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Short-day treatment affects growth, physiological parameters and needle proteome of Chinese pine (*Pinus tabuliformis* Carr.) seedlings

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Abstract
Previous studies have proven that short-day treatment is an effective means to regulate shoot growth and abiotic stress resistance in conifers. However, the effects on Chinese pine (*Pinus tabuliformis*) seedlings are less well known. In this study, short-day treatments were applied to container-grown Chinese pine seedlings by artificially reducing day length to 8 or 10 h for 3 weeks. Growth and physiological responses of the seedlings were evaluated. In addition, proteome analysis using two-dimensional electrophoresis in combination with MALDI-TOF/TOF MS analysis was performed on needles of the seedlings. Short-day treatments significantly affected seedling bud set, height, root collar diameter and number of first-order lateral roots (> 1 mm diameter at junction with taproot). Short-day treatments significantly enhanced the content of total chlorophyll, starch, abscisic acid, and lignin, and increased activity of phenylalanine ammonialyase, polyphenol oxidase, and peroxidase, but did not differ in the content of soluble sugar. We positively identified eight differentially expressed proteins induced by short-day treatments: oxygen-evolving enhancer protein 1, transcription factor VOZ1, ferredoxin-NADP reductase, leaf isozyme 1, protein MET1, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase 1, elongation factor Tu, and ribulose bisphosphate carboxylase/oxygenase activase. These proteins are mainly involved in photosynthesis, carbohydrate metabolism, as well as abiotic and biotic stress resistance. Our results suggest that short-day treatment is a way to produce high-quality Chinese pine seedlings in the nursery.

Keywords Short-day · Physiology · Proteomics · Stress resistance · Chinese pine

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Introduction

High-quality seedlings are essential for successful plantation establishment (Davis and Jacobs 2005; Liu et al. 2012). Shortening of photoperiod (i.e., short-day [SD] treatment) is a dormancy-induction treatment to induce shoot growth cessation and cold hardiness development (Turner and Mitchell 2003). Prior to spring planting, SD treatment is a common routine to increase the hardiness level in seedlings (Peterson 1997). In addition, SD treatment is used to regulate height/diameter ratio creating sturdier seedlings, and it has been applied to Norway spruce (*Picea abies* (L.) Karst.) (Luoranen et al. 2006) and white spruce (*Picea glauca* (Moench) Voss) (Tan et al. 2008) for summer planting, as well as Western red cedar (*Thuja plicata* Donn) (Major et al. 1994), Scots pine (*Pinus sylvestris* L.) (Nilsson et al. 2010) and *Picea abies* (L.) Karst. (Fløistad and Granhus 2013) for autumn planting.

Research on how SD treatment affects seedlings in the nursery has been mainly conducted on the dominant conifers of Canada (e.g., *Picea glauca* (Moench) Voss, black spruce (*Picea mariana* (Mill.) B.S.P.), Sitka spruce (*Picea sitchensis* (Bong.) Carrière)), North America (e.g., Douglas-fir (*Pseudotsuga menziesii* var. menziesii (Mirb.) Franco)) and Scandinavia (e.g., *Picea abies* (L.) Karst., *Pinus sylvestris* L.) for several decades. Previous studies have focused largely on how SD treatment influence bud dormancy and seedling abiotic stress resistance. Reduced photoperiods hasten bud dormancy in *Pinus silvestris* seedlings (Wareing 1950). Height growth cessation and then bud set occurred earlier in seedlings given SD treatment (Heide 1974; Colombo 1997; Grossnickle 2012). In addition, SD treatment increases cold hardiness of *Picea glauca* (Moench) Voss (Bigras and D'Aoust 1993), *Thuja plicata* Donn (Folk et al. 1994), *Picea sitchensis* (Bong.) Carrière (Hawkins et al. 1996), *Picea mariana* (Mill.) B.S.P. (Colombo et al. 2003), *Picea abies* (L.) Karst. (Rostad et al. 2006), *Pseudotsuga menziesii* var. menziesii (Mirb.) Franco (Jacobs et al. 2008), and improves the drought tolerance of *Picea abies* (L.) Karst. (Luoranen et al. 2007) and *Picea glauca* (Moench) Voss (Tan 2007). Research in recent years has attempted to explore the molecular basis behind these above-mentioned morphological and physiological changes in seedlings. Asante et al. (2011) identified genes involved in growth cessation and bud set in *Picea abies* (L.) Karst. seedlings during the SD treatment by combining suppression subtractive hybridization (SSH) and monitoring gene expression by qRT-PCR analysis. Wallin et al. (2017) observed that genes related to dormancy induction and freezing tolerance were activated when SD treatment was prolonged.

