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A THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

Evaluation of abiotic stress tolerance in transplanted *Pinus densiflora* seedlings grown under different water availability

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Evaluation of abiotic stress tolerance in transplanted *Pinus densiflora* seedlings grown under different water availability

Supervised by Professor Hyun Seok Kim

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Abstract

Successful adaptation of seedlings depends on nursery conditions after the transplanted seedlings are exposed to various stresses such as drought, cold and waterlogging. Stress conditions inhibit plant growth and survival, but stress reponse can enhance adaptation in subsequent stress by regulating gene expression. Hardening is able to exert more tolerance to subsequent condition and affect cross-stress condition. However, very few studies have reported the hardening effect on transplant stress in woody plants. Korean red pine (*Pinus densiflora*) is the most important species in Korean forest lands, and mass dieback has been reported due to climate change. This study was conducted to identify a cross-stress hardening effect in *P. densiflora* after transplanting. The aim of this study are (1) to identify the drought hardening effect on transplant stress, (2) to determine the drought hardening effect on transplanted plants subjected to cold stress, and (3) to identify the waterlogging hardening effect on transplant stress. For the first study, three-years-old *P. densiflora* seedlings were grown under different drought hardening intensity for three years, and then transplanted to another site. In order to identify the drought hardening effect, transcriptome responses were compared according to drought intensity before and after transplanting (growing season and winter). Measurement of physiological characteristics was conducted after transplanting (growing season and winter). After transplanting in growing season, moderate drought hardening promoted cell wall organization and defense responses to repair the damaged tissue due to transplanting. In addition, water conservation capacity also improved in moderate drought hardening compared to non-hardening. In first winter after transplanting, moderate drought hardening showed a greater upregulation of gene expression linked to cold stress tolerance such as cyroprotectants, secondary metabolites, and phytohormones compared to severe drought- and non-hardened conditions. Photosynthesis and root collar diameter growth improved under moderate drought hardening after transplanting due to upregulation of the defense response. For the second study, three-years-old *P. densiflora* seedlings were grown under waterlogging and control conditions for

three years, respectively. After three years of waterlogging, hardened seedlings were transplanted to another site. Transcriptional analysis was conducted before and after transplanting and compared between waterlogging- and non-hardened seedlings. Physiological characteristics were measured after transplanting. After transplanting, waterlogging hardening upregulated genes related to cell wall formation and secondary metabolites along with the upregulation of phenylpropanoid biosynthesis genes to alleviate wounding stress caused by root cutting. However, degradation of auxin caused the decreasing tendency in growth under waterlogging hardened seedlings. This study showed that moderate drought hardening enhances cross-stress tolerance after transplanting, and suggests the nursery management strategy to relieve transplant stress and improve various stress tolerances.

Keyword : hardening, stress tolerance, drought hardening, waterlogging hardening, transplant stress, *Pinus densiflora*
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Chapter 1. Introduction

1.1. Research background

Global warming increases the intensity and frequency of drought and heavy precipitation (Trenberth, 2005; Trenberth *et al.*, 2014; Tabari, 2020). In South Korea, the average annual temperature has increased by 1.2 over the last 30 years, indicating a faster rate than in regions (Change, 2014). Heavy precipitation increased in summer between 1973 and 2005 (Chang and Kwon, 2007), and the frequency and intensity of drought also increased in late winter, and early spring and autumn between 1980 and 2015 (Nam *et al.*, 2015; Azam *et al.*, 2018). Extreme weather such as warming, drought and floods has reduced crop production over the last decades all around the world (Boyer *et al.*, 2013; Lesk *et al.*, 2016). In woody species, drought and warming was the main cause of widespread tree mortality in global (Hammond *et al.*, 2022). Severe and long-term drought not only inhibits tree growth or survival, but also activates more frequent or severe forest insect and pathogen occurence (Jactel *et al.*, 2012). These biotic stresses may combine with carbon starvation or loss in hydraulic conductance under drought stress (McDowell *et al.*, 2008).

Drought stress activates abscisic acid (ABA) biosynthesis, which leads to stomatal closure to reduce traspirational loss (Mittelheuser and Van Steveninck, 1969; Agurla *et al.*, 2018). Photosynthesis and plant growth were inhibited by stomatal closure owing to reduction of carbon fixation and overproduction of reactive oxygen species (ROS) (Farooq *et al.*, 2009). ROS causes photoinhibition and downregulation of photosynthesis by damaging chloroplast and deteriorating plant metabolism including hydraulic supply network (Tausz *et al.*, 2001; Adams *et al.*, 2017). Damaged hyrauclic functions, so called the hydraulic faiure, is a key mechanism that is related to tree mortality (McDowell *et al.*, 2008; Choat *et al.*,

2018). On the other hand, carbon starvation due to downregulation of photosynthesis has been suggested as a key mechanism of the tree mortality (McDowell *et al.*, 2008; Sevanto *et al.*, 2014).

Waterlogging is the major inhibitor of plant growth (Dickin and Wright, 2008; Huang *et al.*, 2022). Submerged root plants experience oxygen deficiency, susceptibility to diseases, and inhibition of nutrient and water uptake (Nishiuchi *et al.*, 2012). The limitation of water uptake by waterlogging stress leads to the downregulation of photosynthesis due to stomata closure (Parent *et al.*, 2008; Nishiuchi *et al.*, 2012; Phukan *et al.*, 2016). Prolonged duration of waterlogging leads to production of toxic metabolites such as ROS, ethanol and aldehyes, which leads to cell death and mortality (Copolovici and Niinemets, 2010).

Plants have evolved to survive under stress condtions with change of morphological and physiological characteristics (Bohnert *et al.*, 1995; Lamalakshmi Devi *et al.*, 2017). Stress resistance is an adaptive features to escape, avoid, or tolerate stress (Levitt, 1980). Drought escape is the ability which enable the rapid completion of life-cycle before drought events (Aslam *et al.*, 2015; Kooyers, 2015). Drought avoidance is the ability to maintain water content under drought season. For example, deep and coarse roots, with an increased root/shoot ratio to maximize water uptake and stomatal closure are the traits of drought avoidance. Under waterlogging stress, plants develop adventitious roots and aerenchyma induced by auxins and ethylene (Qi *et al.*, 2019). Tolerance includes the ability of plants to maintain homeostasis by regulation of gene expression such as antioxidant capacity and osmotic adjustment (Touchette *et al.*, 2007; Belhassen, 2013).

Stress can improve the stress tolerance the plants by adaptation mechanisms (Lynch, 2013; Vitasse *et al.*, 2019). Stress hardening enables plants to be more tolerant to subsequent stress exposures (Li *et al.*, 2013; Ghanbari and Sayyari, 2018). Besides, exposed plants to one stress could improve tolerance to an other because many stress pathways were common in various stresses (Iseki *et al.*, 2014; Llorens *et al.*, 2020). This phenomenon is termed cross-resistance or cross-

adpataion (Ghanbari and Kordi, 2019). Hardening can enhance growth or production in various crops. For example, the leaf water potential and growth in drought hardened pepper were higher than non-hardened pepper under drought conditions (Davies Jr *et al.*, 1992). Drought hardened wheat showed higher Rubisco and chlorophyll content and antioxidant activity than untreated wheat (Abid *et al.*, 2016). Pre-treated waterlogging stress mitigate oxidative stress and loss of production under waterlogging stress in soybean (*Glycine max*) (Agualongo *et al.*, 2022). However, in woody species, only few studies on hardening effect have been reported (Villar-Salvador *et al.*, 2004; Thomas, 2009; Saiki *et al.*, 2020).

Successful growth and establishment of seedlings depend on the optimization of physiological attributes such as stress tolerance, height, diameter and root mass, which require appropriate nursery conditions (Grossnickle and MacDonald, 2018). Transplant stress results in massive root and carbon storage loss and inhibits water and nutrient uptake when transplanted seedlings are exposed to various stresses such as drought, cold and waterlogging (Close *et al.*, 2005; Struve, 2009). Drought hardening can support root development, which improves transplant stress tolerance for successful establishment (Landis *et al.*, 1999; Luo *et al.*, 2022). Nevertheless, few research has evaluated the effects of hardening on transplanted trees (Villar-Salvador *et al.*, 2004; Luo *et al.*, 2022).

1.2. Research objectives

This study was conducted to identify the cross stress hardening effect on various stresses after transplanting and evaluate stress tolerance at the physiological and transcriptional level after drought and waterlogging hardening. The aims of the present study were to (1) determine whether drought hardening increases drought stress tolerance; (2) identify the physiological changes according to drought intensity after transplanting and conditions of hardening drought intensity improves stress tolerance after transplanting; (3) determine the waterlogging hardening effect on transplant stress tolerance.

Chapter 2. Drought hardening effect on transplant stress

2.1. Introduction

Drought stress is the major limiting factors for plant growth, and mortality (Boyer *et al.*, 2013; Fahad *et al.*, 2017; Gupta *et al.*, 2020). Over the past decade, global crop loss caused by drought was ~$30 billion (Desa, 2015). Drought was also responsible for ~500,000 ha excess forest mortality in Europe (Senf *et al.*, 2020). Mass drought stress-related dieback of *P. densiflora* has been reported between 2008 and 2017 in South Korea (Kim *et al.*, 2017; Lim *et al.*, 2017; Kim *et al.*, 2020). Under drought conditions, stomatal conductance and photosynthesis were decreased, which leads to tree mortality, and lower crop yield (Breshears *et al.*, 2009; Gill and Tuteja, 2010; Nishiyama and Murata, 2014; Seidl *et al.*, 2017; Lee *et al.*, 2022). However, drought stress also enables plants to adapt to climate change over the long term (Lynch, 2013; Willis *et al.*, 2018; Vitasse *et al.*, 2019). To withstand abiotic stressors, plants modulate their metabolism, morphological characteristics, gene expression, and transcriptomes (Janiak *et al.*, 2018).

Pre-treated drought stress can attenuate the adverse effects of other environmental stressors such as chilling (Li *et al.*, 2013; Ghanbari and Sayyari, 2018) and salinity (Cayuela *et al.*, 2007). Genes encoding cell wall modification, proline biosynthesis, antioxidant enzymes, and ABA by drought stress promote stress tolerance and adaptation (Dudziak *et al.*, 2019; Fei *et al.*, 2020; Ganie and Ahammed, 2021; Khan *et al.*, 2021b; Li *et al.*, 2021). An increase in the content of organic osmolytes such as soluble sugars and proline lowers the water potential and improve drought tolerance in various crops (Yancey *et al.*, 1982; Ashraf and Foolad, 2007; Du *et al.*, 2020; Khan *et al.*, 2021a). Certain key transcription factor (TF) families such as NAM, ATAF, and CUC families (NAC), MYB, WRKY, and basic

leucine zipper domain (bZIP) are implicated in different types of abiotic stress and improvement tolerance in various crops (Qin *et al.*, 2011; Wang *et al.*, 2016). For enhancement of drought tolerance, different chemical treatments have been applied to crops (Farooq *et al.*, 2010; Shan *et al.*, 2011). These techniques and adapting strategies to improve stress tolerance are termed 'drought hardening' (Landis *et al.*, 1999; Khan *et al.*, 2020). Drought hardened wheat displays higher Rubisco and chlorophyll content and antioxidant activity than untreated wheat (Abid *et al.*, 2016). Drought hardening conserves non-embolized xylem and cambium areas in Japanese cedar (*Cryptomeria japonica*) exposed to consecutive drought stress events. The seedling survival rate is improved in drought hardened trees and drought hardening efficiency increases with drought severity (Saiki *et al.*, 2020). By contrast, drought hardening does not reduce mortality or improve relative growth rates in transplanted *Quercus ilex* seedlings (Villar-Salvador *et al.*, 2004).

Drought and cold stresses are the primary causes of tree mortality due to the high risk of embolism (Zanne *et al.*, 2014). Drought and cold stresses tolerance is required for successful transplantation in forest. Before planting, drought stress in the nursery can improve the drought and frost tolerance (Blake *et al.*, 1979; Villar-Salvador *et al.*, 2004; Beikircher *et al.*, 2010). Drought hardening improves root development, which leads to successful growth and establishment of seedlings after nursery growth (Landis *et al.*, 1999; Grossnickle and MacDonald, 2018; Luo *et al.*, 2022). Transplant shock causes massive root and carbon storage loss and reduces water and nutrient uptake (Close *et al.*, 2005; Struve, 2009). Drought stress is the most common cause of transplant stress (Close *et al.*, 2005). Nevertheless, only a few studies have demonstrated enhanced growth or survival in woody seedlings subjected to drought hardening (Fernandez *et al.*, 2006; Saiki *et al.*, 2020).

We endeavored to elucidate the mechanisms by which plants respond to drought and stresses after transplanting in growing season and winter, while monitoring the stress tolerance after drought hardening. To these ends, we analyzed the transcriptional responses of *P. densiflora*. This conifer occupies > 23.5% (1.5 million ha) of all Korean forest lands (Korea Forest Service, 2006). The trees were

grown for two years under three different precipitation conditions, namely, control (100% natural precipitation; dC); moderate drought (40% precipitation blocking; dM); and severe drought (80% precipitation blocking; dS). After three years drought treatment, trees grown under all three precipitation conditions were transplanted to another site and classified into growth conditions (dC : C, dM : M and dS : S) to identify the effects of drought hardening. A transcriptional analysis was conducted during drought stress after two years drought hardening. After transplanting, a transcriptional analysis and comparison of the physiological responses to transplant and first cold stresses were also performed. The aims of the present study were to (i) determine whether drought tolerance increases after two years drought exposure; (ii) identify the drought intensity level that most effectively improves stress tolerance after transplanting; (iii) determine whether drought intensity affects the enhancement of cold stress tolerance after transplanting in the first winter.

