRJEB19683 (https://www.ebi.ac.uk/ena/data/view/PRJEB19683). Statistical analysis of single-gene differential expression within and between the two latitudes in response to SHADE was determined using SUN as control. Analysis was performed using the Bioconductor (v3.3) (Gentleman et al., 2004) DESeq. 2 package (v1.12.0) (Love et al., 2014). False discovery rate (FDR) adjusted *p* values were used to assess significance; a common threshold of 5% was used throughout.

For the data quality assessment and visualization, the read counts were normalized using a variance stabilizing transformation as implemented in DESeq. 2. Differential regulation of genes expressed under SHADE was determined in the populations at the two latitudes separately which is referred to as 'within latitude comparison (within southern latitude population and within northern latitude population)', where SUN condition was used as the control. Comparative analysis of the differentially regulated genes under SHADE (where SUN was used as the control condition) between the northern and southern populations was carried out, which is termed as 'north versus south comparison'. North versus south comparison was performed using DESeq using a two-factor design: design = ~ treatment + latitude + treatment × latitude.

Genes were categorized according to their Gene Ontology (GO) categories, which were determined using Congenie (https://congenie.org/). Pie charts for functional categorization by annotation (GO Biological Process, GO Cellular Component and GO Molecular Function) for the DEGs for respective treatments and comparisons are represented in the Supporting Information material. The percentage of annotation was calculated as the number of annotations to terms in the GOslim category × 100/number of total annotations to terms in the ontology. GO enrichment analysis of the top 30 pathways with a *p* value cut-off of 0.05 FDR was done using ShinyGO (Ge et al., 2020).

3 | RESULTS

3.1 | GO annotations and GO enrichment

As the earlier study revealed the shade response in Norway spruce in general including light signalling pathway genes (Ranade et al., 2019), the current investigation, therefore, focuses on the ecotypic variation under shade in Norway spruce. The details of the overall DEGs including the within and between latitude comparisons have been included in the Supporting Information material (Supporting Information: data 1, Tables S1–S3, Supporting Information: data 2, Figure S2). In general, the percentage of GO annotations was similar across all the comparisons, although the number of DEGs involved in the response to SHADE was diverse (Supporting Information: data 2, Figure S3–S20). GO enrichment analysis is included as Supporting Information material (Supporting Information: data 2, Figure S21–S34). The GO enrichment analysis for Biological Process shows that the phenylpropanoid pathway genes are represented under SHADE and SUN in both the within latitude comparisons (Supporting Information: data 2, Figure S21–22, Figure S27–28), whereas the between latitude (north vs. south) comparison clearly indicates over-representation of phenylpropanoid pathway genes under SHADE in the northern populations (Figure 1). Likewise, the fold enrichment and the number of defense-related genes are higher under SHADE in the northern populations (Figure 1) for the north versus south comparison, whereas both the within latitude comparisons showed enrichment of defense-related genes under SHADE and SUN conditions (Supporting Information: data 2, Figure S21–22, Figure S27–28). In addition, suberin biosynthesis was found to be over-represented under SHADE in the northern populations as compared to the southern ones (Figure 1); suberin acts as a protective barrier in plant cell walls and is involved in plant protection (Pollard et al., 2008). No significant GO enrichment was detected for GO Biological Process for genes having higher expression under SHADE in the southern population as compared to the northern population, for the north versus south latitude comparison. The Cellular Component enrichment shows a higher number of active components under SUN as compared to SHADE in both within latitude comparisons (Supporting Information: data 2, Figure S23–24, Figure S29–30). Interestingly, the north versus south comparison shows a higher number of active cellular components under SHADE in the northern latitude, which includes the Casparian strip and cell wall (Figure 2). The cell wall forms the major barrier to pathogens. Similarly, the Casparian strip is involved in plant protection against biotic and abiotic stress (Holbein et al., 2019).