However, an expanded study to determine effects after SD treatment on seedling physiological processes, specifically on photosynthesis, carbohydrate metabolism and stress resistance, directly at the protein level would provide more useful information than genomics. It is well known that proteins are the final executors of biological processes and that changes in plant physiological function may be associated with protein expression (Chi et al. 2010). Proteomic analysis using two-dimensional electrophoresis (2-DE) in combination with mass spectrometry (MS) has proven to be a powerful and reliable tool to separate and identify differentially expressed proteins and reveal the essence of plant physiological phenomena (Canovas et al. 2004; Anguraj 2015). In recent years, proteomic analysis has become widely applicable in *Oryza sativa* L. (Lee et al. 2010), *Triticum aestivum* L. (Song et al. 2007), *Zea mays* L. (Pechanova et al. 2010), *Glycine max* (L.) Merr. (Sha et al. 2016) and *Arabidopsis thaliana* L. (Severing et al. 2011) to understand the physiological processes of the plants, including primary metabolism, secondary metabolism, tissues and organs differentiation, plant development and abiotic and biotic stress responses

(Heazlewood et al. 2003; Nestler et al. 2011). However, relatively limited information is available concerning proteomic responses in trees. Proteomics research on forest tree has mostly concentrated on *Populus* spp. (Bonhomme et al. 2009), *Pinus* spp. (Garcés et al. 2014) and *Picea* spp. (Blödner et al. 2007), and identified proteins associated with basic metabolism, abiotic and biotic stress responses and wood formation (Kjellsen et al. 2010; Mast et al. 2010; Durand et al. 2011). Thus, proteomic analysis may be a novel and effective method to determine seedling basic metabolism and stress resistance responses to SD treatment.

Chinese pine (*Pinus tabuliformis* Carr.) is one of the most important native afforestation species in North China. In an unpublished study, we evaluated outplanting performance of container-grown Chinese pine seedlings given SDs for 5 years on an afforestation site. We observed that a 3-week SD (8 or 10 h) treatment significantly promoted survival and growth of outplanted seedlings under stressful environmental conditions caused by extreme high temperature, drought and pathogen infection. However, little information is available on how SD treatment affects Chinese pine seedlings in the nursery. The objective of this study was to investigate growth, physiological and proteomic responses in Chinese pine seedlings immediately after SD treatment. We hypothesized that SD treatment would (1) (a) result in height growth cessation and earlier bud set, (b) increase RCD, number of FOLR and root dry mass, and (c) reduce shoot dry mass; (2) increase content of total chlorophyll, soluble sugar and starch; (3) promote ABA and lignin accumulation, as well as increase activity of PAL, PPO and POD; and (4) induce changes in protein expression, involved in regulation of seedling physiological processes.

Materials and methods

Plant material and short-day treatment

Container-grown Chinese pine seedlings were cultivated in a greenhouse at the Jiufeng Experimental Station of Beijing Forestry University (39°54′N, 116°28′E, Beijing, China) under natural day length with day/night temperatures averaging 28/18 °C and 75% relative humidity. On 4 March 2013, Chinese pine seeds sourced from the North Scientific Research Base of Beijing Forestry University (40°24′N, 118°21′E, Hebei province, China) were sown into 98 Ray Leach "Cone-tainers" SC10 Super containers (164 cm^3, top diameter 3.8 cm × depth 21 cm, Stuewe & Sons, Inc., Oregon, USA) with a 3:1 (v:v) mixture of peat (pH 5.5, particle size 0–10 mm, Pindstrup Seedling, Denmark) and vermiculite. Fertilization began on 3 April and continued once per week until 17 July, with irrigation to field capacity as necessary in between. We supplied seedlings with total amounts of 100 mg N, 26.2 mg P, 33.2 mg K, 3 mg EDTA, and 0.9 mg DTPA $seedling^{-1}$. N was applied exponentially following Timmer and Armstrong (1987). The quantity of N to apply for a specific week (N_t) was calculated according to the initial N content in each seed (N_s) = 1.58 mg per seed, the total number of fertilizations (t) = 16 and the desired amount to be added over the number of fertilizer applications (N_T) = 100 mg N. P, K, EDTA and DTPA were evenly split into 16 equal amounts and applied weekly. On 24 July, we randomly selected 9 trays of actively growing seedlings (98 seedlings per tray, 882 seedlings in total) and evenly divided them into three groups, two of which were set to SD-8 h and SD-10 h treatment respectively, and the remaining one was used as control. Seedlings from two SD treatments were covered under a blackout frame made of black plastic, in which SD-8 h and SD-10 h

were respectively provided by artificial reducing day length to 8 h (from 08:00 to 16:00) and 10 h (from 08:00 to 18:00). Seedlings from control were grown under the natural day length, which is about 15 h in July in Beijing. During the 3 weeks period, all seedlings were irrigated with no fertilization. On 15 August, we randomly removed 48 seedlings per tray (432 seedlings in total) for other study. The remaining 50 seedlings per tray (450 seedlings in total) were used in this study. We randomly sampled 30 seedlings per treatment (10 seedlings per replicate, 3 replicates) for measurement of morphological traits, 24 seedlings per treatment (8 seedlings per replicate, 3 replicates) for physiological measurements and 24 seedlings per treatment (8 seedlings per replicate, 3 replicates) for protein extraction. Seedlings used for physiological measurements and protein extraction were immediately frozen in liquid nitrogen and then stored at − 80 °C.

Morphological and physiological measurements

Percentage bud set was assessed. Bud set was defined as the shoot apical meristem constitutes a dome of tissue above the most recently initiated cataphylls (MacDonald and Little 2006). Percentage bud set was calculated as the number of seedlings with bud set divided by the total number of seedlings for each replicate. Seedling height (measured from the root collar to the tip of the terminal bud), and root collar diameter (RCD) were measured. The number of first-order lateral roots (FOLR; > 1 mm diameter at junction with taproot) was counted. Seedlings were separated into needles, stems, and roots, oven dried at 70 °C for 48 h, and finally weighed (± 1 mg) (Oliet et al. 2009).