2.2. Materials and methods

2.2.1 Experimental sites and plant materials

The drought experiment was conducted at Mt. Jiri (127°27′09.8″ N 35°17′09.3″, elevation 282 m a.s.l) in Gurye, South Jeolla Province, Republic of Korea between April 2018 and October 2020. The transplanting experiment was performed at Mt. Taehwa in Central Korea (E 127°18′38.1″ N 37°18′46.6″, 137 m a.s.l.) between October 2020 and August 2021. The drought experiment consisted of control (dC; 100% natural precipitation), moderate drought (dM; 40% precipitation blocking), and severe drought (dS; 80% precipitation blocking) treatments in a metal glasshouse fitted with a transparent Plexiglas® roof 3 m high. The mean annual temperature was 13.1 ± 0.01 ℃ and the average annual precipitation was 1,591 ± 210 mm yr^{-1} between 2018 and 2020. The dM and dS treatments were applied by opening 60% and 20% of the Plexiglas® roof area, respectively, to block precipitation. The total annual precipitation under dC, dM, and dS were 971 ± 261, 792 ± 183 and 290 ± 81 mm yr^{-1} for three years, respectively. Soil moisture content under dC, dM, and dS were 25.6 ± 4.5%, 21.8 ± 4.5% and 17.4 ± 3.7% for three years, respectively. The soil water levels in the top 30 cm were recorded at 15-s intervals and averaged over 30 min (%; n = 36, 12 per treatment; CS-616, Campbell Scientific Inc., Logan, UT, USA). Data for each plot were collected with HOBO stations (HOBO RX3000; Onset Computer Corporation, Bourne, MA, USA).

Twelve, 24, and 24 three-year-old seedlings were transplanted in dC (one plot), dM (two plots), and dS (two plots) in April 2018. Each plot (9.0 m × 9.0 m = 81 m^2) was subdivided into 36 smaller cells (1.5 m × 1.5 m; four seedlings/cell) and *P. densiflora* occupied three cells per plot. The 33 remaining cells were occupied by other tree species. The soil consisted mainly of sandstone, sand, mudstone, and gravel and its pH was 6.5. Additional details and environmental

variables of the site were provided by Bhusal *et al.* (2021). Six seedlings grown at dC and 12 seedlings grown at dM and dS were transplanted at Mt. Taehwa in October 2020. The transplant plot consisted of 90 cells (1.0 m × 3.0 m). Each cell contained seedlings sown 1.0 m apart. *P. densiflora* occupied ten cells while the remainder was occupied by other species. Trees under the same treatment were transplanted in the same cells. Hence, there were two dC cells and four dM and dS cells. In 2021, the mean temperature and total precipitation were 12.7 ℃ and 1,076 mm, respectively. All transplanted seedlings were grown under the same conditions. The seedlings were classified by growth condition (dC:C:WC, dM:M:WM, and dS:S:WS) until 2020. The soil texture was sandy loam and its pH was 5.2 ± 0.1. Its average soil organic matter content was 2.74 ± 0.19% and its average soil N content was 0.16 ± 0.0%. These measurements were taken with an Elemental Analyzer (Flash EA 1112; Thermo Fisher Scientific, Waltham, MA, USA) at the National Instrumentation Center for Environmental Management (NICEM) of Seoul National University. For RNA-Sequencing, leaf samples were collected from three replicate trees per treatment between 07:00 and 08:00 during August 2020 and August 2021. After transplanting in August 2021 and 2022, leaf samples were also collected from six replicate trees per treatment (C, M and S) to analyze nonstructural carbohydrates (NSC), proline, Rubisco, and chlorophyll content. To identify the cold stress tolerance after transplanting, three replicate trees per treatment (C, M and S) to measure cryoprotentant such as NSC and proline in February, 2022. All samples were stored in liquid nitrogen.

2.2.2 Growth and physiological parameters

Root collar diameter and height of six seedlings in each treatment were measured in October 2020 (third year of drought hardening), 2021 and 2022. Root collar diameter was measured using digital calipers (MCD-6CSX; Mitutoyo Co., Ltd., Kawasaki, Japan). In October 2020, four, five and six seedlings were harvested under dC, dM and dS, respectively. The aboveground biomass, underground biomass and root shoot ratio of harvested seedlings were measured. The root biomass was washed and classified as fine root (< 2 mm diameter) and coarse root (> 2 mm diameter). Thereafter, plant biomass was oven-dried at 72 ℃ for 72 hours.

In case of physiological parameters, the predawn and midday water potentials of leaf fascicles were measured in a pressure chamber (No. 1505D-EXP; PMS Instrument Company, Albany, OR, USA) between 04:00 and 05:30 and between 12:00 and 13:00 in August 2021. Leaf gas exchange was measured between 08:00 and 13:00. The net photosynthetic rate (P_{max}) and the stomatal conductance (g_s) of ten needles were measured with a portable infrared (IR) gas analyzer (LI-60; LI-COR, Lincoln, NE, USA) in August, 2021 and 2022. The fixed parameters were the CO_2 concentration (400 µmol mol^{-1}), temperature (25 ℃), relative humidity (RH; 50–60%), airflow rate (500 µmol s^{-1}), and photosynthetic photon flux density (PPFD) (1,400 µmol m^{-2} s^{-1}). The projected leaf area in the chamber was estimated for the leaf gas exchange measurement to recompute the gas exchange variables. All measurements were performed from six replicate trees per treatment.

2.2.3 Leaf biochemical parameters

The nonstructural carbohydrates (NSC) (the sum of soluble sugars and starch) were measured according to the method of Newell *et al.* (2002) using needles collected in August, 2021 and February and August, 2022. Fifteen milligrams dried *P. densiflora* leaf was pulverized with two 5-mm beads and a homogenizer (FastPrep-24; MP Biomedicals, Solon, OH, USA). The powdered samples were suspended in 1.5 ml of 80% (*v/v*) ethanol and incubated in a water bath at 80 ℃ for 30 min. The mixtures were then centrifuged at 14,000 × *g* at 15 ℃ for 10 min. The soluble sugar content was determined colorimetrically at 490 nm according to the phenol-sulfuric acid method (Ashwell, 1966). The soluble sugar was extracted, the supernatants were removed, and the pellets were dried to determine their starch content. Each pellet was suspended in 2.5 ml sodium acetate buffer (0.2 M), incubated at 100 ℃ for 1 h, and cooled to 14–15 ℃. Then 2 ml buffer and 1 ml amyloglucosidase (0.5% [*w/w*]; Sigma A9229-1G; Sigma-Aldrich Corp., St. Louis, MO, USA) were combined and the mixture was incubated at 55 ℃ overnight. The mixture was then centrifuged at 15 ℃ 14,000 × *g* for 10 min and its starch content was determined by phenol-sulfuric acid colorimetry. Two technical replicates for each sample were used for NSC measurement: total 12 and 6 replicates per treatment.

For proline extraction, fresh needles (0.1 g) stored at -80 ℃ were subjected to proline extraction according to the method of Ábrahám *et al.* (2010). Samples were collected in August, 2021 and February and August, 2022. The samples were pulverized with a single 5-mm bead in a homogenizer (FastPrep-24; MP Biomedicals) containing liquid N and 3 ml of 3% (*w/v*) sulfosalicylic acid at 4 ℃. The mixture was centrifuged at 14,000 × *g* and 4 ℃ for 5 min and denatured at 90 ℃ for 5 min. Then 2 ml supernatant was collected and mixed with 2 ml acidic ninhydrin (ninhydrin 1% (*w/v*) in acetic acid 60% (*v/v*), ethanol 20% (*v/v*)), 1 ml glacial acetic acid, and 2 ml of 6 M orthophosphoric acid. The mixture was heated to 100 ℃ for 1 h and cooled to 15 ℃. Then toluene was added to it and the mixture

was vortexed and incubated for 5 min. Absorbance was read at 520 nm. Two technical replicates for each sample were conducted for proline extraction: total 12 and 6 replicates per treatment.

The chlorophyll content was determined by the dimethyl sulfoxide (DMSO) method (Shinano *et al.*, 1996). Briefly, 0.2 g *P. densiflora* leaves in 5 ml DMSO was incubated at 65 ℃ for 6 h and the absorbance was read at 649 nm and 665 nm with a spectrophotometer. Two technical replicates for each sample collected in August, 2021 and 2022 (six samples per treatment) were used for chlorophyll determination. The total chlorophyll content was calculated as follows (Wellburn (1994)):

$$\text{Total chlorophyll content } (\mu g \cdot ml^{-1}) = 21.44\, A_{649} + 5.97\, A_{665}$$

2.2.4 Statistical analysis

Two-way repeated measure of analysis of variance (ANOVA) was used to determine the drought hardening effects on height and root collar diameter and growth, P_{max}, stomatal conductance, NSC, proline, and chlorophyll. One-way ANOVA was conducted for leaf water potential. When differences between treatments were significant, Tukey's test was used for multiple comparisons. All statistical analyses were performed in R v. 4.0.3 (R Core Team, Vienna, Austria).

2.2.5 Transcriptome analysis

For RNA extraction, total RNA was extracted from 40–50 mg needles collected in the growing season and winter after transplanting and three biological replicates for each treatment were used. RNA extraction was performed using Ribospin™ Plant (GeneAll; Seoul, Republic of Korea) according to the manufacturer's instructions. The extracted RNA was sent to Macrogen (Seoul, South Korea) for library preparation and sequencing. The RNA integrity numbers

(RIN) were determined with a Bioanalyzer RNA Pico 6000 Chip Library Preparation (Agilent Technologies, Santa Clara, CA, USA). All samples used in cDNA library construction had RIN > 7. The paired-end nondirectional cDNA libraries (2 × 101-bp) were prepared according to the TruSeq Standard mRNA sample preparation guide and sequenced in an Illumina NovaSeq 6000 system (Illumina, San Diego, CA, USA) at Macrogen.

Prinseq-lite v. 0.20.4 (https://github.com/uwb-linux/prinseq) was used to filter low-quality reads and bases (Schmieder and Edwards, 2011). Reads were subjected to *de novo* assembly to generate a reference transcriptome in Trinity v. 2.13.2 (https://github.com/trinityrnaseq/trinityrnaseq) using its default parameters (Hao *et al.*, 2015). Transdecoder v. 5.5.0 (https://github.com/TransDecoder/TransDecoder) identified the candidate coding regions from the assembled transcripts using its default parameters (Tang *et al.*, 2015). CD-HIT-EST v. 4.8.1 (https://anaconda.org/bioconda/cd-hit) clustered the transcripts using its default parameters (similarity 95%) (Fu *et al.*, 2012). The longest transcripts in each cluster were used. Benchmarking Universal Single Copy-Orthologs (BUSCO v. 3; https://busco-archive.ezlab.org/v3/) was used to assess transcriptome completeness with reference to the Embryophyta_odb10 database (Simão *et al.*, 2015).

Filtered reads were mapped to the assembled transcriptome with Salmon v. 1.8.0 in alignment-based mode (Patro *et al.*, 2017). Differentially expressed genes (DEGs) between treatments were identified with DESeq2 v. 1.34.0 (Love *et al.*, 2014) using false discovery rate (FDR) < 0.05, adjusted *P-value* < 0.05, and $|\log_2$ FoldChange(FC)| > 1. The extracted sequences were compared against those of Arabidopsis thaliana using the Basic Local Alignment Search Tool for proteins (BLASTX; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and e-value threshold = 1×10^{-7} to assign functional annotations (Altschul *et al.*, 1997). The Plant Transcription Factor Database v. 4.0 (http://planttfdb.cbi.pku.edu.cn/) was used to classify the putative *P. densiflora* transcription factor (TF) families and compare them against those of *A. thaliana* (Jin *et al.*, 2016; Lee *et al.*, 2019). Gene ontology (GO)

annotations were used with the PANTHER GO classification system (http://www.geneontology.org) using Fisher's exact test with FDR<0.05 (Mi *et al.*, 2017*)* to explore the functional implications of the genes. The Arabidopsis Information Resource (TAIR) database was used as a reference (Reiser *et al.*, 2016). To overview the regulatory pathways for abiotic tolerance, MapMan v 3.5.1R2 was used with FDR<0.05 and $|\log_2 FC| > 1$. The Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis was performed to identify the pathways related to cold stress tolerance using DAVID (https://david.ncifcrf.gov/) (Huang *et al.*, 2009)

For validation of RNA seq results, total RNA was extracted with Ribospin™ Plant (GeneAll). RNA was then reverse-transcribed with an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed for 16 and 21 genes for the validation of RNA-seq results in transplant stress in August, 2021 and the cold stress in February, 2022), respectively (Table S1). F-box and actin was the internal control in transplant and cold stress, respectively (Zhu *et al.*, 2019). The samples were prepared in a reaction mixture consisting of 10 μl Master Mix (IQ Sybr Green SuperMix; Bio-Rad Laboratories), 10 μM each forward and reverse primer, 1 μl cDNA (50 ng μL^{-1}), and 7 μl DNase- and RNase-free water. Real-time quantitative PCR (RT-qPCR) was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) according to the following program: 95 ℃ for 2 min followed by 40 cycles at 95 ℃ for 10 s, 61 ℃ for 30 s, and 72 ℃ for 30 s. All RT-qPCR reactions were performed in two technical replicates per biological sample. The expression ratios were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Independent T-test was conducted to compare qRT-PCR results.

2.3. Results

2.3.1 Physiological response of drought- and non-hardened trees after transplanting

Under drought conditions, height and root collar diameters tended to be lower under severe drought (dS) condtion compared to control (dC) and mild drought (dM), however, there were no differences between the treatments (Table 2.1). In first year after transplanting, height of seedlings grown severe drought (S) was the highest among treatments (Table 2.1). Root collar diameter of S tended to be higher than moderate drought hardening (M) and control (C). In second year after transplanting, height and root collar diameter of seedlings grown severe drought hardening tended to be higher than control and modeate drought. However, height and root collar diameter of transplanted seedlings showed no differences between treatments for two years (Table 2.2).

