Transcriptome analysis of shade avoidance and shade tolerance in conifers

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Abstract

Main conclusion Gymnosperms respond differently to light intensity and R:FR; although some aspects of shade response appear conserved, yet underlying mechanisms seem to be diverse in gymnosperms as compared to angiosperms.

Shade avoidance syndrome (SAS) is well-characterized in the shade intolerant model species *Arabidopsis thaliana* whereas much less is known about shade tolerance response (STR), yet regulation of SAS and STR with reference to conifers remains poorly understood. We conducted a comparative study of two conifer species with contrasting responses to shade, Scots pine (shade-intolerant) and Norway spruce (shade-tolerant), with the aim to understand mechanisms behind SAS and STR in conifers. Pine and spruce seedlings were grown under controlled light and shade conditions, and hypocotyl and seedling elongation following different light treatments were determined in both species as indicators of shade responses. Red to far-red light ratio (R:FR) was shown to trigger the shade response in Norway spruce. In Scots pine, we observed an interaction between R:FR and light intensity. RNA sequencing (RNA-Seq) data revealed that SAS and STR responses included changes in expression of genes involved primarily in hormone signalling and pigment biosynthesis. From the RNA-Seq analysis, we propose that although some aspects of shade response appear to be conserved in angiosperms and gymnosperms, yet the underlying mechanisms may be different in gymnosperms that warrants further research.

Keywords Gymnosperms · Light intensity · Norway spruce · Phytochrome B · Pigment synthesis · R:FR ratio · RNA sequencing · Scots pine · Seedling morphology

Abbreviations

ATERF *Arabidopsis thaliana* ethylene responsive element binding factors
 COT Cotyledon

ELIP2	Early light-induced protein 2
HYP	Hypocotyl
JA	Jasmonic acid
LHC	Light-harvesting chlorophyll-binding protein complex
Phy/PHY	Phytochrome
R:FR	Red to far-red ratio
ROOT_HYPCOT	Lower (root) to upper (hypocotyl + cotyledon) growth ratio
SA	Salicylic acid
SAS	Shade avoidance syndrome
STR	Shade tolerance response
TF	Transcription factor
TOTAL_LENGTH	Hypocotyl + root

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Introduction

Plants have developed sophisticated mechanisms to sense and respond to light duration, intensity and quality. Multiple wavelengths of the light spectrum are of biological significance in addition to the photosynthetically active radiation (PAR) (400–700 nm). The red (R, 660 nm) to far-red (FR, 730 nm) ratio (R:FR) is an indicator of the degree of shade with low values activating both shade avoidance and tolerance responses (Ballare et al. 1987; Gommers et al. 2013). In a fully light exposed area at midday, the R:FR ratio was reported to be 1.2 (Warrington et al. 1989; Smith 1994), whereas under vegetative shade the R light is largely absorbed by the chlorophyll and other leaf pigments while the FR is transmitted or reflected, resulting in a decrease in R:FR ratio to 0.2–0.8 (Ballare et al. 1987). Similarly, a decrease in R:FR ratio occurs during the twilight hours largely due to the longer path length of the light through the ozone layer which absorbs in the red–green region of the spectrum that results in a relative increase in blue and FR light (Attridge 1990).

Shade is perceived by the plants as decrease in the R:FR ratio, where there is higher FR than R. The study by Fernbach and Mohr (1990) on the Scots pine hypocotyl growth reported the absence of high irradiance response (HIR) (i.e., a lack of hypocotyl growth inhibition under FR), whereas presence of HIR under FR is a common feature in the angiosperms. Thus gymnosperms respond to light quality in a different way as compared to angiosperms. In gymnosperms as well as in angiosperms, the extent of vegetative response to shade differs between species depending on their level of tolerance to shade (Hoddinott and Scott 1996; Humbert et al. 2007). Shade tolerance is a function of the capacity of a particular species to efficiently modify its morphology to adapt and respond to low light intensities and low R:FR ratio, in addition to change the carbon allocation from photosynthetic activity to elongation (Valladares and Niinemets 2008; Giertych et al. 2015). Comparative studies of species with different levels of shade tolerance have been proven as an optimal experimental set up to study the mechanisms governing response to shade in both angiosperms (Sefcik et al. 2006) and gymnosperms (Peer et al. 1999).

A significant amount of research has been devoted to study the eco-physiological consequences of exposure to shade in angiosperms (Valladares and Niinemets 2008) and conifers (Warrington et al. 1989; Hoddinott and Scott 1996). In *A. thaliana*, the shade avoidance syndrome (SAS) is well-characterized and primarily involves the phytochromes, PhyA and PhyB, and the downstream genes required for the integration between the light and hormone signalling pathways (Yang and Li 2017). Shade reduces

phyB activity, which increases the mRNA levels of basic helix–loop–helix (bHLH) transcription factors such as phytochrome interacting factors (PIF) (Lorrain et al. 2008). PIFs regulate a large number of genes encoding metabolic enzymes and genes in the signalling pathways of the phytohormones (Ballare and Pierik 2017). The shade tolerance response (STR) has so far attracted less attention (Gommers et al. 2013). Recently, a RNA sequencing comparative study between two *Geranium* species from different native light environments revealed that STR and SAS share a common phytochrome-mediated mechanism with contrasting patterns of gene expression for the control of immunity and shoot elongation (Gommers et al. 2017). Genes involved in the SAS/STR in conifers remain unexplored, therefore we performed the whole-genome expression study using RNA sequencing (RNA-Seq) to get an overview of the biological pathways and molecular mechanisms that regulate SAS/STR and also to suggest potential candidates involved in the mechanism.

In this study, we aimed to advance our knowledge on the following: (1) Scots pine and Norway spruce as a model system for shade response studies, (2) the effect of R:FR and light intensity on the response to shade in seedlings and (3) the mechanism governing the SAS and the STR in conifers. To address this, seeds from Scots pine and Norway spruce were grown under constant light with contrasting R:FR (SUN-like 1.2 and SHADE-like 0.2) and light intensities. Hypocotyl (HYP), root (ROOT) and cotyledon (COT) lengths were determined in fully developed seedlings under the different light regimes, and the seedlings were analysed for whole genome expression using RNA-Seq.

Materials and methods

Seed material

Scots pine and Norway spruce seeds were collected from 70 unrelated trees from a Swedish natural population in Kaunisvaara (67°N 5′N). To ensure low consanguinity and to capture a representation of the population diversity, trees were collected at a distance of minimum 50 meters from each other. Cones were dried with warm air to force release the seeds. Sound seeds were separated from the empty seeds by flotation. 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 of 98% on a batch of 200 seeds (5 seeds per tree).