There were three replicates per treatment, seedling fresh needles (0.5 g) were sampled from each replicate for measurement of physiological indexes. The following parameters were assessed, content of total chlorophyll (Chl $a+b$), soluble sugar, starch, abscisic acid (ABA), and lignin, and activity of phenylalanine ammonialyase (PAL), polyphenol oxidase (PPO), and peroxidase (POD). The Chl $a+b$ concentration was determined in accordance with Lichtenthaler (1987). The soluble sugar and starch contents were measured as previously described by Yan et al. (2012). The ABA content was quantified using the indirect competitive enzyme-linked immunosorbent assay (icELISA) in accordance with Nhan et al. (2015). We used kits for detection of the phytohormone provided by the College of Agronomy and Biotechnology, China Agricultural University, Beijing. Lignin content was quantified using the method of Wagner et al. (2007). The activity of PAL, PPO, and POD were assayed using the methods described by Hu et al. (2009). The contents of Chl $a+b$, soluble sugar, starch, and lignin, and activity of PAL, PPO, and POD were measured using a Model 722 spectrophotometer (Agilent 8453, Waldbronn, Germany).

Protein extraction

Three replicates per treatment were subjected to proteome analysis. Total proteins were extracted using the phenol method in accordance with Carpentier et al. (2005) with some modifications. For each biological repeat sample, fresh needles (1 g) were ground to a fine powder in liquid nitrogen and resuspended in 3 mL precooled extraction buffer [50 mmol/L Tris-HCl (pH 8.5), 5 mmol/L EDTA, 0.1 mol/L KCl, 1% w/v DTT, 0.9 mol/L sucrose] and incubated at 4 °C for 30 min. The mixture was placed in centrifuge tubes together with an equal volume of ice-cold Tris-phenol (pH 8.5), mixed at 4 °C for 15 min, and centrifuged at 6000 *g* for 3 min at 4 °C. The upper phenol phase was carefully removed and the remaining solution was re-extracted with half the volume of ice-cold Tris-phenol (pH 8.5).

The two extracts were combined by adding 5 times volume of precooled methanol containing 0.1 mol/L ammonium acetate, then stored at − 20 °C overnight to precipitate the proteins. The precipitated proteins were collected by centrifugation at 18,000 *g* for 30 min at 4 °C, washed with methanol three times until the precipitate was white and then twice with precooled 80% acetone containing 0.07% (v/v) β-mercaptoethanol, and finally vacuum dried and stored at − 80 °C. Vacuum-dried pellets (30 mg) were dissolved in 600 µL lysis solution [40 mmol/L Tris-HCl, 7 mol/L urea, 2 mol/L thiourea, 4% w/v CHAPS, 1% w/v DTT, 1 mmol/L EDTA Na_2], incubated at 4 °C for 30 min, and centrifuged at 12,000 *g* for 15 min. The supernatant was collected as the protein extract and used for 2-DE analysis. The protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA), with bovine serum albumin used as the standard (Bradford 1976).

Two-dimensional gel electrophoresis and image comparison

Three gels were run per extract. Proteins (1 mg) extracted from each sample were first separated by isoelectric focusing (IEF) using immobilized pH gradient (IPG) strips (24 cm, 3–10 non-linear pH gradient; Bio-Rad, Hercules, CA, USA). The strips were focused at 20 °C for a total of 63.2 kV-h with IPGphorII (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After IEF, focused strips were equilibrated first in 8 mL equilibration solution I [13.4 mL Tris-HCL (1.5 mol/L, pH 8.8), 14.14 g urea, 120 mL glycerol, 8 g SDS, 0.002% (w/v) bromophenol blue, 160 µL DTT] for 15 min and then in 8 mL equilibration solution II [13.4 mL Tris-HCL (1.5 mol/L, pH 8.8), 14.14 g urea, 120 mL glycerol, 8 g SDS, 0.002% (w/v) bromophenol blue, 0.148 g iodoacetamide] for 15 min. The equilibrated IPG strips were transferred to 13% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels on an Ettan™ DALT twelve System (GE Healthcare Life Sciences, Uppsala, Sweden). Gels were stained with Coomassie Brilliant Blue G-250 (Candiano et al. 2004) and scanned with an image scanner (UMAX, Dallas, TX, USA). Digitized images of the gels were analyzed using ImageMaster 7.0 software (GE Healthcare Life Sciences, Uppsala, Sweden). To detect differentially expressed proteins, we compared the abundance differences between the control and SD treatments. The expression abundance of each protein spot was estimated by the normalized relative percentage volume. Spots of interest with at least 1.5-fold changes in abundance and p value < 0.05 were considered differentially expressed and selected for identification.

Protein identification and analysis based on mass spectrometry

Spots with significant differences in protein abundance were selected for tryptic digestion in accordance with the method of Sommerer et al. (2007). The digested peptides were analyzed using an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonic, Bremen, Germany), which is considered to be more reliable because of its flexibility and unparalleled performance and thus peptide identifications with improved accuracy can be achieved. The MS/MS data were submitted to MASCOT (http://www.matrixscience.com) to search the NCBInr and SwissProt databases for protein identification. Proteins with a MASCOT score that resulted in a confidence interval (CI%) greater than 95% ($p < 0.05$) for the TOF/TOF data were considered positively identified (Tripković et al. 2013). To ensure the accuracy and reliability of the proteomic results, proteins with a matched score at least 1.5-fold higher than the threshold score were selected for functional analysis. Functional

information for the identified proteins was obtained from UniProt (http://www.uniprot.org/).