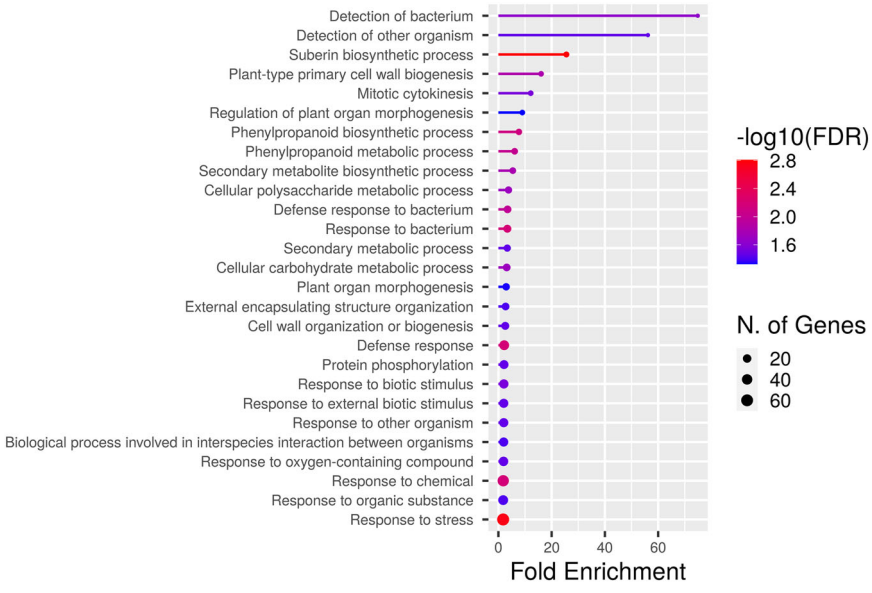

FIGURE 1 Gene Ontology enrichment analysis of top 30 pathways for Biological Process for the differentially regulated genes from the north versus south comparison in Norway spruce populations (*p value* cut-off [FDR]: 0.05). Genes represented have higher expression under SHADE in the northern population as compared to the southern population.