Table 1 Light treatments

Treatment	Light conditions
A	R:FR 0.2 ($36 \mu\text{mol m}^{-2} \text{s}^{-1}$) R ($6 \mu\text{mol m}^{-2} \text{s}^{-1}$):FR ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$)
B	R:FR 1.2 ($65 \mu\text{mol m}^{-2} \text{s}^{-1}$) R ($35 \mu\text{mol m}^{-2} \text{s}^{-1}$):FR ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$)
C	R:FR = 0.2 ($65 \mu\text{mol m}^{-2} \text{s}^{-1}$) R ($11 \mu\text{mol m}^{-2} \text{s}^{-1}$):FR ($54 \mu\text{mol m}^{-2} \text{s}^{-1}$)
D	R:FR 1.2 ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) R ($11 \mu\text{mol m}^{-2} \text{s}^{-1}$):FR ($9 \mu\text{mol m}^{-2} \text{s}^{-1}$)
DARK	Absence of light

Seed germination and light treatment

Stratified seeds (soaked in water at 4 °C overnight) were sown on water-saturated sterile vermiculite in growth boxes (Saveen Werner) and maintained at a constant temperature of 22 °C in Percival (LED-30 Elite series) growth cabinets. 70 sound seeds per treatment, latitude and species (one seed per sampled tree) were germinated under four light treatments (Table 1) and dark (DARK). Treatment A and C represented SHADE-like (SHADE) conditions having the same relative value of R to FR light (R:FR = 0.2) and differed in the total light intensity (36 and $65 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively). Treatment B and D represented SUN-like (SUN) conditions having same R:FR ratio (R:FR = 1.2) but differed in total light intensity (65 and $20 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively). Treatment B (SUN) had the same total light intensity as treatment C (SHADE). Treatment A (SHADE) and B (SUN) had the same FR intensity ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$). Treatment C (SHADE) and D (SUN) had the same R intensity ($11 \mu\text{mol m}^{-2} \text{s}^{-1}$). Treatment D had the lowest total light intensity of all four light treatments ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$). The rationale behind such an experimental design was to investigate whether intensity of R/FR or the ratio between R and FR is the key factor responsible for the effect of shade avoidance response in the two conifer species with contrasting responses to shade. We considered only the R and FR light qualities in this experiment as these are the two main responsible elements that plants use to determine shade conditions and respond accordingly. Plants sense the shade as decrease in R (or decrease in R:FR); R is primarily absorbed by the pigments and FR is reflected thereby decreasing the R:FR ratio. We had technical limitations with attaining the exact sun conditions (with higher total light intensity) to test our hypothesis whether intensity of R/FR or the ratio between R and FR is the key factor responsible for the effect of shade response in conifers. However, this experimental design was able to trigger the shade avoidance and the shade tolerance response in both the conifer species which

is evident from the morphological data analysis included in this work.

Seedling measurements

Length of hypocotyl (HYP), root (ROOT) and cotyledon (COT), the lower (root) to upper (hypocotyl + cotyledon) growth ratio (ROOT_HYPCOT) and hypocotyl + root (TOTAL_LENGTH) were scored at the mm precision for each seedling at the seedling developmental stage where hypocotyl was fully developed (Ranade and García-Gil 2013).

RNA sequencing

Three biological replicates were prepared for each of the light treatments—A (SHADE), B (SUN) and DARK (control), for RNA extraction for Scots pine and Norway spruce. The biological replicates were prepared by pooling three seedlings per sample to reduce variation between replicates and to increase the statistical power of the analysis (i.e. increased sensitivity to detect genes that are differentially expressed between conditions).

Total RNA was isolated using Spectrum Plant Total RNA Kit (Sigma) following the manufacturer's instructions. The mRNA concentration and quality was determined using NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Inc.) and Bioanalyzer 2100 (Agilent Technologies Inc.), respectively. RNA library preparation and subsequent sequencing were performed at SciLifeLab (Stockholm, Sweden). Strand-specific RNA libraries for sequencing were prepared with TruSeq Stranded mRNA Sample prep kit of 96 dual indexes (Illumina) according to the manufacturer's instructions except for the following changes: the protocols were automated in Agilent NGS workstation (Agilent Technologies) using purification steps as described in Lundin et al. (2010) and Borgstrom et al. (2011). Clonal clusters were generated using cBot (Illumina) and sequenced on HiSeq 2500 (Illumina) according to manufacturer's instructions. Bcl to Fastq conversion was performed with bcl2Fastq v1.8.3 from the CASAVA software suite. The quality scale was Sanger/phred33/Illumina 1.8+. The obtained data was deposited to the ENA and is accessible under the accession number RJEB19683.

Pre-processing of RNA-Seq data and differential expression analyses

The data pre-processing was performed as described here: <http://www.epigenesys.eu/en/protocols/bio-informatics/1283-guidelines-for-rna-seq-data-analysis>. Briefly, the quality of the raw sequence data was assessed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastq>

c/). Residual ribosomal RNA (rRNA) contamination was assessed and filtered using SortMeRNA (v2.1; (Kopylova et al. 2012); settings `-log -paired_in -fastx -sam -num_alignments 1`) using the rRNA sequences provided with SortMeRNA (rfam-5s-database-id98.fasta, rfam-5.8s-database-id98.fasta, silva-arc-16s-database-id95.fasta, silva-bac-16s-database-id85.fasta, silva-euk-18s-database-id95.fasta, silva-arc-23s-database-id98.fasta, silva-bac-23s-database-id98.fasta and silva-euk-28s-database-id98.fasta). Data was then filtered to remove adapters and trimmed for quality using Trimmomatic (v0.32; (Bolger et al. 2014); settings `TruSeq 3-PE-2.fa:2:30:10 SLIDINGWINDOW:5:20 MINLEN:50`). After both filtering steps, FastQC was run again to ensure that no technical artefacts were introduced. Filtered reads were aligned to v1.0 of the *P. abies* genome (retrieved from the PopGenIE resource; Sundell et al. 2015) using STAR (v2.4.0f1; Dobin et al. 2013); non-default settings: `-outSAMstrandField intronMotif -readFilesCommand zcat -outSAMmapqUnique 254 -quantMode TranscriptomeSAM -outFilterMultimapNmax 100 -outReadsUnmapped Fastx -chimSegmentMin 1 -outSAMtype BAM SortedByCoordinate -outWigType bedGraph -alignIntronMax 11000`). The annotations obtained from the *P. abies* v1.0 GFF file contain only one transcript per gene-model, and as such, did not need to be modified to generate ‘synthetic’ gene models. This GFF file and the STAR read alignments were used as input to the HTSeq (Anders et al. 2015) htseq-count python utility to calculate exon-based read count values. The htseq-count utility takes only uniquely mapping reads into account. The Scots pine samples were processed similarly, but aligned to the v1.01 of the *P. taeda* genome (Zimin et al. 2014) and its annotation retrieved from <http://pinenome.org/pinerefseq/>. The biological relevance of the data—e.g. biological replicates similarity—was assessed by Principal Component Analysis (PCA) and other visualisations (e.g. heatmaps), using custom R scripts.

Statistical analysis

Seedling morphology traits

Seedling morphology data were converted into percentage of change with respect to DARK (100%). Analysis of variance (ANOVA) was applied to estimate the significance of the light treatment effect. Principal Component Analysis (PCA), ANOVA and Duncan post hoc tests were conducted using R software (R Development Core Team 2015).

Differential expression analysis

Genes involved in shade avoidance syndrome (SAS) were defined as those that were significantly differentially expressed between treatments A (SHADE, R:FR = 0.2)

and B (SUN, R:FR = 1.2) in Scots pine (shade-intolerant), while not being differentially expressed in Norway spruce (shade-tolerant). Genes involved in shade tolerance response (STR) were defined as those that were significantly differentially expressed between the treatments A and B in Norway spruce (shade-tolerant), while not being differentially expressed in Scots pine (shade-intolerant). A third group of genes refers to those that were significantly differentially expressed between the treatments A and B in both species, but in opposite direction (i.e., up-regulated under SHADE in Scots pine and down-regulated under SHADE in Norway spruce (Pine_up_Spruce_down) or down-regulated under SHADE in Scots pine and up-regulated under SHADE in Norway spruce (Pine_down_Spruce_up)). The third group of genes would refer to those genes that were functionally active in shade response in both species.