Statistical analysis

One-way analysis of variance (ANOVA) was used to examine the effects of percentage bud set, height, RCD, FOLR ($D > 1$ mm), dry mass, content of Chl $a+b$, soluble sugar, starch, ABA, and lignin, and activity of PAL, PPO, and POD using SPSS 16.0 (SPSS, Chicago, IL, USA). Percentage bud set was arcsine transformed in order to fulfill normality and homogeneity requirements of ANOVA. When the ANOVA results showed a significant effect, means for morphological and physiological responses were ranked according to Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. Student's t test was used to analyze whether the changes in protein abundance in the needle were statistically significant.

Results

Seedling growth and physiological responses to short-day treatments

Short-day treatments significantly affected percentage bud set, height, RCD, and FOLR ($D > 1$ mm) ($p < 0.05$), but had no significant effect on dry mass of roots, stems, needles, and the whole seedling (Table 1). Compared with the control, SD-8 h and SD-10 h treatments respectively accelerated percentage bud set by 64.6% and 52.7%, decreased seedling height by 10.4% and 12.3%, and increased RCD by 8.2% and 12.1%, and both SD treatments increased FOLR by 66.7%. No significant differences were observed between the SD treatments.

Seedlings accumulated significantly higher amounts of Chl $a+b$, starch, ABA and lignin in response to SD treatments ($p < 0.05$) (Table 2). SD-8 h and SD-10 h treatments significantly increased Chl $a+b$ content by 3.3% and 4.7%, starch content by 5.0% and 3.8%, ABA content by 69.2% and 62.8%, and lignin content by 16.2% and 16.1%, respectively, in comparison with the control. No significant differences were observed between the SD treatments. Soluble sugar content did not differ significantly between the control and the SD treatments (Table 2). A significant increase in the activity of PAL, PPO, and POD was observed in seedlings given SD treatments ($p < 0.05$) (Table 2). SD-8 h and SD-10 h treatments significantly respectively increased PAL activity by 38.7% and 47.0%, PPO activity by 16.7% and 21.3%, POD activity by 28.6% and 35.8%, but no significant difference was observed among seedlings in the SD-8 h and SD-10 h treatments (Table 2).

2-DE image and mass spectrometry protein identification

Approximately 1300 protein spots were detected on each 2-DE gel. On the basis of image comparison analysis, we detected 27 protein spots that showed a significant change in abundance ($p < 0.05$; abundance change > 1.5-fold) (Fig. 1), which were selected for protein identification. Eight differential proteins were positively identified by MALDI-TOF-TOF MS/MS analysis. They were up-regulated oxygen-evolving enhancer protein 1 (OEE1; spot S13), transcription factor VOZ1 (VOZ1; spot S14), protein MET1 (MET1; spot S17), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; spot S22), elongation factor-Tu (EF-Tu;

Table 1 Morphological attributes and *p* values of container-grown Chinese pine seedlings in the control, SD-8 h and SD-10 h treatments

Source	Percentage bud set (%)	Height (cm)	RCD (mm)	FOLR	Dry mass (g)			
					Roots	Stems	Needles	Total seedling
Control	56.7 ± 4.091a	10.6 ± 0.430b	1.82 ± 0.034a	3 ± 0.490a	0.176 ± 0.002a	0.100 ± 0.006a	0.390 ± 0.020a	0.666 ± 0.021a
SD-8 h	93.3 ± 4.091b	9.5 ± 0.242a	1.97 ± 0.044b	5 ± 0.583b	0.167 ± 0.013a	0.101 ± 0.005a	0.431 ± 0.013a	0.698 ± 0.028a
SD-10 h	86.6 ± 3.340b	9.3 ± 0.452a	2.04 ± 0.036b	5 ± 0.775b	0.180 ± 0.016a	0.116 ± 0.011a	0.429 ± 0.019a	0.725 ± 0.033a
p value	0.001**	0.009**	0.008**	0.030*	0.792	0.260	0.283	0.454

Values represent mean ± SE. Different letters within a column indicate a statistically significant difference according to Tukey's HSD test

*Indicates significance at 0.05 level, **indicates significance at 0.01 level

Table 2 Physiological parameters and *p* values of container-grown Chinese pine seedlings in the control, SD-8 h and SD-10 h treatments

Source	Chl a + b (mg/g)	Soluble sugar (mg/g)	Starch (mg/g)	ABA (ng/g FW)	Lignin [OD/(g FW)]	Enzyme activity [U/(g FW min)]		
						PAL	PPO	POD
Control	0.695 ± 0.006a	21.756 ± 1.727a	12.218 ± 0.284a	378.032 ± 6.025a	5.338 ± 0.176a	70.843 ± 5.864a	180.395 ± 2.695a	128.674 ± 14.569a
SD-8 h	0.718 ± 0.003b	21.934 ± 2.065a	13.195 ± 0.204b	639.633 ± 7.311b	6.204 ± 0.115b	98.225 ± 9.556b	210.528 ± 2.099b	165.472 ± 10.528b
SD-10 h	0.728 ± 0.002b	21.780 ± 2.036a	12.975 ± 0.520b	615.513 ± 4.970b	6.195 ± 0.171b	104.147 ± 8.031b	218.740 ± 7.594b	174.683 ± 13.221b
p value	0.014*	0.779	0.041*	< 0.001**	0.012*	0.003**	0.002**	0.003**