Three years after drought hardening, aboveground and underground biomass and root length showed increment under dM and dS compared to dC, but there were no significant differences between treatments (Fig 2.1). Fine root biomass of dM were 93.0 % and 16.7 % higher than dC and dS (P = 0.073, Fig 2.1A). Root shoot ratio of dM tended to be 71.2 % and 28.7 % higher than dC and dS (P = 0.075, Fig 2.1C).

Table 2.1 Averages of height and diameter of *P. densiflora* grown at three different water availability treatments before and after transplanting.

Factor	Treatment	Drought pre-treatment (2018) (Bhusal *et al.*, 2021)	Drought treatemnt (2019) (Bhusal *et al.*, 2021)	Before transplant stress (2020, October)	First year after transplanting (2021, October)	Second year after transplanting (2022, October)
Height (cm)	Control	30.54 ± 1.38 (n=12)	45.06 ± 3.59 (n=12)	63.67±5.89 (n=6)	79.00±6.35 (n=6)	92.42±6.56 (n=6)
	Moderate drought	29.98 ± 0.87 (n=24)	44.29 ± 2.45 (n=24)	66.50±5.04 (n=6)	83.50±3.07 (n=6)	83.57±2.60 (n=6)
	Severe drought	30.06 ± 1.59 (n=24)	42.80 ± 9.41 (n=24)	91.00±5.14 (n=6)	108.38±4.50 (n=6)	122.06±5.4 (n=6)
Root collar diameter (mm)	Control	4.72 ± 0.17 (n=12)	12.67 ± 0.28 (n=12)	11.25±1.07 (n=6)	14.28±1.38 (n=6)	20.82±1.70 (n=6)
	Moderate drought	5.26 ± 0.24 (n=24)	12.58 ± 0.69 (n=24)	13.09±1.53 (n=6)	16.11±1.62 (n=6)	22.82±1.73 (n=6)
	Severe drought	4.50 ± 0.14 (n=24)	11.25 ± 1.02 (n=24)	16.50±1.80 (n=6)	18.90±1.62 (n=6)	23.58±1.67 (n=6)

Table 2.2 Results of two way repeated measure of ANOVA for height, rootcollar dimeter, height and diameter growth, maximum photosynthetic rate (P_{max}), stomatal conductance, foliar soluble sugar and starch content, proline and chlorophyll in *Pinus densiflora* after transplanting.

Factor	*P*-value		
	Drought hardening	Year	Drought hardening × Year
Height	0.016^{*}	0.001^{**}	0.618
Root collar Diameter	0.733	$< 0.001^{***}$	0.801
Height growth	0.065	0.705	0.928
Root collar growth	0.044^{*}	0.008^{**}	0.370
P_{max}	0.002^{**}	0.330	0.667
Stomatal conductance	0.002^{**}	0.300	0.381
Soluble sugars	0.153	0.290	0.075
Starch	0.062	0.012^{*}	0.050
Proline	0.527	$< 0.001^{***}$	0.093
Chlorophyll	0.123	0.275	0.735

Superscript asterisk indicates statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

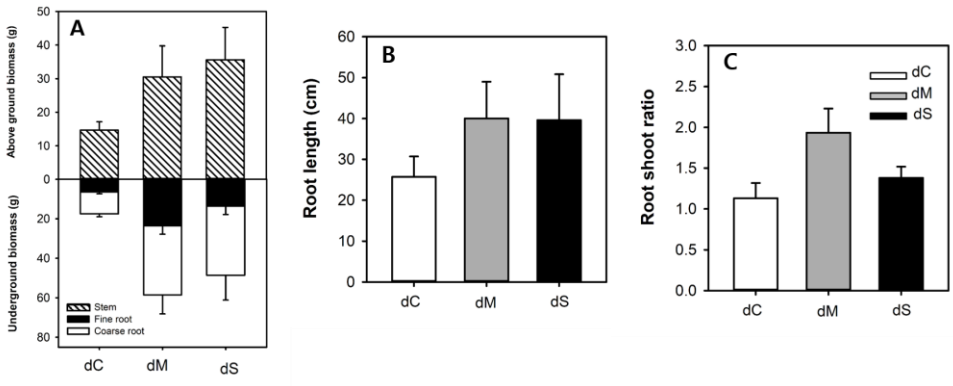

Fig 2.1 Aboveground and underground biomass after 3 year drought hardening. (A). aboveground and underground (fine and coarse root) biomass. (B). root length. (C). root shoot ratio.

Before transplanting, P_{max}, height, root collar diameter and leaf water potential showed no differences between treatments in drought period (Bhusal *et al.*, 2021). After transplanting, root collar diameter growth, P_{max} and stomatal conductance showed significant differences between treatments for two years (Table 2.2). In case of growth, height growth was not significant between treatemtns (Fig 2.2A). Under M, root collar diameter growth showed the highest value between treatments, but it was not significant in post-hoc test (Fig 2.2B). P_{max} under M was 46.6 % and 49.7 % higher than C (P = 0.004) and S (P = 0.004), respectively (Fig 2.2C). Stomatal conductance also increased by 76.3 % and 77.7 % compared to C (P = 0.006) and S (P = 0.007, Fig 2.2D).

Similar to stomatal conductance, the predawn water potential did not significantly differ between treatments, while the midday water potential showed differences between treatments (Fig 2.3). The midday water potential was 37.92 % higher for the trees under M than those under C in one year after transplanting (P = 0.031, Fig 2.3).

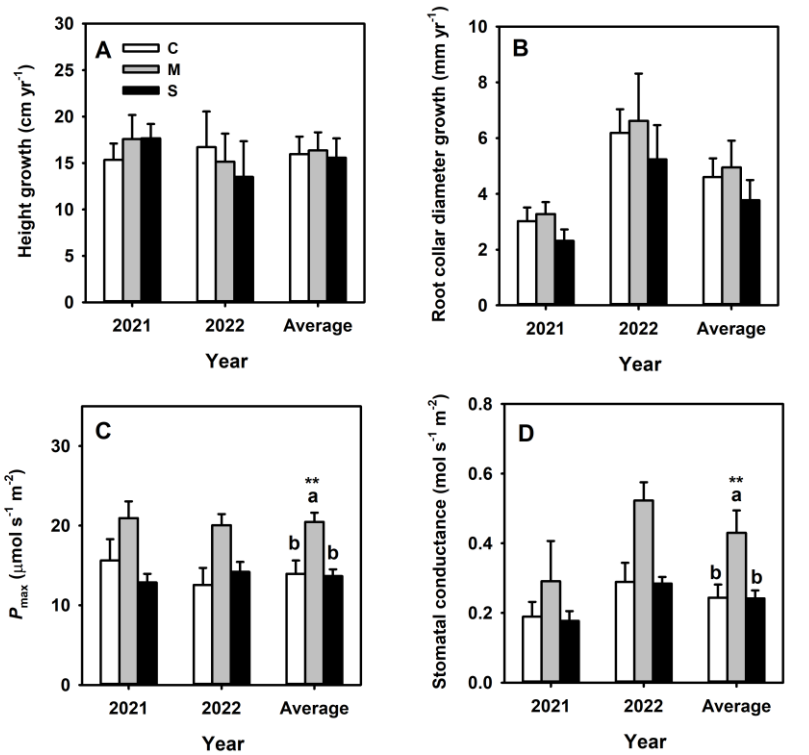

Fig 2.2 Effects of drought hardening on growth and photosynthetic parameters after transplanting. (A). height growth. (B). root collar diameter growth. (C). net photosynthetic rate (P_{max}). (D). leaf stomatal conductance. Asterisk indicates statistical significance (** $P < 0.01$).

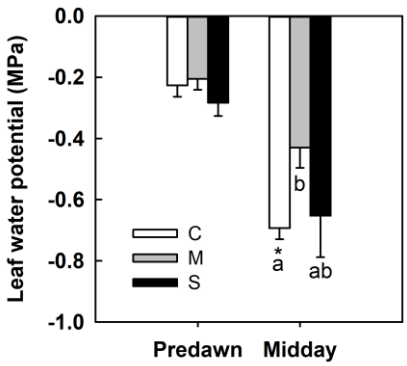

Fig 2.3 Leaf predawn and midday leaf water potentials after transplanting in 2021. Different letters indicate significant difference in drought hardening effect (Tukey's test; $P < 0.05$). Asterisk indicates statistical significance (* $P < 0.05$).

In case of leaf biochemical parmeters, NSC showed no differences between treatments while proline increased by 93.1 % and 87.6 % under dM and dS compared to dC, respectively in previous study in the same site during the drought period (Bhusal *et al.*, 2021). After transplanting, organic osmolytes such as NSC and proline showed no differences between drought hardening treatments (Table 2.2). Starch of M tended to be lower under M in first year after transplanting, however, it was not significant (Tables 2.2, 2.3). Proline in 2021 was 105.7 % higher than 2022 ($P < 0.001$, Table 2.3). In case of chlorophyll, M showed the highest value between treatments but, there were no drought hardening effect (Tables 2.2, 2.3).

Under first cold stress, proline showed differences between treatments by drought hardening effect (Table 2.4). Proline of M was 44.7 % higher than C ($P = 0.010$, Table 2.4). In case of NSC, M showed the highest tendency but it was not significant (Table 2.4).

Table 2.3 Average of water use efficiency (WUE), foliar soluble sugar and starch content, proline content and chlorophyll in *P. densiflora* after transplanting.

Factor	Year Average	Treatment C	M	S
Soluble sugars (mg g^{-1})	2021	77.60 ± 10.97	44.02 ± 7.83	74.88 ± 7.95
	2022	46.64 ± 4.45	50.36 ± 11.31	48.14 ± 4.28
	Average	62.12 ± 7.60	47.19 ± 6.63	61.51 ± 5.90
Starch (mg g^{-1})	2021	87.89 ± 8.86	45.78 ± 6.5	65.79 ± 13.87
	2022	38.74 ± 3.60	40.26 ± 4.06	45.65 ± 3.05
	Average	63.32 ± 9.35	43.02 ± 3.75	55.72 ± 7.42
Proline (μg g^{-1})	2021	20.32 ± 1.75	14.27 ± 1.58	15.47 ± 1.21
	2022	7.80 ± 0.89	8.61 ± 0.74	7.87 ± 0.74
	Average	13.49 ± 2.16	11.88 ± 1.21	11.67 ± 1.31
Chlorophyll (mg g^{-1})	2021	0.41 ± 0.03	0.40 ± 0.03	0.32 ± 0.02
	2022	0.38 ± 0.02	0.45 ± 0.02	0.40 ± 0.02
	Average	0.39 ± 0.02	0.42 ± 0.02	0.36 ± 0.02

Table 2.4 Average levels of proline and NSC of *P. densiflora* grown at three different water availability treatments under the first winter cold stress after transplanting.

Treatment	Proline (μg g^{-1})	NSC (mg g^{-1})
C	8.11 ± 0.58^{b}	345.57 ± 12.80
M	11.74 ± 1.15^{a}	348.09 ± 10.89
S	9.62 ± 0.47^{ab}	315.80 ± 10.13
P-value	0.013^{*}	0.087

Means followed by different lowercase superscript letters are significantly different among the drought hardening level (Tukey's test, $P<0.05$). Superscript asterisk indicates statistical significance ($^{*}P<0.05$).

2.3.2 Gene ontology (GO) terms induced by drought hardening and transplanting

We obtained 622,850,400, 682,999,542 and 609,275,614 raw paired reads (total 131,890,844,142 bp) in the drought experiment (2018–2020), after transplantatinig in the growing season (August, 2021), and the first winter (February, 2022), respectively. We generated 370,135 and 303,411 genes in transplant and cold stresses in Trinity, respectively (Table 2.5). Total Trinity trasncripts were 709,398 and 577,899 in transplant and cold stress, respectively. GC content of transplant and cold stress was 40.7 % and 40.9 %, respectively. Contig N50 length was 1,105 and 1,312 in transplant and cold stress, respectively. The BUSCO analysis revealed 1,465 (90.8%) and 1,472 (91.2%) complete BUSCO genes in transplant and cold stresses, respectively.

Table 2.5 Summary statistics for *de novo* transcriptome assembly.

Assembled contigs	Period	
	Transplant stress	Cold stress
Total Trinity genes (*n*)	370,135	303,411
Total Trinity transcripts (*n*)	709,398	577,899
GC content (%)	40.68	40.94
Contig N50 length (bp)	1,105	1,312
Average contig length (bp)	686.28	776.15
Total assembled bases	486,846,973	486,538,946

Eighty-eight commonly downregulated DEGs were categorized into 19 GO terms under dM and dS. They included photosynthesis, light harvesting, light reaction, and response to light and radiation (Table S2). For the commonly upregulated DEGs between dM and dS, 165 were categorized into 122 GO terms including transport of substances (such as glucose-6-phosphate, hexose phosphate, phosphoenolpyruvate, and water), transpiration, immune responses, defense responses, phytohormone-mediated signaling, and ethylene-activated signaling.

For the transplanted plants, the 271 upregulated DEGs in M compared with C were categorized into 31 GO terms including defense response, phenylpropanoid metabolism, cell wall organization, and terpenoid metabolism (Fig 2.4A). For the 271 downregulated DEGs in M compared with C, and included photosynthesis, dark reaction, sucrose biosynthesis, and metabolism (Fig 2.4B). For the comparison between S and C, the 160 upregulated DEGs were classified into 24 GO terms including cellular response to light intensity, and responses to stress and stimuli (Fig 2.5A). The 232 downregulated DEGs were categorized into 50 GO terms including diterpenoid, terpenoid, and isoprenoid processes and immune processes (Fig 2.5B).