Statistical analysis of single-gene differential expression was performed in R (v3.3.0; R Development Core Team 2015) using the Bioconductor (v3.3; Gentleman et al. 2004) DESeq 2 package (v1.12.0; Love et al. 2014). FDR adjusted *P* values were used to assess significance; a common threshold of 1% was used throughout. For the data quality assessment (QA) and visualisation, the read counts were normalised using a variance stabilizing transformation as implemented in DESeq 2.

Alignment of PHYA and PHYB

Multiple alignment of PHYA and PHYB respectively was carried out using CLUSTAL 2.1 multiple sequence alignment program. We used amino acid sequences from several shade tolerant and shade intolerant species from both angiosperms and gymnosperms.

Results

Effect of shade on seedling morphology

Treatment A and C represent SHADE conditions, and treatments B and D represent SUN conditions (Table 1). The mean (proportion of change from DARK), standard error (SE) and Duncan post hoc test results for hypocotyl (HYP), root (ROOT) and cotyledon (COT) lengths and lower to upper ratio (ROOT_HYPCOT) across light treatments are shown in Fig. 1a and Suppl. Table S. Bar graph with mean raw values of HYP, ROOT, COT lengths and ROOT_HYPCOT in spruce and pine is represented in Suppl. Fig. S1. In Scots pine, both SHADE treatments (A and C) significantly enhanced HYP growth compared to treatment B (SUN) (Fig. 1a). This response is in agreement with the shade-intolerant nature of this species and it also demonstrates that our experimental shade conditions trigger the SAS response.

Fig. 1 **a** Bar plot with mean and $\pm$ SE of the proportion of change from DARK for hypocotyl (HYP), root (ROOT) and cotyledon (COT) lengths, and lower (root) to upper (hypocotyl+cotyledon) growth ratio (ROOT_HYPCOT). Treatments A and C represent SHADE conditions, and treatments B and D represent SUN conditions. **b**

Line plot with mean and $\pm$ SE for patterns of the TOTAL_LENGTH (hypocotyl+root) in response to SHADE (treatments A and C) versus SUN (treatment B) in Scots pine and Norway spruce. *, ** denotes P value=0.01

In Norway spruce, HYP elongation was not enhanced by the SHADE treatments (A and C) when compared to treatment B (SUN), which is also in accordance with the shade-tolerant nature of Norway spruce (i.e., no HYP growth enhancement in response to shade). In Scots pine and Norway spruce, the effect of treatments A (SHADE) and C (SHADE) where the R:FR is 0.2 in both conditions, did not significantly differ. However, treatment D (LOW SUN) where the R:FR is 1.2, significantly enhanced HYP elongation in both species, especially in Norway spruce where HYP was the longest compared to the other three light treatments. HYP enhancement by treatment D could be explained by the low light intensity value ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) of the treatment which was the lowest among all the different light treatments. Together, these results indicate that low R:FR and low intensity both are indicators of shade; however, they may trigger different molecular mechanisms.

Overall, ROOT was longer in Norway spruce than in Scots pine (Fig. 1a). In both species, SHADE (A and C) inhibited ROOT as compared to SUN (B). In Scots pine, treatment A (SHADE) showed a significantly higher inhibitory effect compared to the treatment C (SHADE) indicating that ROOT is more sensitive to the light intensity than HYP. In Norway spruce, however, both SHADE treatments had similar inhibitory effect on ROOT growth. Overall,

ROOT was inhibited by SHADE in both species, which does not support a distinction between species on their level of shade tolerance. In the case of Norway spruce, inhibition of ROOT by treatment D was similar to both SHADE treatments, whereas in Scots pine treatment D inhibitory effect resembled treatment C. Similar to HYP, these results support the importance of light intensity as a factor determining seedling development.

COT length was longer in treatment B and C as compared to treatment A and D in Scots pine (Fig. 1a). In Norway spruce, COT length was similar in all treatments except for treatment B (SUN); COT length was longer under treatment B compared to all other treatments. Further, under treatment A and D, COT length was similar in both species. In Scots pine, COT was inhibited by SHADE, but more intensively by treatment A as compared to treatment C. In Norway spruce, both SHADE treatments equally inhibited COT elongation. As in the case of ROOT, COT response to SHADE did not agree with the species claimed differential response to SHADE. In Scots pine, treatment D inhibited COT elongation similarly to treatment A, whereas in Norway spruce the inhibitory effect of treatment D was equal to that of both SHADE treatments (A and C). Once again, these results support the role of light intensity as a key factor in the control of

Fig. 2 Principal component analysis (PCA) on seedling phenotypic data by treatment (SHADE: A + C versus SUN: B + D) for Scots pine and Norway spruce

seedling growth even under contrasting R:FR values (see the effect of A and D with respect to B).

Irrespective of treatment, the ROOT_HYPCOT was higher in Norway spruce (Fig. 1a). In both species, the ROOT_HYPCOT decreased in a similar manner in response to both SHADE treatments (A and C). Also for this trait (as in case of ROOT), the differential effect of shade and sun did not reveal the difference in the level of shade tolerance between both species. In the case of Norway spruce, the effect of treatment D was significantly higher as compared to treatments A and C, indicating that this trait is very sensitive to low intensity. In the case of Scots pine, treatment D caused a decrease in the trait value at similar and intermediate level between SUN treatment B and the SHADE treatments. Thus, ROOT_HYPCOT also revealed the role of light intensity as a shade factor that interacts with the light quality composition (even if treatment A and D both have low intensity, depending on the trait they show similar or dissimilar effects).

ANOVA and Duncan post hoc test revealed two different patterns of the TOTAL_LENGTH in response to SHADE

(treatments A and C) versus SUN (treatment B) (Fig. 1b; Suppl. Table S2). In Scots pine, treatment C (SHADE) caused a significant increase of the TOTAL_LENGTH as compared to treatment B (SUN) and treatment A (SHADE, low light intensity), both A and B having similar effect on the TOTAL_LENGTH (Suppl. Table S2). Instead, in Norway spruce, both the SHADE treatments decreased the TOTAL_LENGTH equally with respect to the SUN treatment. Overall, it indicates that Scots pine responds to SHADE by reallocating resources (i.e., increase in hypocotyl is compensated by a repression of root elongation), while Norway spruce responds to shade by decelerating seedling growth (i.e., maintaining hypocotyl length similar to sun conditions and repressing root elongation). RNA sequencing was performed using pine and spruce seedlings grown under treatment A (SHADE) and B (SUN) as the phenotypic response (e.g. hypocotyl elongation) under these treatments in both species was determined to be the strongest among all.

Principal component analysis (PCA) results by treatment (A + C versus B + D) for each species were in concordance with the distinct level of the species shade

Fig. 3 PCA including all four groups of genes in **a** Scots pine and **b** Norway spruce. *SAS* genes significantly associated to the SAS, *STR* genes significantly associated to the STR, *Pine_down_Spruce_up* genes down-regulated by SHADE in Scots pine and up-regulated by

SHADE in Norway spruce, *Pine_up_Spruce_down* genes up-regulated by SHADE in Scots pine and down-regulated by SHADE in Norway spruce

Fig. 4 Heatmaps for all four groups of genes in **a** Scots pine and **b** Norway spruce. *SAS* genes significantly associated to the SAS, *STR* genes significantly associated to the STR, *Pine_down_Spruce_up* genes down-regulated by SHADE in Scots pine and up-regulated by

SHADE in Norway spruce, *Pine_up_Spruce_down* genes up-regulated by SHADE in Scots pine and down-regulated by SHADE in Norway spruce

tolerance. PCA did not reveal distinct clusters for SUN and SHADE in Norway spruce, a species considered to be shade-tolerant (Fig. 2), whereas in Scots pine, a shade-intolerant species, the SUN and SHADE clusters show low overlapping (Fig. 2). Hypocotyl (HYP) and growth rate between lower and upper parts of the seedlings (ROOT_HYPCOT) were the factors contributing the most to the SUN and SHADE light clusters separation.