Values represent mean ± SE. Different letters within a column indicate a statistically significant difference according to Tukey's HSD test

*Indicates significance at 0.05 level, **indicates significance at 0.01 level

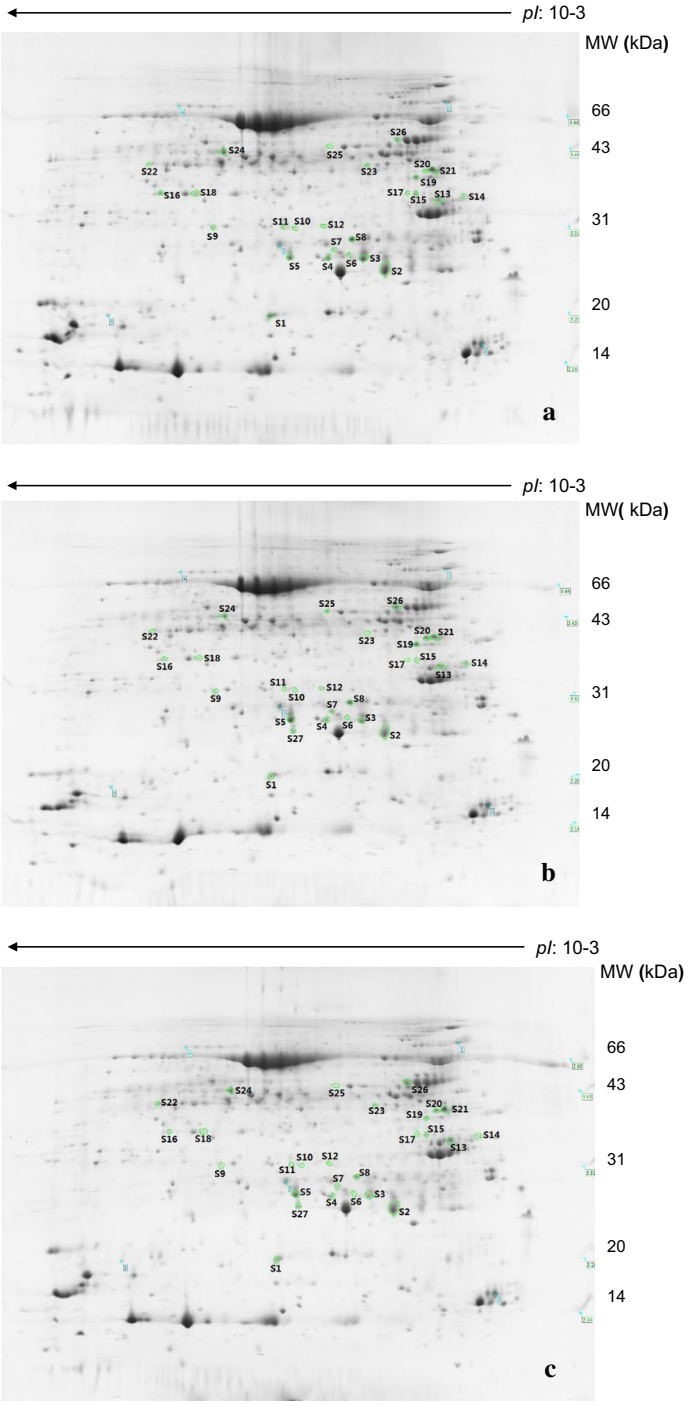

Fig. 1 Two-dimensional electrophoresis (2-DE) images of the control (**a**), SD-8 h treated (**b**) and SD-10 h treated (**c**) Chinese pine seedling needles. *Note* Numbered spots indicate differentially expressed proteins with at least 1.5-fold abundance changes after SD treatments ($p < 0.05$)

spot S25), and ribulose bisphosphate carboxylase/oxygenase activase (RCA; spot S26), and down-regulated ferredoxin-NADP reductase, leaf isozyme 1 (LFNR1; spot S16) and phosphoglycerate kinase 1 (PGK1; spot S24) (Fig. 2, Table 3). Functional information for these eight positively identified proteins is presented (Table 4).

Discussion

Effects of short-day treatments on growth and physiological response

Both SD treatments significantly reduced seedling height, advanced bud set, and increased RCD, which is similar to previous findings on spruce seedlings (Luoranen et al. 2007), but in contrast to Konttinen et al. (2007) with *Picea abies* (L.) Karst.