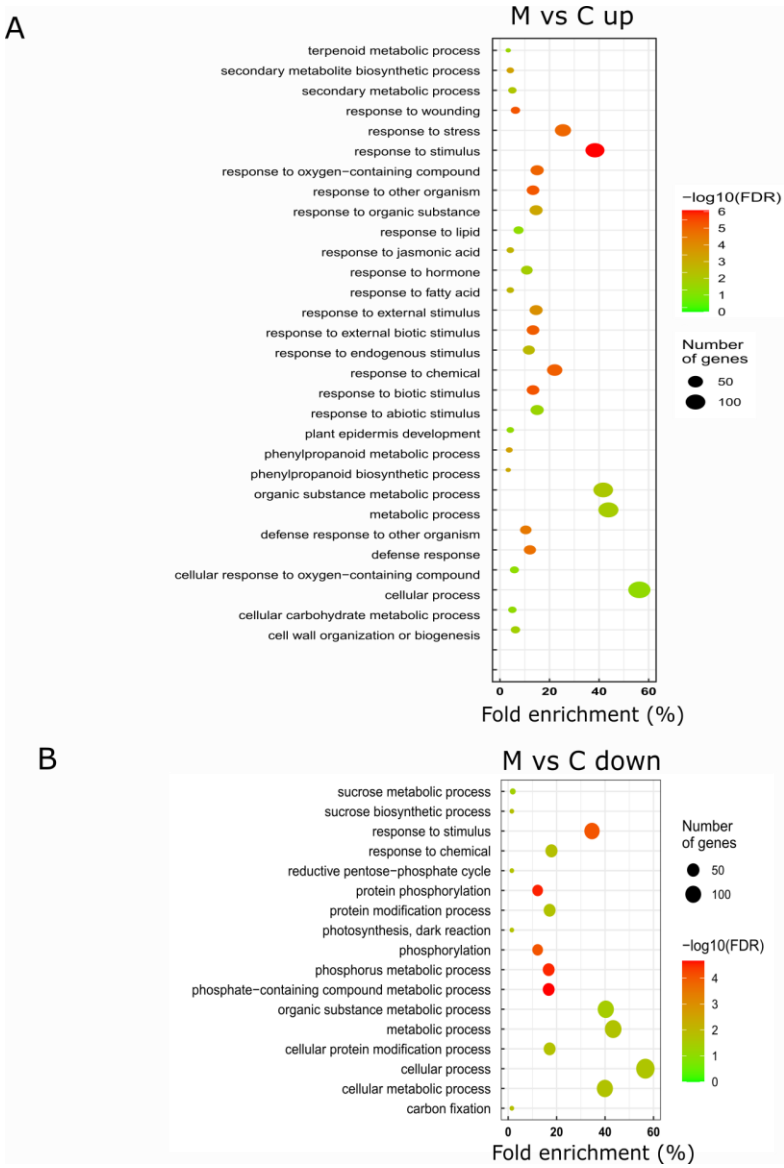

Fig 2.4 Gene ontology (GO) analysis of differentially expressed genes in comparisons between *P. densiflora* grown under moderate drought stress after transplanting. (A). GOBP of upregulated DEGs under M compared to C (B). GOBP of downregulated DEGs under M compared to C. Bubble color indicates P-value (-$\log_{10}$ FDR). Bubble size indicates number of DEGs in GO terms.

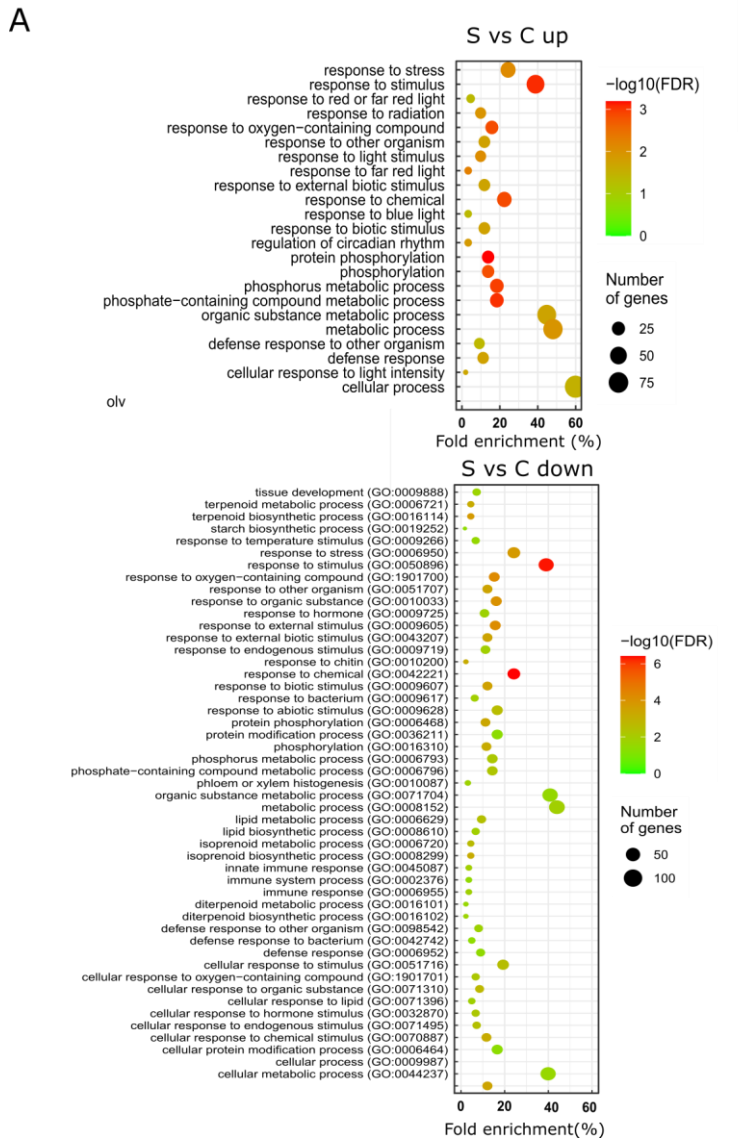

Fig 2.5 Gene ontology (GO) analysis of differentially expressed genes in comparisons between *P. densiflora* grown under severe drought stress after transplanting. (A). GOBP of upregulated DEGs under S compared to C (B). GOBP of downregulated DEGs under S compared to C. Bubble color indicates *P*-value (-$\log_{10}$ FDR). Bubble size indicates number of DEGs in GO terms.

In the first winter after transplanting, 244 GO terms were upregulated under WM compared to WC. The upregulated GO term included defense response, signaling, phytohormone process (JA biosynthesis, auxin transport and SA metabolic process), cell wall organization, secondary metabolites biosynthesis (phenylpropanoid, proanthocyanidin, lignin and flavonoid), alpha-amino acid and carbohydrate metabolic process (Table S3). Additionally, 54 GO terms were downregulated in WM compared to WC, including reproductive process, seed and fruit development, chlorophyll biosynthetic and metabolic processes, response to light intensity, heat and temperature, and reproductive system development (Table S3). In the case of WS compared to WC, 7 GO terms were upregulated, including arabinogalactan protein metabolic process, cell wall hydroxyproline-rich glycoprotein metabolic process, and cell wall proteoglycan metabolic process. The downregulated genes were not classified into GO terms (Table S3).

We compared commonly regulated GO terms between drought vs transplant stress and drought vs cold stress to identify the common stress responses and cross-resistance between stresses.

In transplant stress in summer, C vs. dC, M vs. dM, and S vs. dS, there were 56 upregulated GO terms including lignin biosynthesis, plant-type primary cell wall biogenesis, phenylpropanoid biosynthesis, cellular detoxification of aldehyde, defense response, formaldehyde and aldehyde catabolism, and response to wounding (Table S3). Thirty-nine downregulated GO terms in transplant stress included lignin, terpenoid, isoprenoid, and strigolactone biosynthesis, response to stimulus, and detoxification (Table S3). There were nine upregulated GO terms such as regulation of root development, monovalent inorganic cation homeostasis, and regulation of meristem growth between drought and transplant stress (Table 2.6). For this comparison, there were no common downregulated GO terms.

Table 2.6 Gene ontology (GO) analysis of commonly upregulated genes between drought and transplant stresses.

GO biological process complete	Gene No.	FDR	Fold enrichment (%)
protein phosphorylation (GO:0006468)	11	8.75E-05	33.3
phosphorylation (GO:0016310)	11	1.37E-04	33.3
phosphate-containing compound metabolic process (GO:0006796)	11	3.88E-03	33.3
phosphorus metabolic process (GO:0006793)	11	4.24E-03	33.3
tissue development (GO:0009888)	8	1.77E-03	24.2
regulation of developmental process (GO:0050793)	7	3.15E-02	21.2
regulation of root development (GO:2000280)	4	3.78E-03	12.1
monovalent inorganic cation homeostasis (GO:0055067)	4	3.51E-03	12.1
regulation of meristem growth (GO:0010075)	3	4.93E-02	9.1

Among the three comparisons of cold stress (C vs. dC, M vs. dM, and S vs. dS), 267 GO terms were commonly upregulated, including potassium ion import across the plasma membrane, L-phenylalanine metabolic process (phyenylalanine ammonia-lyase, PAL2, and PAL4), carbohydrate transmembrane transport, glutamine family amino acid and phenylpropanoid biosynthetic process, purine ribonucleoside diphosphate carboxylic acid and jasmonic acid metabolic process, stomatal movement, lipid catabolic process, and transport (Table S5). Contrastingly, 210 GO terms were commonly downregulated, including photosynthesis, dark reaction, light harvesting, carbon fixation, photosystem II repair, photorespiration, chlorophyll biosynthetic process, photosynthetic electron transport chain, and responses to various factors (Table S5). When comparing drought (dM vs. dC and dS vs. dC) and cold stress (C vs. dC, M vs. dM, and S vs. dS), 35 GO terms were commonly upregulated, including leaf abscission, induced systemic resistance, jasmonic acid-mediated signaling pathway, cell communication, signaling, and defense response (Table S6). Conversely, 29 GO terms were commonly downregulated, including glycine catabolic process, photosynthesis, light harvesting in photosystem I, light harvesting, and light reaction in comparison between drought and cold stresses (Table S6).

2.3.3 Differentially expressed transcription factors induced by drought hardening and transplanting

We identified 130 TFs, under drought stress (dM vs. dC and dS vs. dC) the most abundant of which were ethylene responsive element binding factor (ERF, 52 members) followed by NAC (18 members), WRKY (12 members), MYB (nine members), C2H2 (eight members), homodomain-leucine zipper (HD-ZIP, four members), basic helix–loop–helix DNA-binding domain (bHLH), C3H, and SBP (three members each), BES1, bZIP, CO-like, GATA, HSF, and Trihelix (two members each) (Fig 2.6). ERF1, ERF2, and the NAC and MYB families were upregulated under dL. ERF1, ERF2, DREB, NAC, WRKY3, WRKY4, WRKY6, and WRKY20, MYB, and bHLH (MYC2) were upregulated under dS compared with dC (Fig 2.6).

We investigated these TFs after transplanting (M vs. C and S vs. C) to identify the transcriptional patterns. We identified 23 TFs associate with twenty-one upregulated genes in M, two upregulated genes in S, and one upregulated gene common to M and S compared with C. The most highly represented TFs were ERF (10 members) followed by NAC (three members), HB-other and LBD (two members each) and ARR-B, bHLH, C3H, MYB, Trihelix, and WRKY (one member each) (Fig 2.7A). The upregulated TFs under dM and dS increased under M after transplanting. However, the downregulated TFs under M and S were not associated with pattern of seedlings in drought stress (Fig 2.7B).

In the cold stress after transplanting and drought hardening (WM vs. WC and WS vs. WC), 101 upregulated TFs at WM or WS relative to WC were found. Furthermore, the most highly expressed TF families were NAC families (21 members including NAC2, NAC25, NAC35, NAC43, NAC47, and NAC72) followed by MYB (18 members including MYB3 and MYB15), WRKY (17 members including WRKY3, WRKY4, WRKY33, WRKY42, WRKY47, WRKY51, WRKY65, and WRKY71), ERF (15 members including ERF105, ERF2, and ERF1A and B), basic leucine zipper domain (bZIP, 6 members including

bZIP44), Cys2/His2-type (C2H2, 6 members including IDD14), 5 bHLH members including MYC4, lateral organ boundaries domain (LBD, 3 members), BES1 and GATA (2 members each), CO-like, DBB, HD-ZIP, heat stress family (HSF), MIKC_MADS and Trihelix (1 member each) (Fig 2.8). Most TFs upregulated at WM or WS, compared to WC, were highly expressed at WM (99 TFs), except for the two TFs (MIKC-MADS and MYB3) that were upregulated at WS, compared to WC.