Genes involved in shade avoidance (SAS) and tolerance (STS) in conifers

We compared the effect of treatment A (SHADE) and treatment B (SUN) to analyse the genes involved in SAS and STR in the two conifer species as the phenotypic response was the strongest under these treatments. PCA and Heatmaps based on all four gene categories (SAS, STR, *Pine_up_Spruce_down* and *Pine_down_Spruce_up*) (Figs. 3, 4) and

GO Molecular Function

Fig. 5 Gene ontology (GO) molecular function: functional categorization by annotation—pie charts for all four groups of genes. *SAS* genes significantly associated to the SAS, *STR* genes significantly associated to the STR, *Pine_down_Spruce_up* genes down-regulated by SHADE in Scots pine and up-regulated by SHADE in Norway spruce, *Pine_up_Spruce_down* genes up-regulated by SHADE in

Scots pine and down-regulated by SHADE in Norway spruce. Percentage denotes Number of annotations to terms in the GOslim category $\times 100/\text{number of total annotations to terms in the ontology}$. Number in the parenthesis denotes number of genes in the respective functional category

the entire transcriptome (Suppl. Fig. S2 and Suppl. Fig. S3) reveal that both species respond to shade by changing the expression level of multiple genes. In the case of the PCA results, similar conclusion was reached by analysing only the significant genes (*SAS*, *STR*, *Pine_up_Spruce_down* and *Pine_down_Spruce_up*) or the entire transcriptome. We found 905 genes significantly associated to the *SAS* (i.e. genes differentially expressed between SUN and SHADE in Scots pine, while unaffected by SHADE in Norway spruce) (Suppl. Table S3) and 443 to the *STR* (i.e. genes differentially expressed between SUN and SHADE in Norway spruce, but unaffected by SHADE in Scots pine) (Suppl. Table S4).

Chi-square analysis supported a higher number of the *SAS* genes being down-regulated by SHADE (661) as compared to the genes up-regulated by SHADE (271) (P value < 0.0001), whereas there were similar number of the *STR* genes which were found to be down-regulated by SHADE (247) and up-regulated by SHADE (201) (P value = 0.10). We also detected a few genes that were

differentially regulated between the SUN and SHADE conditions in both species. We classified them into two groups, one refers to the genes that were up-regulated by SHADE in Scots pine and down-regulated by SHADE in Norway spruce (*Pine_up_Spruce_down*, Suppl. Table S5); there were 12 such genes. The other group involves genes that were down-regulated by SHADE in Scots pine and up-regulated by SHADE in Norway spruce (*Pine_down_Spruce_up*, Suppl. Table S6); there were 22 genes in this category. An overview of the data, including raw and post-QC read counts, and alignment rates is provided in Suppl. Table S7. A total of 84518 and 70736 expressed genes were sequenced in Scots pine and Norway spruce, respectively.

With reference to the *SAS* and *STR*, GO molecular function (Fig. 5) pie charts reveal similar enrichment for “hydrolase and kinase activity” together with “nucleic acid binding” and “protein binding”. GO biological process (Suppl. Fig. S4) shows increase in the “developmental processes” and “DNA or RNA metabolism” in *STR*. With reference to GO cellular components (Suppl. Fig.

Fig. 6 SAS response. Comparison of the typical SAS responsive gene expression in *A. thaliana* and Scots pine

S4), there is an increase in cell wall, cytosol, ER, extra-cellular, Golgi apparatus, nucleus and plasma membrane involvement in STR as compared to SAS, whereas there is a lower enrichment in the chloroplast, plastid and ribosome activity in the STR.

We compared the SAS response in Scots pine to the well-characterized shade-intolerant *A. thaliana* but since STR appears to be less explored in this context, there are limitations to compare the genes involved in STR with a well-studied model system.

Analysis of the classical SAS genes in conifers

Typical response to shade in model systems such as *A. thaliana* is characterised by decreased accumulation of chlorophyll and reduced expression of distinct genes involved in photosynthesis (e.g. PSI, PSII and LHC). The SAS response model with reference to the findings of the current research (Fig. 6) summarizes the typical genes which are differentially regulated in response to shade in the shade intolerant pine and represents how the final output of shade response in pine is similar to the well-studied shade-intolerant *A.*

thaliana. Modulation of chlorophyll biosynthesis in response to shade is mostly regulated by an overall down-regulation of genes involved in chlorophyll biosynthesis (McLaren and Smith 1978; Smith and Whitelam 1997) and to a lesser extent to an up-regulation of genes associated with chlorophyll degradation (Brouwer et al. 2014) in *A. thaliana*. Similarly, reduced chlorophyll content has also been associated to shade in Scots pine (Razzak et al. 2017). Light-harvesting chlorophyll-binding protein complex I subunit A4 (*LHCA4*), a pigment apoprotein that conforms PSI was found to be down-regulated in Scots pine. This was similar to reduction in the amount of PSI which caused a reduction in chlorophyll content in *Nicotiana tabacum* (Hansson et al. 2007). Likewise, other *LHC* genes which were found to be down-regulated in Scots pine include *LHCA1*, *LHCA3* and *LHCB7*. Chlorophyll a–b binding family protein (*LIL3:1*), high chlorophyll fluorescence phenotype 173 (*HCF173*), protochlorophyllide oxidoreductase C (*PORC*), magnesium-protoporphyrin IX methyltransferase (*CHLM*), magnesium chelatase i2 (*CHL*) and coproporphyrinogen III oxidase (*CPO*), a group of genes involved in chlorophyll biosynthesis (Chatterjee and Kundu 2015), were also detected to be down-regulated in Scots pine. *HCF244* and *HCF136* involved in PSII assembly and photosynthesis/plastid organization, respectively, were found to be down-regulated under shade in Scots pine which earlier reported to be differentially regulated in the two *Geranium* species with contrasting responses to shade (Gommers et al. 2017). In Norway spruce, a chlorophyll a–b binding family protein (*ELIP2*) was down-regulated; while protochlorophyllide oxidoreductase B (*PORB*) which is involved in chlorophyll biosynthesis (Runge et al. 1996) was up-regulated. *ELIP2* was reported to play a protective role in *A. thaliana* exposed to high levels of light and its constitutive expression results in the decrease in the chlorophyll content of the chloroplasts (Tzvetkova-Chevolleau et al. 2007). In summary, pine and spruce show contrasting effects on the expression levels of chlorophyll and photosynthesis-related genes in response to shade where pine demonstrates similar response to what has been described in *A. thaliana*. Thus, a down regulation of photosynthesis appears to be a conserved SAS response between angiosperms and conifers.