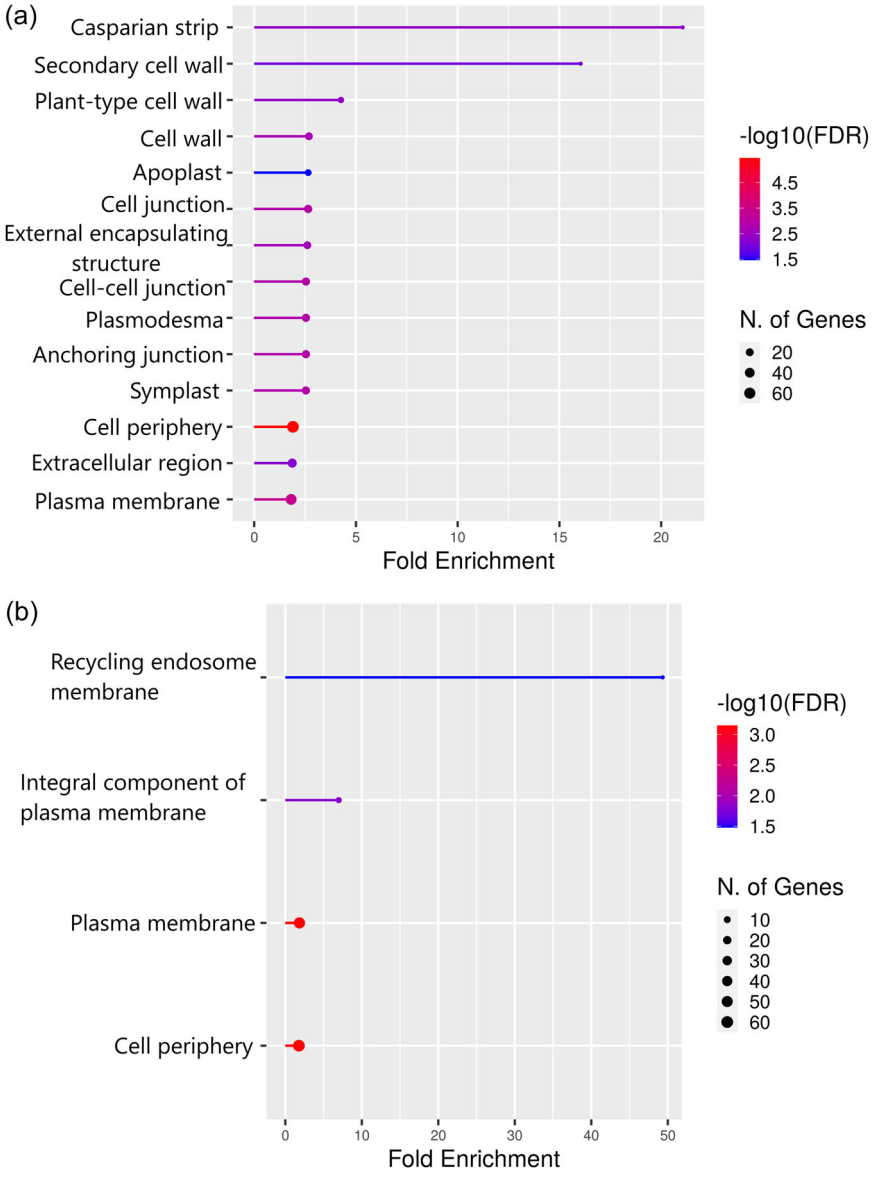

FIGURE 2 Gene Ontology enrichment analysis of top 30 pathways for Cellular Component for the differentially regulated genes from the north versus south comparison in Norway spruce populations (*p value* cut-off [FDR]: 0.05). (a) Genes represented have higher expression under SHADE in the northern population as compared to the southern population. (b) Genes represented have higher expression under SHADE in the southern population as compared to the northern population. [Color figure can be viewed at wileyonlinelibrary.com]

Therefore, higher fold enrichment of Casparian strip and cell wall under SHADE could be regarded as the adaptation of northern trees to local FR-enriched or shade-like conditions for better survival. Moreover, a higher number of active cellular components under SHADE in the northern trees (Figure 2a) as compared to the southern ones (Figure 2b), indicates that northern trees interpret a higher amount of FR as a signal for growth and development, which is again an adaptation to the local light conditions.

3.2 | Latitudinal variation in DEGs involved in the light signalling pathway in response to SHADE

Light signalling pathway genes in association with shade tolerance in Norway spruce are identified (Ranade et al., 2019), but whether their differential regulation follows a latitudinal cline is not known. Therefore, in the current work, we focused on the latitudinal variation in the DEGs involved in the light signalling pathway. PHOTOSYSTEM II REACTION CENTER PSB27 PROTEIN (*PSB27*), a thylakoid protein that enables adaptation to changes in light intensity (Hou et al., 2015) and DRACULA2 (*DRA2*), involved in the control of shade induced gene expression (Gallemí et al., 2016), were found to be up-regulated in the northern Norway spruce as compared to the south under SHADE. PHYTOCHROME RAPIDLY REGULATED2 (*PAR2*), which is up-regulated after simulated shade perception and plays a role in photomorphogenesis (Zhou et al., 2014) was up-regulated in the northern population.

3.3 | Latitudinal variation for defense-related DEGs in response to SHADE

Genes involved in defense mechanisms were analysed for differential expression in response to SHADE. For this purpose, the genes

categorized under defense response in the GO were considered (e.g., GO:0042742, GO:0002229, GO:0050832). The within latitude comparisons revealed an equal number of defense-related DEGs up-regulated and down-regulated in response to SHADE in the northern and southern populations respectively ($p > 0.05$). Thirty-six defense-related were up-regulated and 46 defense-related were down-regulated under SHADE in the southern population, while 119 defense-related were up-regulated and 128 defense-related were down-regulated under SHADE in the northern population. This finding agrees with the shade-tolerant nature of Norway spruce (Ranade et al., 2019). Interestingly, the north versus south comparisons showed a significantly higher number of defense-related genes up-regulated in the northern population under SHADE ($p < 0.05$). Forty-four defense-related genes were up-regulated under SHADE in the northern population while 27 defense-related genes were up-regulated under SHADE in the southern population. This analysis is also supported by the GO enrichment analysis discussed in the previous section of the article (Figure 1, Supporting Information: data 2, Figure S21–22, Figure S27–28).