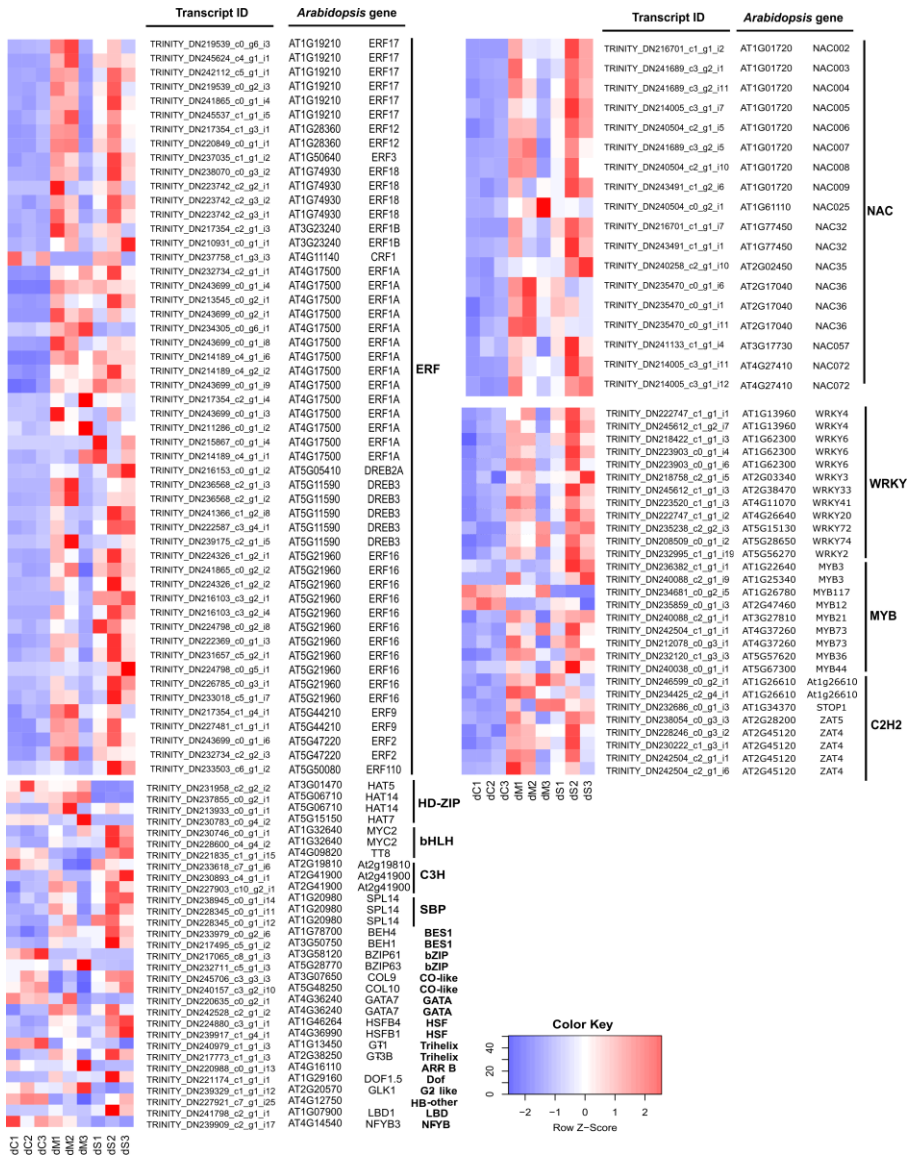

Fig 2.6 Heatmap of relative transcription factor (TF) genes expression in drought experiment. Heatmap colors indicate Z-scores of TMM-normalized TPM values. Red and blue color indicate upregulated and downregulated expression level, respectively.

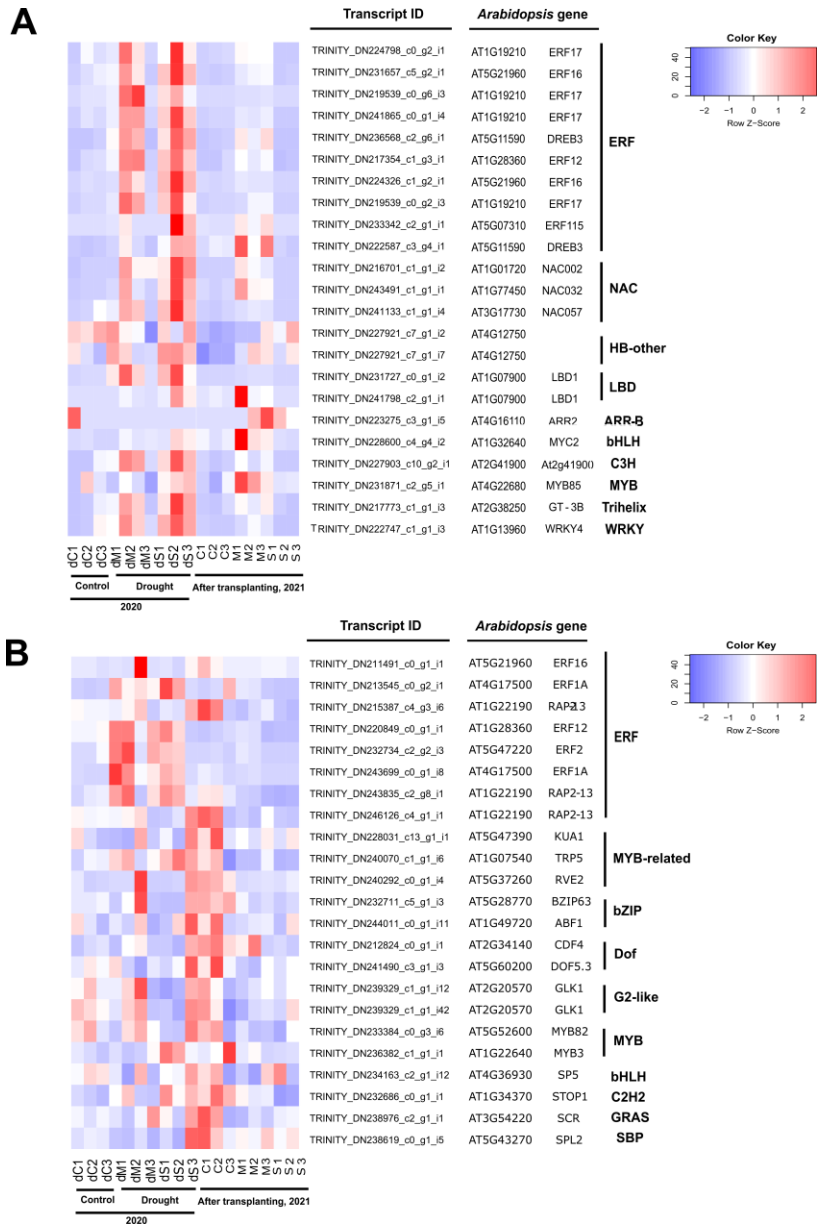

Fig 2.7 Heatmap of expression of (A) upregulated TFs and (B) downregulated TFs. Heatmap colors indicate Z-scores of TMM-normalized TPM values. Red and blue color indicates upregulated and downregulated expression level, respectively.

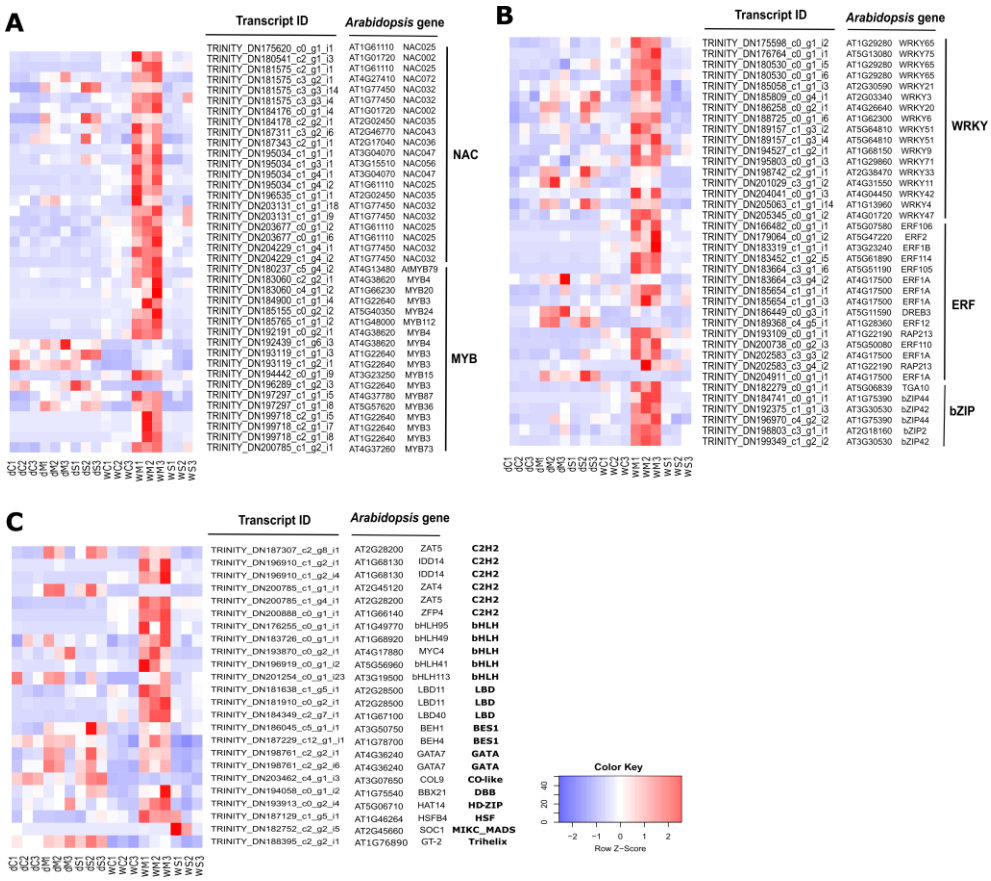

Fig 2.8 Heatmap of expression of (A) NAC and MYB TFs, (B) WRKY, ERF, and bZIP TFs, (C) C2H2, bHLH, LBC, other TFs. Heatmap colors indicate Z-scores of TMM-normalized TPM values. Red and blue color indicates upregulated and downregulated expression level, respectively.

2.3.4 Pathway analysis of drought hardening before and after transplanting

The MapMan analysis revealed that auxins, brassinosteroids, ethylene, salicylic acid (SA), mitogen-activated protein kinase (MAPK), and genes related to signaling in response to environmental stimuli were upregulated under dM rather than dC (Fig 2.9A). Under dS, SA, cell wall, heat shock proteins, peroxidases, and glutathione *S*-transferase were more highly expressedthan dC (Fig 2.9A). After transplanting, most of the genes involved in phytohormone, cell wall, glutathione *S*-transferase, were upregulated under M relative to C but not S (Fig 2.9B). By contrast, the genes controlling redox state and peroxidases were downregulated under M rather than C.

In the cold stress in winter, plant hormones, including auxins, brassinosteroid, ABA, ethylene, salicylic acid (SA), JA, MAPK, peroxidase, glutathione-S-transferase, cell wall, secondary metabolites were upregulated in WM compared to WC (Fig 2.9C). However, in WS, genes related to auxins, brassinosteroid, JA, MAPK, signaling, glutathione-S-transferase were downregulated relative to WC (Fig 2.9C).

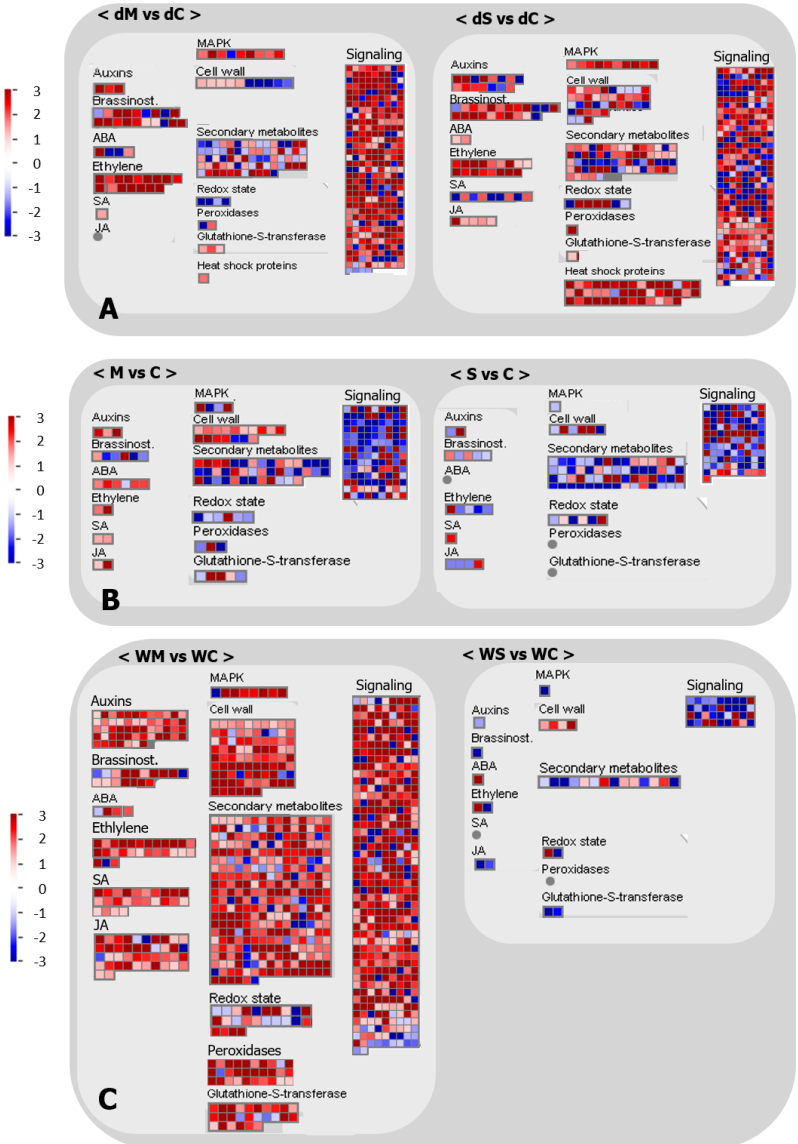

Fig 2.9 MapMan analysis of stress-related DEGs in *P. densiflora* in response to (A) drought stress (B) transplanting, and (C) cold stress in winter. Different colors represent $\log_2$ TPM values for gene expression. Red indicates upregulation and blue indicates downregulation. ABA, abscisic acid; brassinost., brassinosteroid; HSP, heat shock protein; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid.

2.3.5 Validation of RNA-seq results

We validated the accuracy of the RNA-Seq by comparing them against those for the qRT-PCR on 16 genes in M vs. C and in WM vs WC. Correlations between the $\log_2$ fold change values of RNA-Seq and those of qRT-PCR showed significant linear relationships (R^2 = 0.810 and 0.784, Fig 2.10A,B). In case of RT-PCR results, most of phenylpropanoid biosynthesis genes were relatively upregulated under M in transplant and cold stresses, similar to RNA-seq results (Fig 2.10C,D). Among them, UDP-glycosyltransferase 72B1 (UGT72B1) was commonly upregulated under M in both stresses.