Multiple studies have shown a decrease in anthocyanin synthesis in response to low R:FR in angiosperms (Alokam et al. 2002; Cagnola et al. 2012). In gymnosperms, anthocyanin synthesis in response to shade seems to be species dependent; reduced anthocyanin accumulation was observed in *Sequoia sempervirens* (Peer et al. 1999) in contrast to increased anthocyanin accumulation reported in Scots pine (Razzak et al. 2017). Razzak et al. (2017) demonstrated that anthocyanin level increased gradually with increase in light intensity for both R and FR light in Scots pine (monochromatic light treatments); anthocyanin accumulation increased

significantly under SHADE condition (R:FR 0.25) as compared to the SUN condition (R:FR 1.2). Likewise, dihydroflavonol 4-reductase (*DFR*), involved in anthocyanin biosynthesis pathway was found to be up-regulated in pine, whereas it was downregulated in spruce under shade. Ribonuclease 1 (*RNS1*) was reported to inhibit anthocyanin accumulation in *A. thaliana* (Bariola et al. 1999); congruently, we found *RNS1* to be down-regulated in Norway spruce. On the other hand, flavanone 3-hydroxylase (*F3H*) and leucoanthocyanidin dioxygenase (*ANS/LDOX*) which are involved in anthocyanin biosynthesis (Petroni and Tonelli 2011), were down-regulated by shade in Norway spruce. Overall, our results are not conclusive about the possible role of anthocyanin in shade tolerance in conifers.

None of the candidate genes selected by Gommers et al. (2017) from the transcriptome analysis in response to low R:FR in *Geraniums*, were found to be associated with SAS or STR in the current study in conifers. Yet, few genes that were listed to be differentially expressed in the two *Geranium* species were found to be associated with SAS in Scots pine; genes that were down-regulated under shade in Scots pine include—*ONE-HELIX PROTEIN 2* (associated with PSI), *PHOTOSYSTEM II SUBUNIT P-1* (associated with PSII), *RIBULOSE BIPHOSPHATE CARBOXYLASE SMALL CHAIN 1A* (photosynthetic activity) and *SIGMA FACTOR E* (plastid gene regulation).

Transcription factors (TF) involved in SAS and STR

Figure 7 represents the number transcription factors (TF) (GO:0003700) grouped according to the TF family which were found to be involved in SAS and STR. Higher number of TFs were found to be associated with SAS (Total 51: 33 up-regulated under shade and 18 down-regulated under shade) as compared to STR (Total 33: 17 up-regulated under shade and 16 down-regulated under shade). Transcription factors from the family MYB and bHLH were mainly found to be associated to SAS and STR in both the conifer species.

Expression of *PHY* genes in the conifer species, the major contributors to SAS in *A. thaliana*

The SAS molecular control is well-characterized in *A. thaliana* (Devlin et al. 2003; Franklin et al. 2003; Martinez-Garcia et al. 2014), whereas little is known about the molecular mechanisms that control shade tolerance, and the lack of knowledge is even more evident with reference to both types of shade responses (tolerance and avoidance) in conifers. Gommers et al. (2013) suggested three hypothetical models for the molecular regulation of shade tolerance, one of which involves alterations in the molecular structure of *PHYA* to increase phyA stability and, consequently, suppression of hypocotyl elongation antagonistically to the PhyB-mediated

Fig. 7 Transcription factors involved in SAS in Scots pine and STR in Norway spruce

SAS response. In addition, in *A. thaliana* both *PHYA* and *PHYB* expressions are known to be rapidly up-regulated following exposure to shade (Devlin et al. 2003; Martinez-Garcia et al. 2014). Our RNA-Seq data revealed no changes in *PHYN/A* expression in either species, while *PHYP2/B* was found to be down-regulated by shade in the shade-intolerant species (Scots pine) and up-regulated in the shade tolerant species (Norway spruce). The results are in contrast to what has been reported in *A. thaliana* thus leaving the intriguing possibility that the phytochrome differentially regulates shade response in angiosperms and gymnosperms. Association of differentially expressed *PHYB* under low R:FR light in the two *Geranium* species with contrasting responses to shade was reported by Gommers et al. (2017). We conducted an alignment of both *PHYN/A* and *PHYP/B* gene sequences where we included multiple angiosperm and gymnosperm species from both the shade response categories—shade tolerant and shade intolerant (Suppl. Fig. S5). This exercise revealed multiple non-synonymous polymorphic sites associated to their shade response category rather than to their phylogenetic one, thus indicating that even if there is no co-relation between the mode of phytochrome expression in response to shade between angiosperms and gymnosperms, it is still possible that they share a similar mode of action

which warrants further research. Moreover, a few amino acid positions in the *PHYA* and *PHYB* were conserved among the angiosperms and among the gymnosperms, irrespective of the shade response category. A polymorphism I143L in *PHYB* of *A. thaliana* is associated with variation in red light response (Filiault et al. 2008); presence of leucine at the corresponding position in all the gymnosperm species included in the alignment indicates that gymnosperms will respond to red light in a different manner than the angiosperms. This suggests that due to the sequence variability, phytochromes may attain a different conformation leading to distinct biochemical properties and functions in the two groups.

Jasmonic acid (JA) and salicylic acid (SA) pathways

Suppression of JA-mediated defence against biotic stress is a strategy to prioritize the SAS over other stresses (de Wit et al. 2013; Leone et al. 2014; Xu et al. 2016). The Skp1/Cullin/F-box E3 ubiquitin ligase complex (SCF^{COI1}) containing coronatine-insensitive protein 1 (COI1) activates JA-dependent responses (Devoto et al. 2002). SCF^{COI1} degrades jasmonate-zim-domain (JAZ) proteins, which are known repressors of JA signalling (Kazan and Manners 2012). Jasmonate-zim-domain protein 3 (JAZ3), known to

act as a negative regulator of JA signalling (Kazan and Maners 2012) was found to be down-regulated in Scots pine in response to shade which seems contradictory to the shade intolerant nature of the species.

Arabidopsis thaliana ethylene responsive element binding factors (*ATERFs*) are primarily involved in biotic stress responses; however, some *ERFs* have also been characterized as being responsive to abiotic stress such as temperature, drought and wounding (Fujimoto et al. 2000). *ATERF1* is a downstream element of the ethylene and jasmonate pathways, and participates in the regulation of defence-related genes (Lorenzo et al. 2003). Like-wise, basic endochitinase B (*CHI-B*) encodes a basic chitinase involved in ethylene/JA-mediated signalling pathway, which renders defence against chitin-containing fungal pathogens (Thomma et al. 1998). Down-regulation of *ATERF5* in spruce and up-regulation of *ATERF1* expression in pine in response to shade again contradicts the suppression of JA-mediated defence in SAS. However, up-regulation of *CHI-B* in spruce and insignificant *CHI-B* expression in pine under shade is in accordance with the suppression of defence mechanism during SAS response.

Salicylic acid (SA) pathway is also known to be repressed under shade (de Wit et al. 2013). *Arabidopsis thaliana* methyl esterase 1 (*ATMES1*) and other members of the *ATMES* gene family regulate interaction between SA and JA through regulation of the levels of their respective methylated derivatives (Dempsey et al. 2011). *ATMES1* which is involved in SA hydrolysis (Vlot et al. 2008) was found to be down-regulated in Scots pine, challenging the known suppression of SA in the SAS. *Arabidopsis thaliana* phytoalexin deficient 4 (*ATPAD4*) functions in SA-dependent defence signalling by interacting with another lipase-like protein (*A. thaliana* enhanced disease susceptibility 1, AtEDS1), in a positive feedback loop to promote SA biosynthesis (Glazebrook et al. 1997). *ATPAD4* was down-regulated by shade in the shade-intolerant Scots pine supporting its role in the SAS activation through diminishing SA signal. To the best of our knowledge, this gene has not been previously associated to the SAS. In Norway spruce, phenylalanine ammonia-lyase 2 (*PAL2*), a gene that mediates SA synthesis (Chen et al. 2009), was detected to be up-regulated by shade suggesting a role of SA in the STR.