3.4 | Effect of SHADE on lignin content

FTIR spectroscopy is based on molecular vibrations and informs primarily about the functional groups of the chemical compounds that are present in the sample. Since each IR active compound has unique FTIR spectra, FTIR spectroscopy is an excellent chemical fingerprinting tool, with the recorded spectra reflecting the entire chemical matrix of the sample, in situ (without the need for extraction) and without external agents (dyes, labels, markers). To highlight differences, individual OPLS-DA models were created, facilitating interpretation and direct comparisons. Supporting Information: Table S4 summarizes these models and Figures 3–4 give a graphical overview of them. While the recorded FTIR spectra contain information about the entire chemical fingerprint of these trees, we focus only on the lignin-related changes (marked by asterisks in the Loadings plots. All bands with a higher than 50% correlation are marked in the Loadings plots, using the colours of their classes, e.g., light blue for SUN grown seedlings and dark blue for SHADE grown seedlings). Higher lignin synthesis was detected under SHADE conditions as compared to the SUN treatments at both latitudes (Figures 3–4). Both lignin-related spectral bands (aromatic -C = C- vibrations at ca. 1508 and 1595 cm^{-1}, marked by asterisks in all Loadings plots) show consistently higher intensity for SHADE grown trees at both locations. The predictive ability of the models (Q2(cum) values in Supporting Information: Table S4) appears similar, indicating comparable differences in the chemical composition between SUN-SHADE conditions. The southern model has considerably higher predictive ability than the corresponding northern model, indicating a much more pronounced effect of SUN-SHADE conditions in the south (Supporting Information: Table S4). The SUN versus SHADE model from the northern population appears to be weak among both the models, but it still indicates higher lignin synthesis under SHADE as compared to the SUN condition. One contributing factor could be an elevated lignin baseline in general (i.e., Norway spruce trees in the north already produce more lignin in the SUN, thus while SHADE conditions further increase lignin content, the difference is less in relative terms). In most angiosperms, light promotes lignin synthesis, whereas shade decreases lignin production. In contrast to this, FTIR data from Norway spruce showed enhanced lignin synthesis under shade irrespective of location, which is a novel finding that this study reports.

3.5 | DEGs involved in lignin biosynthetic process

Genes specific to lignin synthesis were analysed for differential expression in response to SHADE. For this purpose, the genes categorized under GO:0009809 were considered. Few of the key genes specific to the lignin biosynthetic process (Herrero et al., 2013; Liu et al., 2018) that were up-regulated under SHADE include *CINNAMOYL COA REDUCTASE* (*CCR1*, *CCR2*), *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD2*, *CAD6*, *CAD9*), *CAFFEOYL COENZYME A O-METHYLTRANSFERASE* (*CCoAOMT*), *CAFFEIC ACID O-METHYLTRANSFERASE* (*COMT*), *HYDROXYCINNAMOYL-COA SHIKIMATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE* (*HCT*), *LACCASE 4* (*LAC4*), *PHENYLALANINE AMMONIA-LYASE* (*PAL2*, *PAL4*) and *PEROXIDASE* (*PRX52*, *PRX72*). The differentially expressed *MYB3/MYB DOMAIN PROTEIN 4* (*MYB4*) repressors were not included in the lignin-related gene analysis but analysed separately, as they negatively regulate lignin synthesis and their higher expression implies down-regulation of the lignin pathway (Behr et al., 2019; Ma & Constabel, 2019; Xiao et al., 2021)

The FTIR data supports an increased lignin content in response to SHADE in both the Norway spruce populations, although the transcriptomic analysis with reference to key genes in the lignin biosynthesis pathway did not reveal a significant higher number of lignin genes up-regulated under SHADE. With reference to suppressors of the lignin biosynthesis pathway, more than one copy/homolog of MYB3/MYB4 repressors were found to be differentially regulated under SHADE; some homologs were up-regulated while some were down-regulated, where down-regulation of the repressor indicates up-regulation of lignin pathway and vice versa (Behr et al., 2019; Ma & Constabel, 2019; Xiao et al., 2021). MYB3 and MYB4 down-regulates *CINNAMATE 4-HYDROXYLASE* (*C4H*); MYB4 also targets *CAD* and *CCR* to suppress them, which leads to the suppression of the lignin synthesis pathway (Xiao et al., 2021). Lower expression of *MYB3/MYB4* leads to the up-regulation of the lignin-related genes targeted by these MYB factors. None of the repressors of the lignin pathway was found to be differentially regulated under SHADE for the within latitude comparison in the southern populations, while lignin specific *CCR1*, *PAL2* and a homolog of *PRX52* were found to be up-regulated under SHADE. In the northern Norway spruce population, an equal number of repressors were found to be up-regulated and down-regulated under SHADE for the within latitude comparison; two out of the three copies of *MYB3* were down-regulated under SHADE and one out of the three copies of