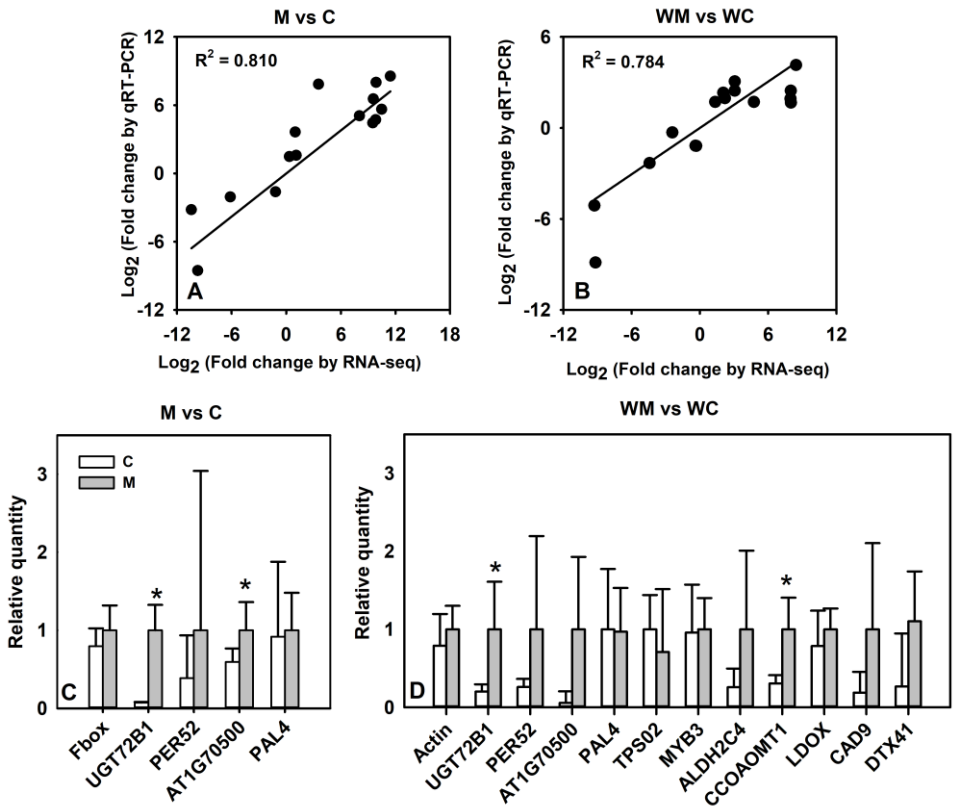

Fig 2.10 Validation of RNA-seqeuncing results using quantitative real-time PCR (qRT-PCR). (A) regression of between RNA-seq and qRT-PCR value of M vs C (B) regression of between RNA-seq and qRT-PCR value of WM vs WC. The relative expression of phenylpropanoid biosynthesis (UGT72B1, PER52, AT1G70500, PAL4, TPS02, MYB3, ALDH2C4, CCOAOMT1, LDOX, CAD9, and DTX41) genes in (C) transplant and (D) cold stresses**.** Asterisks indicate statistically differences (Independent t-test, $P<0.05$).

2.4. Discussion

2.4.1 Regulated common genes between drought and transplant stress

The more shared stress signaling pathway, the more improved hardening effect between different stresses (Liu *et al.*, 2021). Drought stress shows the most similar symptoms with transplant stress (Close *et al.*, 2005; Struve, 2009). Here, the defense response genes included secondary metabolites biosynthesis and cell wall organization were upregulated, and 39 including isoprenoid genes were downregulated in response to transplant stress (Table S3). Secondary metabolites such as lignin biosynthesis was activated to recovery wounded tissue occurred in the transplanting procedure (Hawkins and Boudet, 2003). Similar to this results, wounding stress cause the downregulation of the isoprenoid pathway by inhibiting DXS (Mitra *et al.*, 2021).

The 132 genes were commonly expressed under both transplant and drought stresses and the GO terms were associated with nine upregulated including root development and no downregulated genes (Table 2.6). Root system development and meristem size are vital to drought tolerance and maintain plant productivity under drought stress (Díaz-Espejo *et al.*, 2012; Verslues and Longkumer, 2022). Similar to drought stress, water and nutrient upatake rate reduced in transplant stress due to root loss (Struve, 2009). Based on the foregoing results, we concluded that root wounding explained transplant stress and only the root development pathways were common to both transplant and drought stresses.

2.4.2 Drought tolerance after drought hardening

After two year drought hardening, moderate and severe drought hardening improved the drought tolerance. In long-term drought stress, plants have changed physiological and morphological mechanisms and these processes are controlled by phytohormoens (Manavalan *et al.*, 2009; Alhaithloul *et al.*, 2020). In this study, the photosynthesis genes were downregulated in two drought conditions (Table S2). Generally, drought stress caused generation of ROS and oxidative stress is the cause of downregulation of photosynthesis (Asada, 2006; Gill and Tuteja, 2010). On the other hand, downregulation of photosynthesis may be an adaptation to stress preventing damage to the photosynthetic apparatus under long-term drought stress (Degenkolbe *et al.*, 2009). Similar to the previous study, our results also showed the downregulation of photosynthesis genes under drought condition without reduction in height and diameter growth for three years (Bhusal *et al.*, 2021).

Phytohormone such as, ethylene, ABA, JA, and SA activate processes including stomatal closure, enhancement of root growth, and accumulation of osmolytes to resist drought stress (Fahad *et al.*, 2015; Alhaithloul *et al.*, 2020). Generally, ABA induced stomatal closure and inhibition of transpiration under drought stress to decrease water loss (Mittelheuser and Van Steveninck, 1969; Agurla *et al.*, 2018). However, in our previous study, ABA showed no differences between treatments after two year drought and it resulted in no reduction in stomatal conductance, leaf water potential, A_{max} and growth under drought stress (Bhusal *et al.*, 2021). Besides, there was no improvement in root biomass growth under drought condition (Fig 2.1) and transcriptional analysis showed the upregulation of substance transport and transpiration genes under dM and dS (Table S2). The maintenance of substance transport and transpiration under drought stress improves the drought tolerance, water use efficiency, and recovery after the drought season (Wade *et al.*, 2000; Siopongco *et al.*, 2006; Aleem *et al.*, 2021).

On the other hand, JA of dS increased by 65.3 % than dC (Bhusal *et al.*,

2021) and genes involved in JA also upregulated under dS than dC after 2 year drought hardening (Fig 2.9A). Increased JA under drought led to accumulation of proline and improve antioxidant capacity in same site, which enhanced drought stress tolerance (Bhusal *et al.*, 2021; Kim *et al.*, 2021). Consistent with previous results, our results showed upregulation of JA genes induced secondary metabolites and heat shock proteins genes by activating MYC, WRKY, NAC and ERF TFs under dS (Fig 2.6, 9A). Corresponded with these results, there was no decreases in growth not only in moderate drought but also in severe drought condition (Ruan *et al.*, 2019; Bhusal *et al.*, 2021). Increases in secondary metabolites such as flavonoids induce antioxidant enzymes and maintain cellular redox balance (Brunetti *et al.*, 2013; Bose *et al.*, 2014; Nadarajah, 2020) and heat shock proteins (HSPs) also protect other proteins against irreversible damage (Gong *et al.*, 2001; Al-Whaibi, 2011). To sum up, severe drought hardening improved drought tolerance to a greater extent than moderate drought stress for two years by upregulation of JA genes to induce defense response.

2.4.3 Drought hardening effect after transplanting

Moderate drought hardening improved transplant stress after drought hardening. The increases of defense response genes under M were induced by stimulation of JA and SA by activation of TFs such as NAC and ERF (Figs 2.4A, 2.7A, 2.9B). However, there were no differences in upregulation of TFs between S and C (Fig 2.7A). Genes related to phytohormones induced plant defensive responses to resist environmental and pathogen stresses (Verma *et al.*, 2016) and NAC and ERF TFs were involved in defense responses (Gutterson and Reuber, 2004; Zhang *et al.*, 2007; Seo *et al.*, 2010; Mao *et al.*, 2012). Under M, upregulation of JA genes induced secondary metabolites genes such as phenylpropanoid biosynthesis, cell wall organization and terpenoid metabolism (Fig 2.4A). The upregulation of phenlypropanoid genes including lignin and flavonoid and terpenoid metabolism recovered wounded tissue by increasing antioxidant enzymes and cell wall modification (Dixon and Paiva, 1995; Barku, 2019).

Increased secondary metabolites lead to improve growth through the structural and functional stabilization (Edreva *et al.*, 2008). Under M, root collar diameter, photosynthesis and stomatal conducatance increased than S and C for two years after transplanting and leaf midday water potential was also higher in M (Figs 2.2, 2.3). Similar to our result, drought-hardened *Eucalyptus pilularis* showed higher leaf water potential and stomatal conductance than non- hardened trees (Thomas, 2009). Under prolonged drought stress, drought- hardened potato seedlings had higher stomatal density, and biomass than the control under prolonged drought stress (ZHANG *et al.*, 2018). Drought- hardened plants may alter their metabolism in response to drought stress to accelerate photosynthesis and growth (Galle *et al.*, 2011). Moderate drought hardening improved defense response and water conservation capacity, which lead to enhance productivity after transplanting.

2.4.4 Regulated common genes between cold and drought stress conditions

In this study, the GO terms upregulated by cold stress were similar to the general metabolic processes, such as JA, secondary metabolites, L-phenylalanine metabolic process, and stomatal movement (Table S7) (Knight *et al.*, 2004; Ortega-García and Peragón, 2009). Under cold stress, ABA-induced stomatal closure was reportedly accompanied by downregulation of photosynthesis (Acharya and Assmann, 2009; Agurla *et al.*, 2018), which was also pronounced in our results (Table S5).

When common stress responses exist between different stresses, hardeing effect improved (Liu *et al.*, 2021). Earlier studies revealed that cold stress showed similar physiological responses to drought stress, such as accumulation of solutes, stomatal closure, and the downregulation of photosynthesis (Farooq *et al.*, 2009; Verma *et al.*, 2016). Similarly, in our study, drought and cold stresses showed commonly upregulated GO terms, including JA-mediated signaling pathway and downregulated GO terms, such as photosynthesis, photosystem I, light harvesting, and reaction (Table S6).

2.4.5 Cold stress tolerance after drought hardening according to drought hardening intensity

Our results showed that moderate drought hardening improved the cold stress tolerance by inducing the CORs genes such as secondary metabolites, cell wall organization and antioxidants genes, while most of stress genes showed no differences between WS and WC (Fig 2.9C). These CORs genes were induced by DREB/ERF, MYB, bHLH, and WRKY TFs and phytohormones such as JA and ABA (Stockinger *et al.*, 1997; Hu *et al.*, 2013; Jin *et al.*, 2018). Similar to previous study, TFs related to CORs genes, such as MYB, bHLH, ERF, WRKY, bZIP, and NAC, were upregulated in WM compared to WS and WC (Fig 2.8A,B). Under WM compared to WC, including cryoprotectant (alpha amino acid and carbohydrates), secondary metabolites (phenylpropanoid and flavonoid), and phytohormone (SA, auxins, ethylene, and JA) metabolic process upregulated, while 7 GO terms were upregulated at WS compared to WC (Table S6). Proline content also increased in WM compared to WC (Table 2.4). The downregulated GO term at WM included response to light and radiation, including chlorophyll and pigment processes (Table S6). Cold stress downregulates the photosynthesis, such as chlorophyll and enzyme activities (Meryman *et al.*, 1977; Suzuki *et al.*, 2008), however, downregulated photosynthesis might presumably improve the cold tolerance by increasing antioxidant enzymes, as in the results observed at WM (Turk *et al.* 2020). In addition to proline, the phenylpropanoid pathway also plays a crucial role in promoting stress tolerance (Isah, 2019; Singh *et al.*, 2021). The phenylpropanoid pathway includes the formation of secondary metabolites, such as flavonoids, anthocyanins, and pro-anthocyanidins (PA), and ROS scavenging enzymes (Winkel-Shirley, 2002; Apel and Hirt, 2004; Hernández *et al.*, 2009; Brunetti *et al.*, 2013). Moderate drought hardening alleviate physiological damage by inducing CORs genes, which led to improve photosynthesis and productivity (Figs 2.2B,C).

Chapter 3. Waterlogging hardening effect on transplant stress

3.1. Introduction

Climate change affects the global hydrological cycle and a heavy precipitation is expected to occur more frequently in response to global warming (Dore, 2005; Olsson *et al.*, 2015). Intensification of heavy precipitation observed across the world (Fischer and Knutti, 2016; Zhou *et al.*, 2016). Increase of water vapor in the atmosphere by global warming leads to more heavy precipitation events, despite global average annual precipitation is reduced (Tabari, 2020; Armon *et al.*, 2022). In recent years, unprecedented flooding have been observed frequently due to heavy rainfall all over the world (Wilby and Keenan, 2012; Garner *et al.*, 2017). In the 2016 Louisiana flood, rainfall about 72 hours resulted in widespread flooding (Watson *et al.*, 2017). India also experienced large floods during monsoon season in 2013 (Ranalkar *et al.*, 2016). Wang *et al.* (2014) predicted that intensities of rainfall extreme increase over time in Toronto: in 2030s (5-17%), in 2050s (11-22%) and in 2080s (25-50%).