Genes related to defence response

Analysis of the genes that belong to GO categories such as defence response, immune response and acquired resistance shows a higher number of defence-related genes being down-regulated by SHADE (48) as compared to the genes up-regulated by SHADE (28) (P value = 0.02) in pine, whereas similar number of genes were down-regulated (18)

and up-regulated by SHADE (26) (P value = 0.23) in spruce. Therefore, from the expression analysis we speculate that SAS in conifers is associated with reduced defence response making the plant prone to diseases which is in accordance with the studies in *A. thaliana* where it was demonstrated that low R:FR light down-regulates the defence pathways making the plant more susceptible to pathogens (Cerrudo et al. 2012; de Wit et al. 2013; Chico et al. 2014).

Comparison between gene expression patterns in response to shade in *A. thaliana* and conifers

Table 2 summarises the genes which have well-defined expression patterns with reference to shade response in *A. thaliana* and the related genes in conifers, which were found to be significant in response to shade. The regulation of some genes [e.g. auxin response factor (*ARF*), gibberellin (GA)-related, *ATERF*] appeared to be conserved in the shade intolerant pine and *A. thaliana*. Expression of few genes in spruce was in opposite direction (e.g. *A. thaliana* home box gene, *ATHB*) or insignificant [e.g. Gretchen Hagen 3 (*GH3*), *ARF*] as compared to *A. thaliana*, which may be due to the contrasting response to shade in the two species.

Discussion

Shade avoidance and shade tolerance are different plant strategies to optimize photosynthesis in response to shade. These two shade-response strategies are also found in conifer species, where pine species are considered to have a lower level of shade tolerance compared to spruce species (Warrington et al. 1989; Hoddinott and Scott 1996). R:FR ratio is considered a reliable indicator of the degree of shade and its low values are associated with shade avoiding and shade tolerance responses. Here we demonstrate the distinct morphological and transcriptional changes in response to shade in two conifer species with contrasting levels of shade tolerance; Scots pine, shade-intolerant, and Norway spruce, shade-tolerant.

In our comparative system, Scots pine versus Norway spruce, hypocotyl and total seedling elongation in response to shade reflect the level of the species shade tolerance, whereas this is not the case for root, cotyledons and root to aerial tissue ratio responses, suggesting that hypocotyl and seedling elongation should be the shade response markers for further physiological studies in conifers. Previous studies with simulated shade reported plant elongation associated to shade intolerant species as compared to tolerant ones in angiosperms (Gommers et al. 2017) and gymnosperms (Hoddinott and Scott 1996). Furthermore, our results indicate that differences in shade tolerance between both conifer species could be related to differences in carbon re

Table 2 Comparison between genes involved in shade response in *A. thaliana* and conifers

Gene	Function	<i>A. thaliana</i>	Norway spruce	Scots pine	References
Auxin-related	Plays role in shade-induced elongation growth	↑ Auxin biosynthesis			Yang and Li (2017)
<i>AUX/IAA</i> -responsive genes— <i>SAUR</i> -like auxin-responsive proteins	Regulate plant growth and development	↓ <i>SAUR32</i> under light enriched in FR ↑ <i>SAUR76</i> under light enriched in FR <i>SAUR15</i> , <i>SAUR67</i> under shade	↓ <i>SAUR32</i> , <i>SAUR76</i> , <i>SAUR70</i> under shade ↑ <i>SAUR12</i> under shade	↓ <i>SAUR70</i> under shade	Ren and Gray (2015), Leivar et al. (2012a)
<i>ATHB</i> —HD-Zip class II subfamily transcription factor	Promotes hypocotyl growth by modifying the responsiveness to <i>AUX/IAA</i>	↑ <i>ATHB2</i> and <i>ATHB6</i> under low R:FR	↓ <i>ATHB6</i> and <i>ATHB15</i> under shade	Not significant	Steindler et al. (1999), Sessa et al. (1993)
Gretchen Hagen 3 (<i>GH3</i>)	Involved in auxin homeostasis	↑ <i>GH3.3</i> and <i>GH3.4</i> after 1 h under FR ↓ <i>GH3.3</i> and <i>GH3.9</i> after 24 h under FR	Not significant	↑ <i>GH3.17</i> under shade	Leivar et al. (2012b)
ATP binding cassette subfamily B (<i>ABCB</i>)	ABC family members mediate auxin efflux	ABC family members required for shade-induced hypocotyl elongation	Not significant	↓ <i>ABCB4</i> , <i>ABCB19</i> under shade	Ge et al. (2017)
Auxin1/like AUX (<i>AUX/LAX</i>)- <i>LAX2</i>	LAX2-transporter of auxin		↑ <i>LAX2</i> under shade	Not significant	Swarup and Péret (2012)
Auxin response factors (<i>ARFs</i>)	Mediate auxin regulation of multiple processes	↓ <i>ARF11</i> , <i>ARF18</i> under FR	Not significant	↓ <i>ARF16</i> under shade	Leivar et al. (2012b)
Brassinosteroid (BR)-related	Brassinosteroid (BR) regulates photomorphogenesis, promotion of stem growth under shade	BR biosynthesis			Yang and Li (2017)
<i>BRI1</i> -EMS-SUPPRESSOR 1 (<i>BES1</i>) homolog protein 1- (<i>BEH1</i>)	<i>BEH1</i> mediates brassinosteroid-induced growth		Not significant	↓ <i>BEH1</i> under shade	Wang et al. (2002), Ciolfi et al. (2013)
<i>MYB30</i> -transcription factor	Promotes expression of BR target genes		Not significant	↑ <i>MYB30</i> under shade	Yin et al. (2005), Li et al. (2009)
Gibberellins (GAs)-related	Promotes growth	↑ GA under shade	↑ Gibberellin-regulated family proteins under shade- (AT1G74670, AT1G22690)	↑ Gibberellin-regulated family proteins under shade- (AT2G18420, AT2G39540)	Yang and Li (2017)

↑ Represents up-regulation of the gene and ↓ represents down-regulation of the gene

allocation. In Scots pine, carbon accumulates in the stem at the expense of root and cotyledon development. On the contrary, Norway spruce seems to respond to shade with an overall decrease in biomass production. This interpretation agrees with the previously proposed “carbon gain hypothesis” that defines shade-tolerant species and involvement of maximization of light capture (i.e., increase in specific leaf area and chlorophyll content) together with a scant trunk elongation and growth as compared to the shade-intolerant species where stem elongation aims to outreach light as a strategy to survive (Valladares and Niinemets 2008; Hallik et al. 2009; Modrzyński et al. 2015).