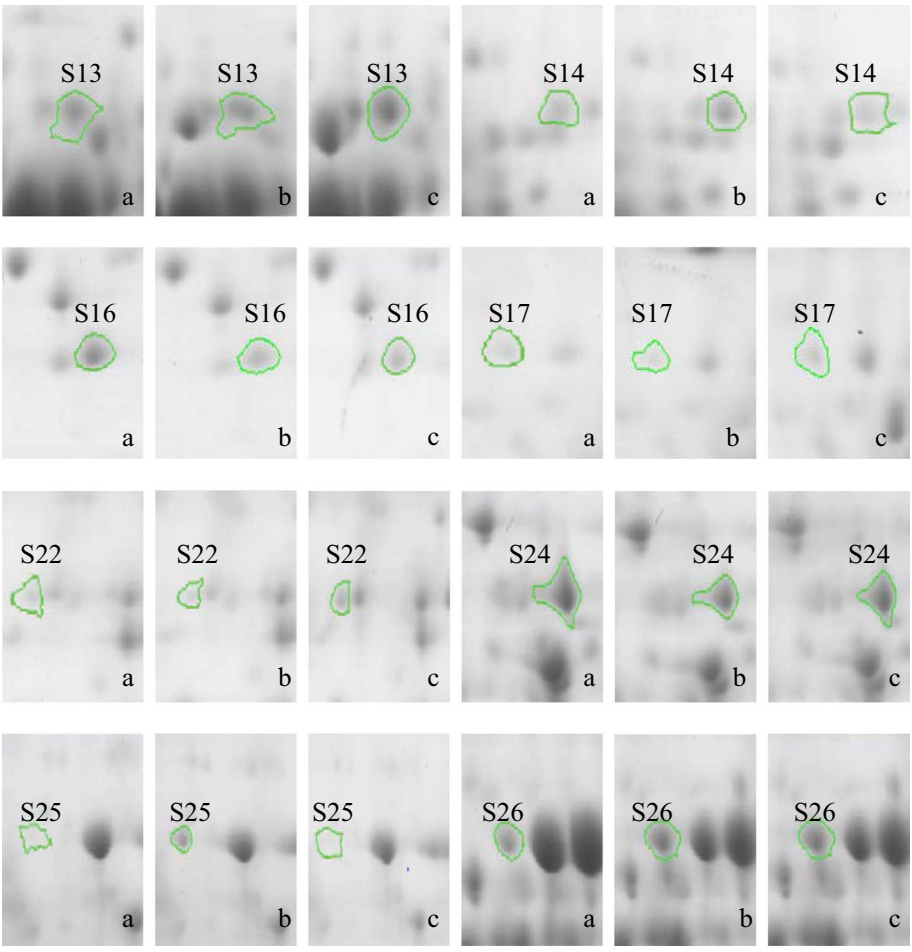

Fig. 2 Enlarged views and expression patterns of the positively identified proteins among the (**a**), SD-8h treated (**b**) and SD-10h treated (**c**) Chinese pine seedling needles

Table 3 Identification of differentially expressed proteins in needles of SD-treated Chinese pine seedlings by MALDI-TOF/TOF analysis

Spot no.	Source database	Accession no.	Protein name	Species	Mowse score[a] (threshold score[b])	EpI[c]/Emw (kDa)[d]	Expect[e] MS/MS	Matched peptides[f]
S13	NCBIprot	AJG36442.1	Oxygen-evolving enhancer protein 1	*Pinus sylvestris*	211 (51)	5.56/36.035	1.3e−06	RLTYDEIQSQ-TYLEVK
							2.9e−08	GGSTGYDNAVAL-PAGGR
S14	SwissProt	VOZ1_ARATH	Transcription factor VOZ1	*Arabidopsis thaliana*	52 (28)	5.73/54.787	0.00031	QIMVEFGGLK
S16	SwissProt	FENR1_ORYSI	Ferredoxin-NADP reductase, leaf isozyme 1	*Oryza sativa subsp. indica*	50 (30)	8.72/40.358	0.001	RLVYTNDQGEIVK
S17	SwissProt	MET1_ARATH	Protein MET1, chloroplastic	*Arabidopsis thaliana*	57 (29)	8.19/37.558	0.00012	EIQMQNYLKK
S22	SwissProt	G3PC_PINSY	Glyceraldehyde-3-phosphate dehydrogenase	*Pinus sylvestris*	184 (30)	6.67/36.678	0.0031	TVDGPSNKDWR
							0.013	GAGFNIIPSSTGAAK
							1.9e−09	LVSWYDNEWGYSSR
S24	NCBIprot	AIZ74346.1	Phosphoglycerate kinase 1	*Pinus massoniana*	265 (51)	8.84/53.026	1.9e−12	ADLNVPLDENQNIT-DDTR
							0.023	VILTSHLGRPK
							0.014	YSLKPLVPR
S25	SwissProt	EFTU_ARATH	Elongation factor Tu, chloroplastic	*Arabidopsis thaliana*	218 (30)	5.84/51.883	1.4e−08	KYDEIDAAPEER
							4.2e−11	GITINTAT-VEYETENR

Table 3 (continued)

Spot no.	Source database	Accession no.	Protein name	Species	Mowse score[a] (threshold score[b])	EpI[c]/Emw (kDa)[d]	Expect[e] MS/MS	Matched peptides[f]
S26	SwissProt	RCA_ARATH	Ribulose bisphosphate carboxylase/oxygenase activase	*Arabidopsis thaliana*	129 (29)	5.87/52.347	4.4e−07	SFQCELVMAK
							0.0012	FYWAPTREDR

[a]Statistical probability of true positive identification of the predicted protein calculated by MASCOT using MS/MS data

[b]Threshold score of credible identification using MS/MS data

[c]Isoelectric point (pI) of the predicted protein

[d]Molecular mass (mw) of the predicted protein

[e]Value of identification using MS/MS data

[f]Start and end positions of the identified peptide fragment in the protein sequence using MS/MS data