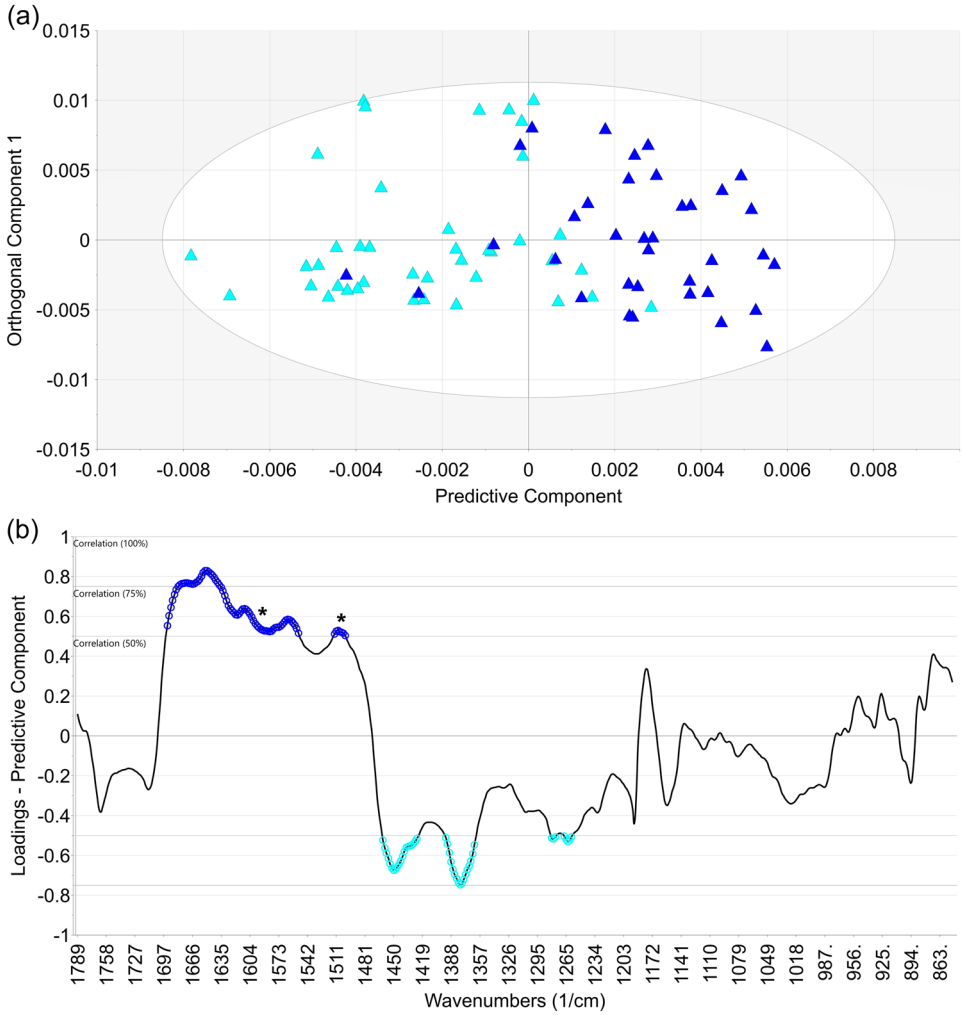

FIGURE 3 (a) OPLS-DA scores plots of FTIR spectra from northern Norway spruce samples grown under SUN (light blue) and SHADE (dark blue) conditions. Each symbol represents one sample. (b) The corresponding correlation scaled Loadings plot for the predictive component. Bands more intense in SUN and SHADE grown trees are marked by light and dark blue, respectively. Only bands with more than 50% correlation are marked with their respective colours. Asterisks denote aromatic -C = C- bands, associated with lignin. FTIR, fourier-transform infrared; OPLS-DA, orthogonal projections to latent structures–discriminant analysis. [Color figure can be viewed at wileyonlinelibrary.com]

MYB4 were down-regulated under SHADE. In addition, seven genes specifically involved in lignin were also up-regulated under SHADE—*PRX72*, *CAD9*, *LAC4*, *PAL2* and one homolog each of *PRX52*, *COMT* and *PAL4*. With reference to the north versus south comparison in Norway spruce, both copies of *MYB3* transcripts were found to be down-regulated by SHADE in the north as compared to the southern population, coupled with up-regulation of two lignin specific genes—*CCR2* and *CAD2*.

To summarise, we propose that lower expression of *MYB3*/*MYB4* coupled with the higher expression of some lignin synthesis specific genes may have contributed to the higher levels of lignin detected by the FTIR results under SHADE in Norway spruce. However, a further experimental investigation is required to validate the association of the expressed genes toward elevated lignin levels under the SHADE conditions. Validation of the expression data by RT-qPCR was not performed for two reasons—firstly, the study includes robust statistical analysis with the RNA-seq data (Coenye, 2021), and secondly, the FTIR analysis presents a solid proof of enhanced lignin synthesis under SHADE.