We investigated the relative effect of R:FR and light intensity in seedling development in both conifer species. Scots pine and Norway spruce were exposed to two simulated shade conditions (SHADE, R:FR=0.2) with different light intensities (36 and 65 $\mu\text{mol m}^{-2} \text{s}^{-1}$, treatment A and C, respectively) and two simulated sunny conditions (SUN, R:FR=1.2) with contrasting light intensities (65 and 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$, treatment B and D, respectively). Our results suggest that response to shade is essentially triggered by changes in R:FR; low light intensity under shade (low R:FR) can amplify the shade response, at least in the case of Scots pine, and can also trigger the shade response even under low light intensity with sunny conditions (high R:FR). In a recent study in Scots pine, the role of light intensity in the modulation of response to FR and R wavelengths was demonstrated (Razzak et al. 2017). In Norway spruce, however, the low R:FR value overrides the effect of light intensity, at least for the assayed conditions, as the seedling growth responds similarly to both SHADE treatments (despite differences in light intensity).

Our third research question was to identify the potential candidate genes involved in the regulation SAS and STR in conifers; we compared the expression profiling (RNA-Seq) between seedlings of Scots pine (shade intolerant) and Norway spruce (shade tolerant). As a first exploratory approach of the RNA-Seq data, the gene count already revealed interesting results. Firstly, 67% of the genes showing a significant response to shade were associated to the SAS; secondly, among those genes, 63% were down-regulated, whereas among the STR genes the proportion of up-regulated and down-regulated genes was similar. These two outcomes provide evidences that the shade-tolerant species also adapts to shade by altering the transcriptional level of multiple genes (see PCA and Heatmaps). However, there are clear differences in the extent and main direction of those transcriptional alterations.

Our transcriptome analysis provides evidences about the involvement of pigment and hormone biosynthetic pathways in SAS and STR responses in conifers in a comparable way to what has been previously described in angiosperms (Nozue et al. 2015; Gommers et al. 2017; Yang and Li 2017).

Although we suggest a few potential candidates involved in the SAS/STR mechanisms in conifers, further investigation is required to resolve the phenomenon. We have identified a number of major gene family players associated to SAS or STR in conifers which were previously found to be associated to the SAS in angiosperms, e.g. pathways involving auxin/indole-3-acetic acid (AUX/IAA), brassinosteroid, ethylene, gibberellins (GAs), JA and SA. Some of those gene families, such as SAURs and auxin response factors (ARFs) from the AUX/IAA pathways, seemed to involve a highly dynamic feedback mechanism that regulates the auxin cellular levels characterized by a rapid and transient enrichment in these genes (Ciolfi et al. 2013; Ren and Gray 2015) which indicates that the experimental design also in terms of duration of the light treatment will affect the level of expression. Similar feed-back regulatory evidences were also detected in gene families from the other hormonal and anthocyanin biosynthesis pathways. For example, up-regulation of *GA20OX3* is described as conditional to the amount of gibberellin hormone, where high levels of gibberellin inhibit *GA20OX3* expression (Sun 2008). Association of SAS with suppression of JA and SA mediated responses involved in plant defence against insect/pathogen and disease resistance is well characterised in *A. thaliana*; JA-mediated defences are repressed by low R:FR in shade-intolerant but enhanced in shade-tolerant wild species (Gommers et al. 2017). In the current work, although the expression of few genes (*JAZ3*, *ATERF*, *ATMES1*) related to JA/SA-pathway was found to be contradictory with reference to SAS/STR, analysis of genes that belong to the GO categories related to defence response supports that SAS in conifers is associated with reduced defence response, whereas defence mechanism remains unaffected in STR. Thus, the suppression of defence mechanism appears to be conserved in conifers but the underlying regulatory network differs from angiosperms. Although significant progress have been made to decipher the complex regulatory mechanisms that operate through the interaction of JA–SA signalling which controls the defence strategies in angiosperms, the molecular mechanisms in conifers still needs to be explored.

Consistently with previous studies where reduction in chlorophyll content has been associated to shade avoidance in *A. thaliana* (McLaren and Smith 1978; Smith and Whitelam 1997) and in Scots pine (Razzak et al. 2017), we have found several genes from the chlorophyll biosynthesis pathway, which were down-regulated in pine: *LHCA1*, *LHCA3*, *LHCA4*, *LHCB7*, *LIL3:1*, *HCF173*, *PORC*, *CHLM*, *CHL* and *CPO*. Moreover, down-regulation of pheophytinase (*PPH*) and accelerated cell death 2 (*ACD2*) in Scots pine, two genes known to mediate chlorophyll-breakdown (Eckhardt et al. 2004; Zhang et al. 2014), suggests that chlorophyll synthesis during the SAS is also affected by feedback regulatory mechanisms.

The LHC genes associated with SAS in the current study were different than those which were found to be differentially expressed under low R:FR light in the two *Geranium* species with contrasting responses to shade (*LHCA6*, *LHCB4.1*, *LHCB5*) (Gommers et al. 2017). Our study has also contributed with evidences about chlorophyll accumulation during the STR, concluded from the expression pattern observed for *ELIP2* and *PORB* genes. In the literature, increased chlorophyll content has been suggested as an adaptive mechanism exhibited by the shade-tolerant species which involves maximization of light capture (Valladares and Niinemets 2008). Increase in chlorophyll content contrasts with a lower number of GO cellular components such as chloroplast activity in STR as compared to SAS. One possible interpretation is that during the process of plant response to shade the aim is to optimize light acquisition while maintaining growth to a low rate (Loach 1967; Valladares and Niinemets 2008; Hallik et al. 2009; Modrzynski et al. 2015). In *A. thaliana*, *ELIP2* was reported to be associated to FR response (Leivar et al. 2012a), whereas, to our knowledge, *HCF173*, *CHL*, *LHCA4*, *CPO*, *PORB*, *LIL3:1*, *CHLM* and *PORC* have not been previously linked to shade response.

LATE ELONGATED HYPOCOTYL (LHY), a TF from MYB family was found to be repressed by shade in association with SAS in pine; *LHY* plays key role in the circadian oscillator in *A. thaliana* and its involvement in shade avoidance in *A. thaliana* is not established (Mizoguchi et al. 2002), but an ortholog of *LHY* was down-regulated under shade in maize (Wang et al. 2016). *PHYTOCLOCK 1 (PCL1)*, a G2-like TF family member, which is characterised as circadian clock gene (Onai and Ishiura 2005), is found to be up-regulated by shade with reference to SAS. *LEAFY* (LFY TF family) is a meristem identity regulator and it targets *LATE MERISTEM IDENTITY2 (LMI2)* (MYB TF family) that has a role in the meristem identity transition. Thus, *LFY* plays a role in floral meristem development and promotes transition from vegetative growth to flowering (Pastore et al. 2011). Both *LEAFY* and *LMI2* were up-regulated in pine in response to shade. *STERILE APETALA (SAP)* is a transcription regulator essential for the maintenance of floral identity acting in a similar manner as *APETALA1* (Byzova et al. 1999), which was found to be co-expressed with *LHY* under shade in pine. Changes to the circadian clock and early flowering are one of the major components of the shade avoidance response as reported in *A. thaliana* (Adams et al. 2009; Ciolfi et al. 2013); thus involvement of circadian and flowering genes in shade avoidance response appears to be conserved in pine and *A. thaliana*. Circadian rhythm associated TF was not detected in association to STR. However, *TEMPRANILLO (TEM) 1* was found to be down-regulated and, one of the homolog of *TEM1* and *APETALA 2 (AP2)* were up-regulated by shade in

spruce. *TEM1* along with *TEM2* delays flowering in *A. thaliana* until the plant is ready for this process by accumulating enough reserves or has reached the appropriate growth stage (Matias-Hernandez et al. 2014). Involvement of *AP2* is not restricted to establishment of the floral meristem and floral organ identity, but it also plays a general role in the control of *A. thaliana* development (Jofuku et al. 1994). This can be interpreted as postponing the process of flowering associated to STR in spruce at the seedling stage.