Flooding and waterlogging are environmental stress triggering reduction of growth in crop and woody species around the world (Urquhart, 2004; Parent *et al.*, 2008; Onda *et al.*, 2010; Muhammad, 2012). The cost of damage by flooding was 3.4 times higher than by drought in 2016 in United States (Jia *et al.*, 2021). In case of cotton (*Gossypium hirsutum* L.), waterlogging stress reduced yield from 10% to 40% (Hodgson, 1982; Bange *et al.*, 2004). In Texas, sudden vegetation dieback occurred as a result of extreme precipitation event (Stagg *et al.*, 2021). Flooding reduce plant productivity and leads to plant death because of reduction of stomatal conductance (Voesenek and Bailey-Serres, 2015). Low O_2 (oxygen) level in submerged root decline ATP synthesis, respiration and leaf stomatal closure (Drew,

1997; Rodriguez-Gamir *et al.*, 2011). Decrease of stomatal closure leads to inhibition of transpiration, photosynthesis and respiration due to accumulation of reactive oxygen species (ROS) (Yan *et al.*, 1996; Chen *et al.*, 2014).

Phytohormones play a crucial role in physiological mechanisms to survive under oxidative stress conditions (Ahmad *et al.*, 2010; Alhaithloul *et al.*, 2020). Ethylene, and abscisic acid (ABA) have the most important roles in waterlogging stress by regulating stomatal opening and closing (Bashar *et al.*, 2019). Ethylene can enhance photosynthesis by increasing stomatal conductance (Iqbal *et al.*, 2011; Yamauchi *et al.*, 2014). High stomatal conductance is a key indicators of tolerance in flooding stress by reducing accumulation of ROS (Arbona *et al.*, 2009; Caudle and Maricle, 2012). Flooding tolerant species *Zea mays* and *Vicia faba* (bean) maintained photosynthesis rate and high stomatal conductance in flooding stress, whereas *Phaseolus vulgaris* and *Pisum sativum* reduced stomatal conductance and photosynthesis rate (Caudle and Maricle, 2012). Besides, ethylene induced adventitious root production to adapt flooding stress (Qi *et al.*, 2019). Jasmonic acid (JA) also regulates waterlogging stress by inducing antioxidants to scavenge ROS (Kamal and Komatsu, 2016; Raza *et al.*, 2021).

Plants can improve stress tolerance by the adaptation process to prior stress exposure, which is called ‘hardening’ (Borges *et al.*, 2014; Hilker and Schmülling, 2019). Hardening enhances various abiotic stresses such as drought (ZHANG *et al.*, 2018; Khan *et al.*, 2020; Saiki *et al.*, 2020), chilling (Ghanbari and Sayyari, 2018) and salinity (Cayuela *et al.*, 2007). Previous studies have reported that pre-treated waterlogging hardening could improve production of wheat in post waterlogging stress (Li *et al.*, 2011). Plus, waterlog hardening reduced the oxidative damage and yield loss in soybean (*Glycine max*) in waterlogging stress (Agualongo *et al.*, 2022). Waterlogging hardened wheat had higher chlorophyll content and photosynthesis rate than non-hardened wheat (Li *et al.*, 2011). Besides, abiotic stress hardening could induce cross-stress tolerance to subsequent stress (Hossain *et al.*, 2018; Llorens *et al.*, 2020; Thomas *et al.*, 2020).

Transplanting shock has negative effects on plant growth and survival when

seedling are moved to other environment (Close *et al.*, 2005). Transplant shock limit water and nutrient uptake due to root pruning similar to drought and waterlogging stress (Close *et al.*, 2005; Lee *et al.*, 2022). Seedlings adapted nursery condition where irrigated frequently and protected from wind due to higher tree density (Close *et al.*, 2005). Allen *et al.* (2017) have reported nursery production system affect growth, tree survival, root formation and drought tolerance. Therefore, it is necessary to nursery management to improve stress tolerance. However, there have been few studies related to hardening effect to transplanting stress in tree (Villar-Salvador *et al.*, 2004).

Here, to understand the transcriptional response to waterlogging and transplant stress and to identify the stress tolerance after waterlogging hardening, we analyzed transcriptional responses of conifer species *P. densiflora*, which occupied more than 23.5% in Korean forest (Korea Forest Service, 2006). Trees were grown for three years at two different water availability: control, (100% natural precipitation; C); and waterlogging (30% additional irrigation, W). After three years of waterlogging treatment, trees were transplanted to another site to study the waterlogging hardening effect. To investigate waterlogging hardening effect on transplant stress tolerance, transcriptome analysis was performed after transplanting. This study aims (i) to determine whether waterlogging stress improve stress tolerance after transplanting; (ii) to identify the waterlogging memory on transplanted seedlings; and (iii) how transplant stress has a common gene expression with waterlogging stress.

3.2. Material and Methods

3.2.1 Experiment sites and plant materials

Waterlogging hardening was conducted at Mt. Jiri (127°27′09.8″ N 35°17′09.3″, elevation 282 m a.s.l) in Gurye, South Jeolla Province, Republic of Korea for three years (April, 2018 to October, 2020). Waterlogging experimental site consisted of two treatments: control (C) and waterlogging treatment (W). In C treatment, plants were grown at natural precipitation, and in W treatment, irrigation was supplemented by sprinklers. A total of 48 sprinklers were installed at a 3 m height and 70 cm interval. The additional irrigation was added as follows. If the weekly precipitation was lower than the 20 year average (1345.7 mm, from 1997 to 2017), additional irrigation was conducted by program (Bhusal *et al.*, 2022). In case of high precipitation in a week, irrigation was not added.

The mean annual temperature is 13.1 ± 0.01 ℃ and annual precipitation of ‘C’ was 1392, 1495, and 1891 mm and, that of ‘W’ was 1811, 1891 and 2128 mm in 2018, 2019 and 2020, respectively. The average soil water content in ‘C’ was 21.54%, 24.24% and 26.69%, and, those of ‘W’ were 27.48%, 30.12% and 32.95%, respectively. Soil water contents of the top 30 cm were recorded using soil moisture sensor (%, CS-616, Campbell Scientific Inc.; 12 in each treatment) with 15s interval and averaged by 30 min. The soil moisture data were collected by HOBO stations (HOBO RX3000; Onset Computer Corporation) in each plot.

Twelve three-years old seedlings of 11 species included five gymnosperm species: *Abies holophylla* Maxim, *Pinus densiflora* Siebold & Zucc, *Pinus thunbergii* Parl, *Pinus koraiensis* Siebold & Zucc, and *Larix kaempferi* (Lamb.) Carrière, and sixangiosperm species: *Acer pictum* Thunb. subsp. mono (Maxim.) H.Ohashi, *Betula platyphylla* Sukaczev, *Chamaecyparis obtusa* (Sibold & Zucc.) Endl, *Prunus sargentii* Rehder, *Quercus acutissima* Carruth, and Fraxinus rhynchophylla Hance. were transplanted in each treatment in April, 2018. Each

treatment consisted of 36 cells (each cell 1.5m × 1.5m) and 4 seedlings in each cell transplanted at 80 cm distance between seedlings. *P. densiflora* occupied three cells in each treatment. The remaining 11 species occupied the rest of cells. The soil texture was of sandy clay loam, with a pH of 6.5. Additional details and environmental variables of the site are provided in Bhusal *et al.* (2022).

The hardened trees were transplanted to Mt. Taehwa in central Korea (E 127°18′38.1″ N 37°18′46.6″, 137m a.s.l.) in October, 2020. A total of 6 *P. densiflora* seedlings grown at each treatment were transplanted to Mt. Taehwa in October, 2020. The transplant experimental site was consisted of 90 cells (1.0 m × 3.0 m). Each cell was consisted of 3 seedlings at 1.0 m distance between seedlings. *P. densiflora* was grown at 2 cells per treatment and the rest of the cells were used for other species. Trees grown under the same treatment were transplanted in same cells. The mean temperature and annual precipitation were 12.7 ℃ and 1076 mm in 2021, respectively. The seedlings were categorized based on the growth condition until 2020 (grown at C : TC and W : TW). The soil texture used was sandy-loam with a pH of 5.2 ± 0.1. Soil organic matter and soil N is 2.74 ± 0.19 % and 0.16 ± 0.0%, respectively. Soil biochemical analysis was analyzed using an Elemental Analyzer (Flash EA 1112; Thermo Electron, Waltham, MA, USA) at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University. Leaf samples were harvested between 7:00 a.m. and 8:00 a.m. from three replicate trees in each treatment for transcriptomic analysis in August, 2020 and 2021. All the collected samples were put in liquid nitrogen, immediately.

3.2.2 Physiological parameters

After transplanting, root collar diameter and height measurement was conducted in October, from 2020 to 2022. Measurement of root collar diameter and height from 6 seedlings per treatment was conducted using digital calipers (Mitutoyo vernier calipers 100mm, Mitutoyo, Japan) and height rod.

Leaf gas exchange measurement was conducted on 6 seedlings of each treatment between 08:00 and 13:00 in August, 2021. The net photosynthetic rate (P_{max}), stomatal conductance (g_s) and transpiration rate were measured from ten needles of each seedling using a portable infrared (IR) gas analyzer (LI-6400; LI-COR, Lincoln, NE, USA). The fixed factors of photosynthetic measurement were as follows: CO_2 concentration (ambient CO_2 concentration: 400 μmol mol^{-1}), temperature (25 ℃), photosynthetic photon flux density (PPFD) (1,400 μmol m^{-2} s^{-1}), relative humidity (RH; 50–60%), and airflow rate (500 μmol s^{-1}).The instantaneous water use efficiency (*i*WUE) was calculated as P_{max} divided by g_s. The projected leaf area of measurement chamber was measured to recalculate the gas exchange variables considering leaf area. After leaf gas exchange measurement, needles were fully collected in a 15 ml tube and put in the liquid nitrogen tank immediately for analyzing NSC, proline and chlorophyll content.

3.2.3 Leaf biochemical parameters

The leaf nonstructural carbohydrates (NSC) was analyzed by measurement of soluble sugars and starch follwed the method of Newell *et al.* (2002). Fresh 15 mg *P. densiflora* needles from six seedlings was dried for 3 days at 70 ℃. Dried samples were powdered with two 5-mm beads using homogenizer (FastPrep-24; MP Biomedicals, Solon, OH, USA). The powdered samples were added in 1.5 ml of 80% (*v/v*) ethanol and the mixture was incubated in a water bath at 80 ℃ for 30 min. Then, the mixtures were centrifuged at 14,000 × *g* at 15 ℃ for 10 min. The

soluble sugar content was measured at 490 nm using spectrometer (Optizen 2120UV; KLAB., Korea) the phenol-sulfuric acid colorimetric method (Ashwell, 1966). After soluble sugar extraction, the remained pellets were dried to measure starch content. Each pellet was added in 2.5 ml sodium acetate buffer (0.2 M) and the mixture was incubated at 100 ℃ for 1 h, and cooled at 15 ℃. And then, 2 ml sodium acetate buffer and 1 ml amyloglucosidase (0.5% [*w/w*]; Sigma A9229-1G; Sigma-Aldrich Corp., St. Louis, MO, USA) were added with the mixture at 55 ℃ overnight. The mixture was centrifuged at 15 ℃ 14,000 × *g* for 10 min. The starch content measurement was measured at 490 nm and followed phenol-sulfuric acid colorimetry method. Two technical replicates per seedlings were conducted.

Fresh 0.1 g needles at -80 ℃ were used to proline extraction according to the method of Á brahám *et al.* (2010). The samples were powdered with a single 5-mm bead using a homogenizer (FastPrep-24; MP Biomedicals) at liquid nitrogen. The powdered samples were added in 3 ml of 3% (*w/v*) sulfosalicylic acid at 4 ℃. The mixture was centrifuged at 14,000 × *g* at 4 ℃ for 5 min and incubated at 90 ℃ for 5 min. Then, 2 ml supernatant was mixed with 2 ml acidic ninhydrin (ninhydrin 1% (*w/v*) in acetic acid 60% (*v/v*), ethanol 20% (*v/v*)), 1 ml glacial acetic acid, and 2 ml of 6 M orthophosphoric acid. The mixture was incubated to 100 ℃ for 1 h and cooled to 15 ℃. Toluene was added to the mixture and incubated for 5 min. The proline content was determined at 520 nm using spectrometer (Optizen 2120UV; KLAB., Korea). Two technical replicates per seedlings were conducted.

The chlorophyll content was measured using dimethyl sulfoxide (DMSO) method (Shinano *et al.*, 1996). Fresh 0.2 g needles in 5 ml DMSO was incubated at 65 ℃ for 6 h. The chlorophyll content was read at 649 nm and 665 nm with a spectrophotometer (Optizen 2120UV; KLAB., Korea). Two technical replicates per seedlings were conducted. The total chlorophyll content was calculated as follwed (Wellburn (1994)):

$$\text{Total chlorophyll content } (\mu g \cdot ml^{-1}) = 21.44\, A_{649} + 5.97\, A_{665}$$

3.2.4 Statistical analysis

Two-way repeated-measures ANOVA was performed for height, root collar diameter, height and root collar diameter growth with the fixed factors “treatment” and the random factor “Year”. Independent t-test was used to identify the waterlogging hardening effect on transplant stress on height and root collar diameter growth, P_{max}, g_s, *i*WUE, NSC, proline, and chlorophyll. All statistical analyses were conducted in R v. 4.0.3 (R Core Team, Vienna, Austria).