4 | DISCUSSION

4.1 | Local adaptation to extended FR-enriched light results in enhanced defense-related gene expression

A higher requirement of FR to maintain growth northwards in Norway spruce has been reported previously (Clapham et al., 1998; Ranade & García-Gil, 2021). This has been interpreted as the result of local adaptation to the light conditions at the northern latitudes, which receive extended periods of FR during the growth season. The expression analysis of DEGs in the light pathway adds support to Norway spruce

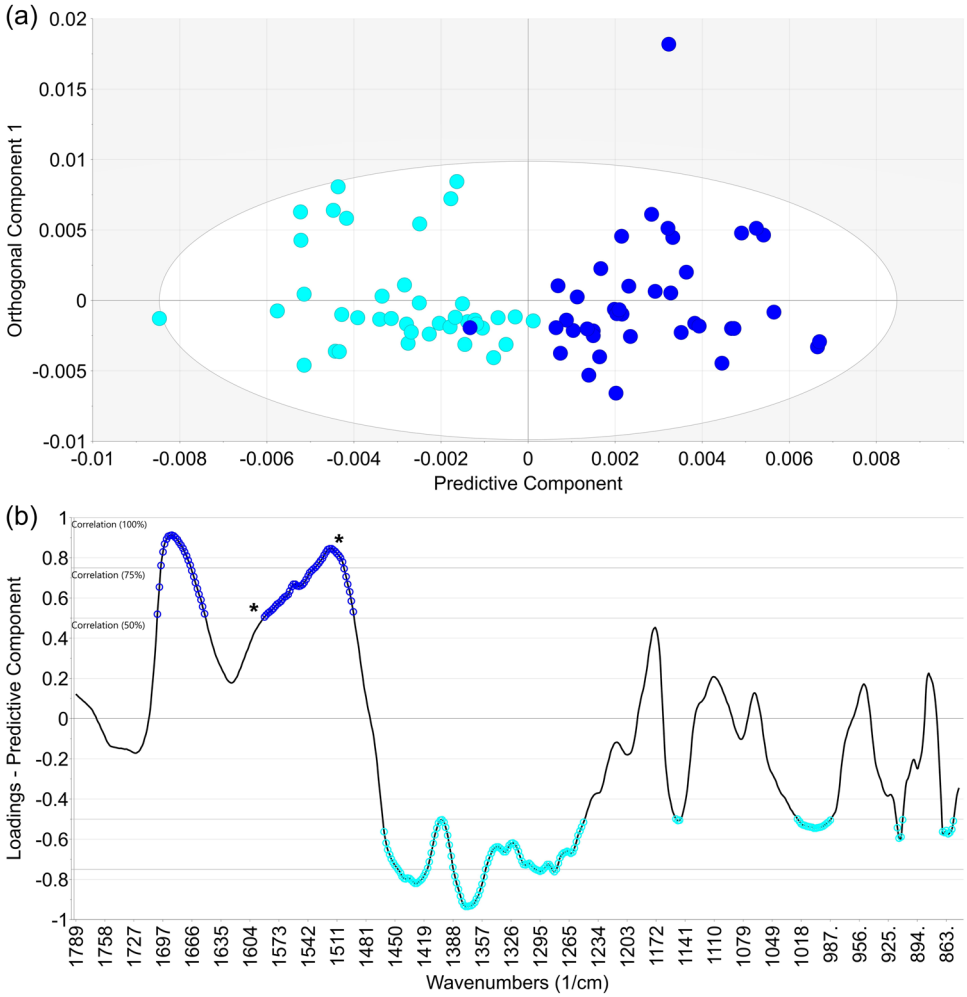

FIGURE 4 (a) OPLS-DA scores plots of FTIR spectra from southern Norway spruce samples grown under SUN (light blue) and SHADE (dark blue) conditions. Each symbol represents one sample. (b) The corresponding correlation scaled Loadings plot for the predictive component. Bands more intense in SUN and SHADE grown trees are marked by light and dark blue, respectively. Only bands with more than 50% correlation are marked with their respective colours. Asterisks denote aromatic -C = C- bands, associated with lignin. FTIR, fourier-transform infrared; OPLS-DA, orthogonal projections to latent structures–discriminant analysis. [Color figure can be viewed at wileyonlinelibrary.com]