From previous studies it is known that low R:FR triggers the SAS morphological and physiological changes to improve light capture; a response mediated primarily by the phytochromes (Martinez-Garcia et al. 2010). Shade reduces phyB activity and, consequently, increases the mRNA levels of the bHLH transcription factors such as phytochrome interacting factors (PIF) (Lorrain et al. 2008). PIFs regulate a large number of genes encoding metabolic enzymes and genes in the phytohormones signalling pathway such as auxin (*AUX/IAA*) (Hersch et al. 2014), ethylene (Jeong et al. 2016), brassinosteroids (BRs) (Oh et al. 2012), and gibberellins (Schwechheimer 2011), which control low R:FR-induced shade avoidance responses.

In *A. thaliana*, role of *PHYA* and *PHYB* in shade avoidance is well-documented and involves up-regulation of *PHYA* and *PHYB* in response to low R:FR (Devlin et al. 2003; Franklin et al. 2005; Martinez-Garcia et al. 2014). Our study reveals that *PHYP2/B* is down-regulated by low R:FR light in Scots pine (shade intolerant) and up-regulated in Norway spruce (shade tolerant), whereas no change was detected in the expression level of *PHYN/A*. In Scots pine, down-regulation of *PHYP2/B* could result in a decrease in the absolute value of phyP2/B active form leading to the activation of the downstream cascade that mediates hypocotyl elongation. On the controversy, in Norway spruce, up-regulation of *PHYP2/B* could result in an increase in the absolute value of phyP2/B active form and the consequent prevention of the SAS activation. In contrast to *A. thaliana*, where *PHYA* and *PHYB* are rapidly up-regulated in response to shade (Devlin et al. 2003; Martinez-Garcia et al. 2010, 2014), our expression study does not support a role for *PHYN/A* in shade avoidance or tolerance in conifers.

Moreover, in *A. thaliana* up-regulation of the *PHYs* does not result in an increase in phyA and phyB activity, instead, SAS is initiated with the FR-mediated conversion of the phyB into its inactive form and the degradation of its antagonistic protein, phyA (only under very low R:FR values phyA is stabilized mediating the so-called FR high irradiance response (FR-HIR) (Martinez-Garcia et al. 2010, 2014). It could be speculated that, despite the expression pattern of the two *PHYs* differ between *A. thaliana* and the two conifer species, they may still share similar patterns of protein activity regulation. In fact, alignment of *PHYA* and *PHYB* gene sequences from five

different species (*A. thaliana*, *Populus*, *Eucalyptus*, *Glycine max*, Scots pine, *Picea glauca* and Norway spruce) reveals multiple non-synonymous polymorphisms associated to the species level of shade tolerance (tolerant versus intolerant) rather than to their phylogenetic classification (angiosperm versus gymnosperm). It remains to be investigated if this is an evidence of a shared functional role of the phytochrome proteins in angiosperms and gymnosperms with reference to shade tolerance. Conserved amino-acids positions in *PHYA* and *PHYB* within angiosperms and within gymnosperms suggests that there is a possibility of altered function due to sequence variability as in the case of cryptochrome 2 in *A. thaliana* (El-Assal et al. 2001), due to which the mode of action of phytochromes may differ in the two groups. Thus, from the phytochrome (phyA and phyB) amino-acid sequence patterns that either categorise the plant species by their type of shade response (shade tolerant or shade intolerant) or by the plant group (angiosperm or gymnosperm), we conclude that the phytochromes may possess a common functional role with reference to shade response and simultaneously, also mediate shade response in a different fashion due to altered conformation. In this context, the polymorphism I143L in *PHYB* of *A. thaliana* is associated with variation in red light response (Filiault et al. 2008); and from the alignments it is seen that in all the gymnosperm species there is Leucine at the corresponding position indicating that gymnosperms will respond to red light in a different manner than the angiosperms.

Whereas a lack of correspondence between the expression level and protein activity may be the case in *PHYB2/B*, it is interesting to mention that *PHYN/A* gene expression was previously shown to remain steady in dark- and light- grown spruce seedlings (Clapham et al. 1999). This apparent lack of induced *PHYA* expression in response to shade could be related to the absence of FR high irradiance response (FR-HIR) (known to be regulated by phyA) previously described in several conifer species including pine and *Ginkgo* (Fernbach and Mohr 1990; Mathews and Tremonte 2012) or presence of mild FR-HIR in Scots pine (Razzak et al. 2017). Clapham et al. (1998) suggested the *PHYA* light-mediated self-regulation as a character which was acquired late in phytochrome evolution, after the separation of the gymnosperms and angiosperms lineages, which together with the results of this study suggest that such a role may not have been acquired by the *PHYN/A* in conifers. In this respect, phylogenetic study of FR-HIR distribution by Mathews and Tremonte (2012), supports FR-HIR to be ancestral in living seed plants. The authors also presented evidences of several episodes of positive selections in *PHYA*, early in the history of angiosperms that resulted in the acquisition of new roles of FR-HIR, such as the control of seedling de-etiolation, and that could have resulted in a divergent evolution of

PHYA function between gymnosperms and angiosperms. In contrast, *PHYB* lineage has been less affected by selection (Mathews et al. 2003; Mathews 2010). In the present study, we contribute with the evidence that *PHYA* did not change its expression level in response to shade, but a change in the expression of *PHYB* was detected in both the conifer species. In conclusion, our study supports a major putative role of *PHYB* in response to shade in conifers, where further research must be conducted to solve the role of *PHYA*.

Conclusion

Our comparative study of two conifers with contrasting levels of shade tolerance unveils conifer seedlings as a suitable system to study the mechanism of shade response, where hypocotyl elongation is the morphological feature that better reflects the differences in shade tolerance between both species. Seedling total length reveals that the shade tolerant species (Norway spruce) has adopted a mechanism to improve light capture while decreasing biomass production, whereas in the shade intolerant species (Scots pine), light use optimization is achieved through elongation to escape the shade. We also conclude that low light intensity can trigger similar morphological shade response as low R:FR. In Norway spruce, R:FR is the main factor determining seedling development under shade, whereas in Scots pine we observed an interaction between light intensity and R:FR. The comparative RNA-Seq analysis sheds light on our understanding of the involvement of light and hormone signalling pathways that control both processes, the SAS and the STR. Most of the genes show a role that was exclusive to one of the processes and a large proportion of them are novel while genes expressed in opposite directions in each species seem less abundant. There are other interesting features that also differentiate both responses such as the higher number of genes found in relation to the SAS and a clear preference towards gene down-regulation in SAS as compared to the STR. Our results revealed that *PHYB* may play a central role in the control of SAS and STR in conifers as compared to *A. thaliana* where both *PHYA* and *PHYB* are known to respond to shade. Overall, the SAS and STR seem to be regulated by a complex mechanism where negative feedback loops and antagonistic effects play an important role to modulate the extent of the response to shade, especially, in the control of the hormone levels. It is therefore sensible to conclude that the full understanding of the mechanism behind shade response in conifers warrants further research.

Author contribution statement SSR contributed with experiment performance, data collection, data analysis and interpretation, and manuscript writing. ND contributed with experimental design and bioinformatics support. MRGG

contributed with experimental design, data analysis and interpretation, and manuscript writing. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interests sAuthors declare that they have no competing interest.

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