Table 4 Functional information of positively identified differentially expressed proteins

Spot no.	Change[a]		Protein name	UniProt accession no.	Biological process molecular function
	SD-8 h	SD-10 h			
S13	↑	↑	Oxygen-evolving enhancer protein 1	A0A0B5KFY1	Photosynthesis PhotosystemII stabilization
S14	↑	↑	Transcription factor VOZ1	Q9SGQ0	Response to heat Response to water deprivation
S16	↓	↓	Ferredoxin-NADP reductase, leaf isozyme 1	A2Y8E0	Photosynthesis
S17	↑	↑	Protein MET1, chloroplastic	Q94BS2	Photosynthesis PhotosystemII assembly PhotosystemII repair
S22	↑	↑	Glyceraldehyde-3-phosphate dehydrogenase	P34924	Glycolysis
S24	↓	↓	Phosphoglycerate kinase 1	A0A0A7M5T4	Glycolytic process
S25	↑	↑	Elongation factor Tu, chloroplastic	P17745	Protein biosynthesis
S26	↑	↑	Ribulose bisphosphate carboxylase/oxygenase activase	P10896	ATP binding Response to cold

[a]Change ↑ or ↓ means up-regulation or down-regulation of identified protein after SD (8 h and 10 h) treatments

seedlings in which diameter was reduced by SD-8 h treatment. Previous studies have shown that more FOLR contributed to larger well-developed root system that improved seedling survival and growth after field planting (Davis and Jacobs 2005; Dey et al. 2012). We noted that SD-8 h and SD-10 h treatments significantly increased number of FOLR, which suggested that seedlings exposed to SD treatments might have a good quality root system for greater water and nutrient uptake. Thus, we accept our first hypothesis that SD treatment would result in height growth cessation and earlier bud set, as well as increase RCD and number of FOLR. However, differing from the previous study with *Picea glauca* (Moench) Voss (Tan 2007), no significant effect of SD treatments on dry mass of roots, stems, needles, and the whole seedling was observed in Chinese pine seedlings. This led us to reject parts of the first hypothesis about root and shoot dry mass.

Differences in the photosynthetic efficiency of trees can be explained by changes in the chlorophyll content (Vogg et al. 1998). Increases in photosynthetic rate correspond to higher chlorophyll contents of seedlings (Zawaski and Busov 2014). Under the SD-8 h and SD-10 h conditions, the total chlorophyll content of Chinese pine seedlings was significantly increased, which suggested that SD treatments might enhance photosynthetic rates. Similar to the findings of Gibon et al. (2004), who observed a larger proportion of fixed carbon is retained as starch instead of sugars in the leaf under SD conditions, we noted that both SD treatments significantly increased starch accumulation in the needle, with no difference in soluble sugar content, in Chinese pine seedlings. Consequently, we accept our second hypothesis that SD treatment would increase the content of total chlorophyll and starch. We also reject parts of the hypothesis about soluble sugar content. Increased ABA level has been shown to play an important role not only in development of bud dormancy in response to shortening photoperiod (Olsen 2010), but also in adaptive responses of plants against abiotic and biotic stresses (Lee and Luan 2012). We observed that SD-8 h and SD-10 h treatments resulted in a significant increase in ABA levels in of Chinese pine seedlings, which suggested that SD treatments might have benefited bud development and dormancy. In addition, increased ABA levels suggested that SD treatments might also promote abiotic and biotic stress resistance. Deposition of lignin occurs during secondary cell wall thickening of tracheid (Rossi et al. 2006). Further, there was a tendency towards formation of latewood tracheids with thick walls in SD-treated plants (Heide 1974). Furthermore, Liu (2012) observed that lignin plays crucial roles in plant responses to abiotic and biotic stresses. In the current study, a significant increase in lignin content was observed in SD-treated Chinese pine seedlings, which suggested that SD treatments might be beneficial to the secondary cell wall thickening to provide a physical barrier against abiotic and biotic stresses. PAL is the first enzyme in the phenylpropanoid pathway involved in lignin biosynthesis (Möller et al. 2006). PAL activity is correlated with lignin content and disease resistance in plants (Ye et al. 2006). The production of PPO-catalyzed toxic phenolic secondary metabolites is beneficial to enhance plant resistance to pathogen infection and reduce damage to plant caused by insect herbivore (Baldwin and Preston 1999). Induction POD involved in lignin biosynthesis pathway has been shown to occur due to abiotic and biotic stresses in plants (Moura et al. 2010). We observed that SD-8 h and SD-10 h treatments significantly increased PAL, PPO and POD activity in Chinese pine seedlings, which might account for higher lignin content and abiotic and biotic stress resistance in SD-treated seedlings. Thus, we accept our third hypothesis that SD treatment would promote ABA and lignin accumulation, as well as increase activity of PAL, PPO and POD.

Effects of short-day treatments on proteomic response

In the present study, we positively identified eight differentially expressed proteins induced by both SD treatments. The proteins were OEE1 (spot S13), VOZ1 (spot S14), LFNR1 (spot S16), MET1 (spot S17), GAPDH (spot S22), PGK1 (spot S24), EF-Tu (spot S25), and RCA (spot S26). Because these proteins are mainly involved in photosynthesis, carbohydrate metabolism, as well as abiotic and biotic stress resistance, we accept our fourth hypothesis.