populations' adaptation to the local light quality conditions. For example, *PAR2*, *PSB27* and *DRA2* are found up-regulated only in the northern population. The expression of these genes is known to be induced by shade (Gallemí et al., 2016; Hou et al., 2015; Zhou et al., 2014). We have also detected a higher number of immunity/defense-related genes up-regulated in the northern populations. In this context, a study by Hansson, in 1998 reported that the northern populations of Norway spruce showed the strongest resistance to *Gremmeniella abietina* (fungus) attacks (Hansson, 1998). The productivity of the forests in the northern part of Sweden is comparatively lower than in the south, owing to the harsh climatic conditions and soil quality. However, the fast-growing southern forests are affected by lower survival rates than the northern ones (Andersson et al., 2003). Considering the results of the current study, one reason for this feature may be the negative correlation between growth and defense processes, the so-called growth-defense trade-offs (Xie et al., 2018).

4.2 | FR-enhanced light activates lignin synthesis in Norway spruce

FTIR data supports higher lignin synthesis under SHADE in Norway spruce. This can be interpreted as an adaptation under shade conditions to defend itself from pathogen attack. The differential regulation of lignin and defense-related genes in response to shade was associated with cline in the SNPs in Norway spruce (Ranade & García-Gil, 2021). Hence, these genes seem to play a potential role in contributing to local adaptation to light quality. Overall, with the results from the current analysis (expression of a higher number of defense genes coupled with higher lignin synthesis) and earlier reports of higher disease resilience and higher survival rates (although slow-growing) (Andersson et al., 2003; Hansson, 1998) in the northern Norway spruce populations, we hypothesize that these populations have adapted to the prolonged exposure to twilight during the northern growth season by modifying their growth

patterns to increase survival by being more disease resilient, a hypothesis that warrants further experimental validation.

MYB3 and MYB4 negatively regulate the lignin synthesis pathway (Behr et al., 2019; Ma & Constabel, 2019; Xiao et al., 2021). Higher expression of a few key lignin-specific genes under SHADE coupled with the down-regulation of lignin pathway suppressors (MYB3/MYB4) under SHADE supports the finding from FTIR data regarding higher lignin synthesis in Norway spruce under SHADE. For example, MYB4 targets suppression of lignin-specific gene *CAD*, and therefore, higher expression of *MYB4* under SUN as compared to SHADE suggests suppression of *CAD* under SUN as compared to SHADE, leading to higher lignin synthesis under SHADE (northern Norway spruce population). However, few other genes involved in the lignin pathway were also found to be down-regulated under SHADE coupled with up-regulation of *MYB3*/*MYB4* (Supporting Information: Tables S1–S3) and yet FTIR data suggests higher lignin content under SHADE. One explanation for this could be the involvement of complex feedback loops in the lignin biosynthesis pathway that may be present, particularly in conifers, which requires further investigation for validation. The posttranslational modifications in response to SHADE cannot be overlooked, which also requires further research. Another reason for *MYB3*/*MYB4* expression and lignin synthesis not being in accordance may be explained by variation in the binding capacity of the different MYB family members. Further, the competition between the coexpressed MYB members, the possible interaction between them or with other transcription factors, and the feedback loops may also contribute to the mechanism of the regulation of the phenylpropanoid pathway that remains to be explored further in conifers. Similar reasons were accounted for the abundance of grass MYB4 homologs that were bound to the promoter of lignin biosynthetic genes, which were not correlated with the expression level of the *MYB4* genes (Agarwal et al., 2016; Miyamoto et al., 2020). A recent study in gymnosperms reports that the regulatory process of *Ginkgo biloba MYB* (*GbMYBR1*), a homolog of *MYB4*, differs from that of the MYB4-type repressor genes in *Arabidopsis* (Su et al., 2020). The same study reports that *GbMYBR1* lacks the key repressor motifs—EAR/TLLLFR, in the C-terminal region of the gene but still functions as a repressor of the lignin pathway repressing key lignin synthesis genes—*HCT*, *PAL*, 4-COUMARATE: CoA LIGASE (*4CL*) and *CAD*. In this context, it would be interesting to analyse the motifs in the MYB factors detected in the current study and analyse their association with repression with experimental support. Moreover, the possibility of the presence of alternative pathways specific to conifers could also be explored in this context, which has been discussed in the latter part of this section.

Light quality regulates the expression of R2R3 MYB members in *Arabidopsis* (Jin et al., 2000; J. Zhao et al., 2007; Mondal & Roy, 2018). A recent study reports that MYB30 which belongs to the R2R3 MYB family, functions as a key negative regulator of photomorphogenic

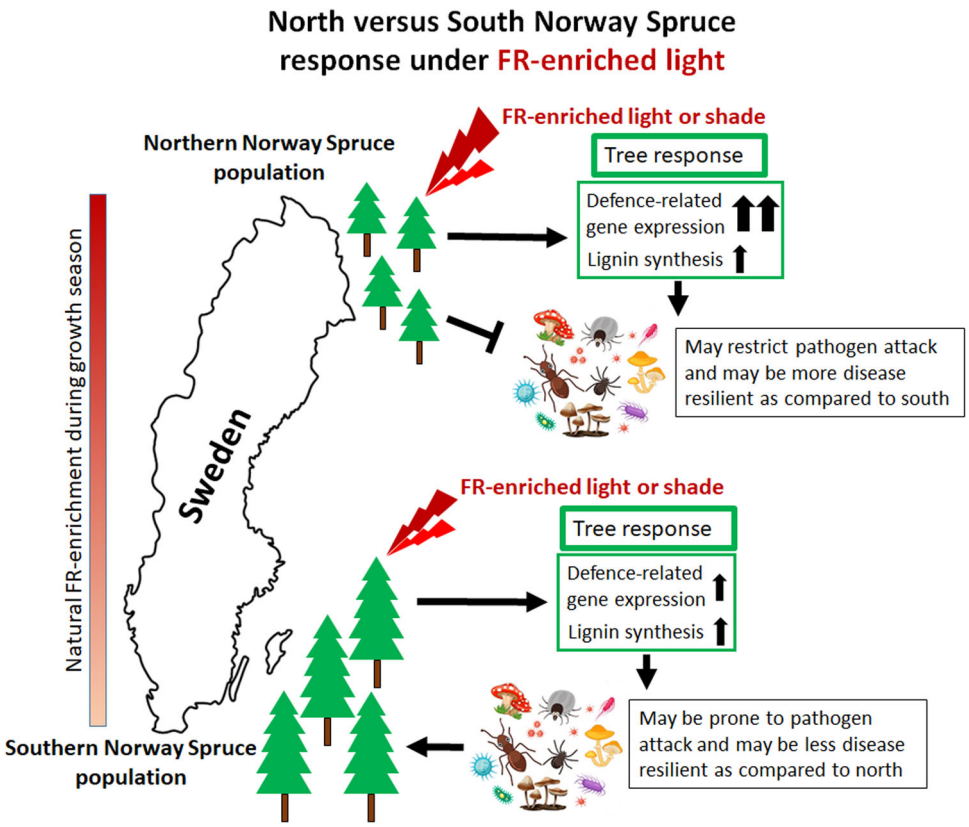

FIGURE 5 Northern Norway spruce populations in Sweden are adapted to the prolonged exposure to FR-enriched twilight during the growth season by being more disease resilient for better survival. FR, far-red. [Color figure can be viewed at wileyonlinelibrary.com]

development in *Arabidopsis* through preferential interaction with the Pfr (active) forms of the PHYA and PHYB, and PHYTOCHROME-INTERACTING FACTORS (PIFs) to repress photomorphogenesis under prolonged R light irradiation (Yan et al., 2020). In this context, it would be interesting to further explore whether the underlying mechanism of higher lignin synthesis under low R:FR light is mediated by the involvement of R2R3 MYB members and their interaction with light perceiving photoreceptors in conifers.

5 | CONCLUSIONS

We propose that the lignin pathway in conifers may be regulated by complex feedback loops and may function by following an alternative mechanism unlike the angiosperms, which needs further validation. Additional analysis is also required to determine the functional characterization of the different MYB3/MYB4 members detected in this study to understand the molecular process involved in the lignin pathway in conifers in response to shade or low R:FR ratio. This analysis reports a novel finding of increased lignin synthesis under shade in Norway spruce, which is very different from what is known in flowering plants. However, further research is required to comprehend the mechanism involved. In addition, shade revealed ecotypic variation in defense-related gene expression in Norway spruce. Finally, we hypothesize that the northern Norway spruce populations in Sweden have adapted to the prolonged exposure to FR-enriched twilight during the growth season by modifying their growth patterns to increase survival by being more disease resilient (Figure 5), a hypothesis that warrants further experimental validation. Together, these findings could be applied to produce disease resilient trees in the context of sustainable forestry and climate change.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The RNA-seq data were deposited to the ENA and are accessible under the accession number PRJEB19683 (https://www.ebi.ac.uk/ena/data/view/PRJEB19683). All other data are included in the supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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