Proteins involved in photosynthesis

We detected four photosynthesis-related proteins, namely OEE1 (spot S13), LFNR1 (spot S16), MET1 (spot S17) and RCA (spot S26). OEE1 is a component of the oxygen evolution complex of photosystem II (PS II) (Liu et al. 2014), involved in combination with the manganese cluster and water splitting reaction (Wang et al. 2013), which plays a key role in maintaining the stability of PS II (Bonhomme et al. 2009). LFNR1 (spot S16) is involved in the pathway of photosynthesis, which regulates the linear electron transport in chloroplasts by transferring electrons from ferredoxin to $NADP^+$ (Hanke et al. 2005). MET1 (spot S17) functions as a PS II core protein CP43 and CP47 chaperone on the stromal side of the membrane, which was essential for PS II assembly and repair (Bhuiyan et al. 2015). RCA is an essential photosynthetic protein in plants, which promotes and maintains the activity of ribulose bisphosphate carboxylase oxygenase (Rubisco) that catalyses the first step in photosynthetic CO_2 assimilation (Boex-Fontvieille et al. 2014). In the present study, SD-8 h and SD-10 h treatments induced the up-regulated expression of OEE1 (spot S13), MET1 (spot S17) and RCA (spot S26), which suggested that SD treatments might promote PS II repair and maintain normal photosynthetic rate and efficiency of carbon assimilation. In contrast, down-regulated LFNR1 (spot S16) could have an adverse effect on photosynthetic electron transfer, whereas prevent excessive NADPH causing by high light and reduce risk of damage to photosynthetic system due to excessive reduction of the thylakoid membrane.

Proteins involved in carbohydrate metabolism

Two proteins involved in glycolysis, GAPDH (spot S22) and PGK1 (spot S24), were identified in the present study. Plant GAPDH is a crucial enzyme that catalyzes the sole redox reaction of glycolysis (Valcu et al. 2008). PGK1 is an ATP-generating glycolytic enzyme. Changes in the expression level of PGK1 may affect the generation of products in the glycolytic pathway and plant secondary metabolism processes (Kosová et al. 2011). We observed that SD-8 h and SD-10 h treatments decreased the abundance of PGK1 (spot S24) and increased the abundance of GAPDH (spot S22), which suggested that SD treatments might inhibit the glycolytic pathway to some extent. The down-regulated PGK1 and up-regulated GAPDH may decrease the carbon flux through the glycolytic pathway (Kanno et al. 2017), leading to increased pentose phosphate pathway carbon sources, and increase the accumulation of secondary metabolites such as lignin in SD-treated seedlings. A similar result was obtained by Lee et al. (2014), who found that there were increases in secondary metabolites as well as sugars of *Picea abies* (L.) Karst. seedlings after SD treatment.

Proteins involved in stress resistance

The phytochrome-interacting VOZ1 (spot S14) is a plant specific one-zinc-finger type DNA-binding protein, which plays role in regulating adaptation to abiotic and biotic stress conditions in *Arabidopsis* (Nakai et al. 2013). GAPDH (spot S22) in plant decreases reactive oxygen species levels and promotes resistance to oxidative stress (Baek et al. 2008). EF-Tu (spot S25) protein is involved in protein biosynthesis implicated in stress adaptation (Momcilovic and Ristic 2007). RCA (spot S26) recovers the activity of Rubisco under heat stress, which is the main mechanism for continued carbon dioxide fixation for a plant to adapt to stress (Weston et al. 2007). Stress resistance in plants is closely associated with expression of stress-regulated proteins (Kosová et al. 2011). In the present study, up-regulation of VOZ1(spot S14), GAPDH (spot S22), EF-Tu (spot S25) and RCA (spot S26) was observed in seedlings under SD-8 h and SD-10 h treatments, which suggested that SD treatments might positively relate to cross-adaptation to stresses and would improve the abiotic and biotic stress resistance.

Limitations

Our investigations were limited to assessments that were made immediately after the 3-week SD treatments, further research should be conducted to examine what happens when these seedlings are returned to natural photoperiods either in the nursery or after planting. The effect of different duration of SD treatments was not studied in these experiments. However, responses associated with various SD durations of seedlings should be further examined. It is a possible limitation of this study to investigate the effect of SD treatments on seedling physiology by only using some photoperiod-related parameters. Our protein identification was also constrained by the incompletion of the present protein database. A deeper investigation of the relationship between protein expression induced by SD treatment and field performance is required, however, to explore whether the differentially expressed proteins might be used as attributes of seedling quality to predict the adaptability of seedling response to reforestation site.

Conclusions

Both SD treatments can reduce height growth, advance bud set, as well as increase RCD and number of FOLR for Chinese pine seedlings. Under abiotic and biotic stress conditions, SD-treated seedlings may be at an advantage because of increased ABA levels and lignin content, as well as PAL, PPO and POD activity. Based on our proteomic analysis, SD treatment may affect the photosynthesis, carbohydrate metabolism, as well as abiotic and biotic stress resistance.

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Author's contributions LJ, YL, RKD and GL conceived and designed the experiments; PL in was responsible for laboratory work; LJ performed the experiments, analyzed the data and wrote the paper; YL and RKD guided manuscript writing and editing.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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