3.2.5 Transcriptome analysis

Total RNA was extracted from about 40 mg needle of 6 samples using Ribospin™ Plant (GeneAll, Seoul, Republic of Korea). Extracted RNA was analyzed by Macrogen (Macrogen, Seoul, South Korea) to conduct library construction and sequencing. RNA Integrity Number (RIN) was evaluated on Bioanalyzer RNA Pico 6000 chip and RIN of all sample were higher than 7 for cDNA library construction. cDNA library (paired-end non directional, 2 × 101 bp) was constructed using the TruSeq Standard mRNA libarary prep kit and sequenced by Illumina NovaSeq 6000 system (Macrogen, Seoul, South Korea). The raw read data was filtered using Prinseq-lite version 0.20.4 (Schmieder and Edwards, 2011). Clean reads were assembled using *de novo* assembly (Trinity v.2.13.2) (Hao *et al.*, 2015). To find the candidate coding regions of assembled transcripts, Transdecoder v.5.5.0 was used with default parameters (Tang *et al.*, 2015). CD-HIT-EST v.4.8.1 was performed to cluster of transcripts (similarity 95%) (Fu *et al.*, 2012). To assess the quality of assembled transcriptome, Benchmarking Universal Single Copy-Orthologs (BUSCO, v.3) was used with the Embryophyta_odb10 database (Simão *et al.*, 2015).

Salmon v.1.8.0 was used to map clean reads to the assembled transcriptome on alignment-based mode (Patro *et al.*, 2017). After mapping, DESeq2 v.1.34.0 was

applied to normalize the read counts and compare differential expression genes (DEGs) between treatments (Love *et al.*, 2014) with lower than 0.05 of a false-discovery rate (FDR) – adjusted *P-value* and $|\log_2$ fold change (FC)$| > 1$. For gene ontology analysis, Basic Local Alignment Search Tool for proteins (BLASTX) compared the sequences against with those of *Arabidopsis thaliana* (*A. thaliana*) using with e-value threshold of 1×10^{-7} to reveal gene functional annotations (Altschul *et al.*, 1997). PANTHER GO classification system was used to identify the cellular component, molecular function and biological process using Fisher's exact test with FDR < 0.05 **(**http://www.geneontology.org) *(Mi et al., 2017)*. TFs of *A. thaliana* in Plant Transcription Factor Database v4.0 **(**http://planttfdb.cbi.pku.edu.cn/) was used as reference to identify the transcription factors (TF) families of *P. densiflora* (Jin *et al.*, 2016; Lee *et al.*, 2019). Pathway analysis of DEGs was conducted using MapMan v 3.5.1R2.

To validate RNA-seq results, RNA extraction was performed using Ribospin™ Plant kit (GeneAll, Seoul, Republic of Korea). Extracted RNA was synthesized to cDNA using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Primers of 10 genes were designed with Primer3 (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table S11). The reference gene was F-box for normalization (Zhu *et al.*, 2019). Each mixture for qRT-PCR contained 10 μl of mastermix (IQ Sybr Green SuperMix; Biorad, USA), 10 μM forward and reverse primer, 1 μl cDNA (50 ng μl^{-1}) and 7 μl DNase /RNase free water. Then, qRT-PCR was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as followed cycle : 95 ℃ for 2 min, followed by 40 cycles at 95 ℃ for 10 s, 61 ℃ for 30 s and 72 ℃ for 30 s. qRT-PCR reactions were carried out with two technical replicates per seedlings. Expression of qRT-PCR were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A correlation analysis of RNA-seq and qRT-PCR results was calculated using v.R 4.0.3.

3.3. Results

3.3.1 Physiological response of waterlogging hardening after transplanting

Before transplanting, photosynthetic rate, leaf water potential and height and rootcollar diameter growth decreased W compared to C for two years (Bhusal *et al.*, 2022). After transplanting, height and root collar diameter showed differences between TW and TC (Table 3.1). However, height of TW tended to be higher than TC in 2022, however it was not significant in post-hoc test (Table 3.1). Root collar diameter also showed no significant differences in post-hoc test (Table 3.1). Height and root collar diameter growth were not significant different between TW and TC, however, there was interaction between treatment and year in height growth (Table 3.1). Height growth tended to be lower under TW than TC in 2021, while there was no differences between TW and TC in 2022 (Table 3.1). There was no statistical significance in post-hoc test (Table 3.1). In root collar diameter growth, there was no significant tendency (Table 3.1). P_{max} and stomatal conductance tended to be higher at TW than TC, but it was not significant. Similar tendency to P_{max}, chlorophyll also showed increased tendency at TW, however, it was not significant. Soluble sugar and starch were decreased 40.5% and 50.3 % at TW compared to TC, respectively and only starch was significantly reduced (P = 0.007, Table 3.2).

Table 3.1 Average of height, root collar diameter and growth of height and root collar diameter of *P. densiflora* grown at control and waterlogging treatment after transplanting.

		Year			*P-value*		
Factor	Treatment	2020	2021	2022	Treatment	Year	Treatment × Year
Height (cm)	Control	63.67 ±5.89	79.00 ±6.35	92.42 ±6.56	0.018*	0.038*	0.432
	Waterlogging	63.02 ±5.73	75.15 ±5.85	95.41 ±6.20			
Root collar diameter (mm)	Control	11.25 ±1.07	14.28 ±1.38	20.82 ±1.70	0.001**	<0.001***	0.686
	Waterlogging	12.62 ±0.91	15.68 ±0.09	20.10 ±0.46			
Height growth (cm yr^{-1})	Control		15.33 ±1.76	19.88 ±2.28	0.836	0.171	0.045*
	Waterlogging		12.15 ±1.19	20.25 ±3.71			
Root collar diameter growth (mm yr^{-1})	Control		3.02 ±0.49	6.54 ±0.80	0.245	0.004**	0.570
	Waterlogging		3.05 ±0.17	4.43 ±0.70			

Table 3.2 Results of independent t-test and average of the maximum photosynthetic rate (P_{max}), stomatal conductance, water use efficiency (*i*WUE), leaf soluble sugars and starch, and chlorophyll content measured in *P. densiflora* after transplanting.

	Treatment		*P-value*
	TC	TW	
P_{max}	15.63 ± 2.67	21.05 ± 2.98	0.217
Stomatal conductance	0.19 ± 0.04	0.32 ± 0.07	0.126
*i*WUE	88.11 ± 10.88	70.71 ± 13.16	0.334
Soluble sugars	77.60 ± 10.97	46.15 ± 7.22	0.059
Starch	87.89 ± 8.86	43.48 ± 7.22	0.007**
Chlorophyll	0.41 ± 0.03	0.49 ± 0.05	0.201

The superscript asterisk and indicates statistical significance (Independent t-test $P < 0.05$).

3.3.2 Comparison of differentially expressed genes between waterlogging and non-hardened seedlings

Total 406,823,434 and 368,384,228 raw paired reads were generated in the waterlogging experiment and after transplanting, respectively. We produced 269,984 transcripts and 134,369 genes after filtering and *de novo* assembly in Trinity (Table 3.3). GC content was 41.3 % and contig N50 length was 1,501. Total assembled bases were 236,105,474. To assess assembly quality, we conducted BUSCO analysis and 1483 (91.9 %) complete BUSCO genes was predicted with 49 (3.0%) fragmented and 82 (5.1%) missing genes.

Table 3.3 Summary statistics for *de novo* transcriptome assembly.

Assembled contigs	Number
Total Trinity genes (*n*)	134,369
Total Trinity transcripts (*n*)	269,984
GC content (%)	41.32
Contig N50 length (bp)	1,501
Average contig length (bp)	874.52
Total assembled bases	236,105,474

Of these DEGs, 3 up- and 12 downregulated genes were not categorized into GO term under W compared to C. After transplanting, 233 upregulated genes at TW were categorized into 66 biological process, 21 molecular function and cellular component (Table S8). Among them, it is worthy to mention biological process that are related to stress tolerance such as oxylipin, lignin, flavonoid, phenylpropanoid biosynthesis, carbohydrate metabolic process, immune system, defense response to fungus and bacterium, and response to bacterium, fungus, wounding, water deprivation, jasmonic acid, and salicylic acid (SA) (Fig 3.1A). In phenlypropanoid biosynthesis process, phenylalanine ammonia-lyase 4 (PAL4), UDP-glycosyltransferase 72B1 (UGT72B1) and probable cinnamyl alcohol dehydrogenase 9 (CAD9) were upregulated in TW compared to TC. The upregulated flavonoid biosynthesis process included dihydroflavonol 4-reductase (DFRA), leucoanthocyanidin dioxygenase (LODX), chalcone-flavanone isomerase 1 (CHI1), and anthocyanidin reductase (BAN). In upregulated molecular function, ABC-type transporter activity, oxidoreductase activity, quercetin 7-O-glucosyltransferase, quercetin 3-O-glucosyltransferase, UDP-glucosyltransferase, hydrolase activity and hydrolyzing O-glycosyl compounds. For cellular component, cell wall, chloroplast and membrane were upregulated. On the other hand, 156 genes were downregulated at TW and these were categorized into 5 molecular functions such as oxidoreductase activity, anion binding and catalytic activity (Fig 3.1B).

Transplant stress, which commonly regulated TW vs W and TC vs C 794 genes were upregulated and categorized into 198 biological processes, 49 molecular functions and 71 cellular components (Table S9). By transplant stress, various response stresses were found such as response to wounding, mechanical stimulus, bacterium, fungus, osmotic stress and reactive oxygen species. Cell wall related genes were the most significant biological process such as lignin, glucuronoxylan, cellulose, hemicellulose, galacturonan, xyloglucan and cuticle. To resist transplant stress, phenlypropanoid biosynthesis and flavonoid metabolism were upregulated. On the other hand, 303 downregulated genes were identified in

transplant stress and categorized with 72 biological proceses, 53 molecular functions, and 46 cellular components (Table S9). Genes related to photosynthesis were downregulated such as photosystem I, light harvesting, carbon fixation and dark reaction.

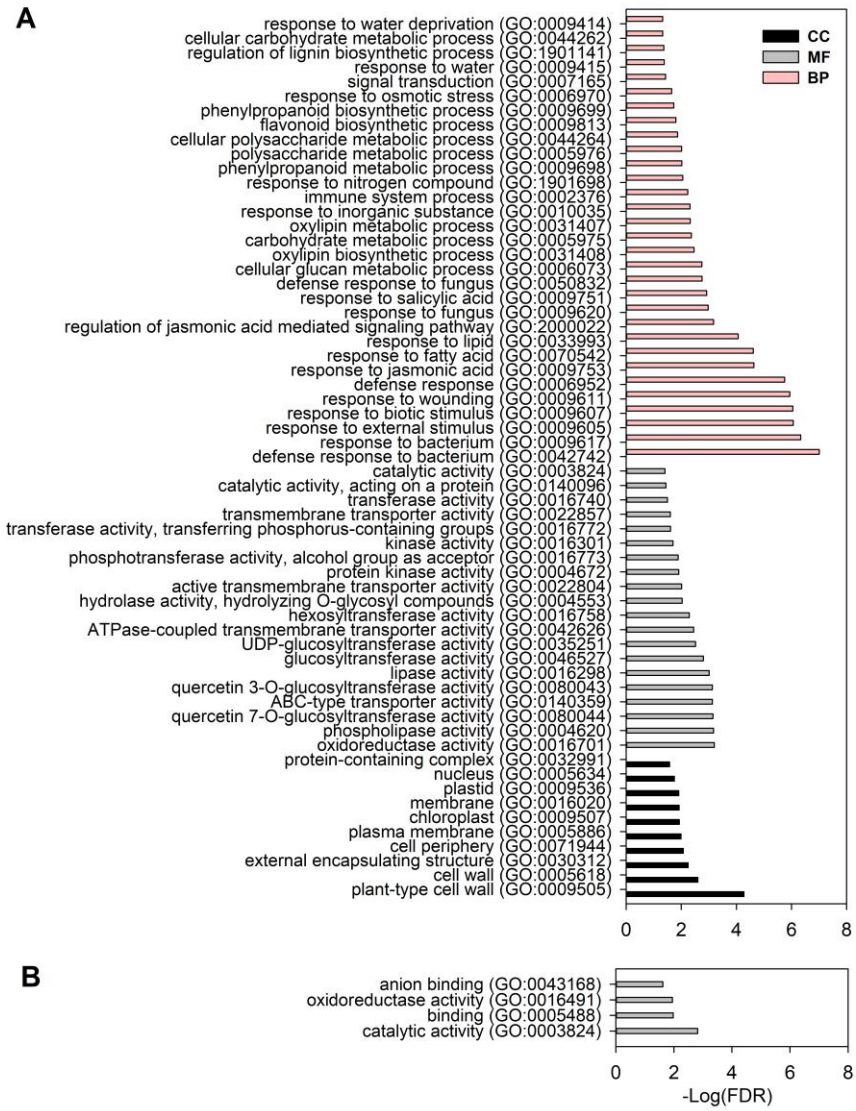

Fig 3.1 Gene ontology (GO) analysis of differentially expressed genes in comparisons between *P. densiflora* grown waterlogging stress after transplanting (TW and TC). (A). upregulated GO term at TW (B). downregulated GO term at TW. The x axis indicates p-value $-\log_{10}$ (FDR). The GO terms associated with Fisher's exact test with FDR corrected P-value < 0.05. The CC, MF and BP indicate cellular component, molecular function and biological process, respectively.

3.3.3 Transcription factors and pathway analysis after tranplanting

Before transplanting, there were no differentially expressed genes between C and W. However, we identified 8 downregulated and 7 upregulated TFs at TW compared to TC (Fig 3.2). Downregulated TFs at WT were two ERF families (ERF9 and RAP2-13), RAV, bZIP, ARF, C3H, GRAS and Trihelix. The highly expressed TFs at WT included three LBD family (LBD1), 2 MYB familes (MYB3 and MYB5), bZIP29 and ERF017.

In mapman analysis, genes related to cell wall, MAPK and secondary metabolites were upregulated at TW than that of TC. Among them, log_2FC value of jasmonate.synthesis-degradation.lipoxygenase, SA synthesis-degradation, cell wall proteins, phenylpropanoid, lignin biosynthesis and flavonoid, redox thioredoxin, peroxidase, glutathione-S-transferase were higher than 7. Log_2FC value of genes related to auxin, ethylene signal transduction and MAPK were lower than -7 at TW compared to TC (Fig 3.3).

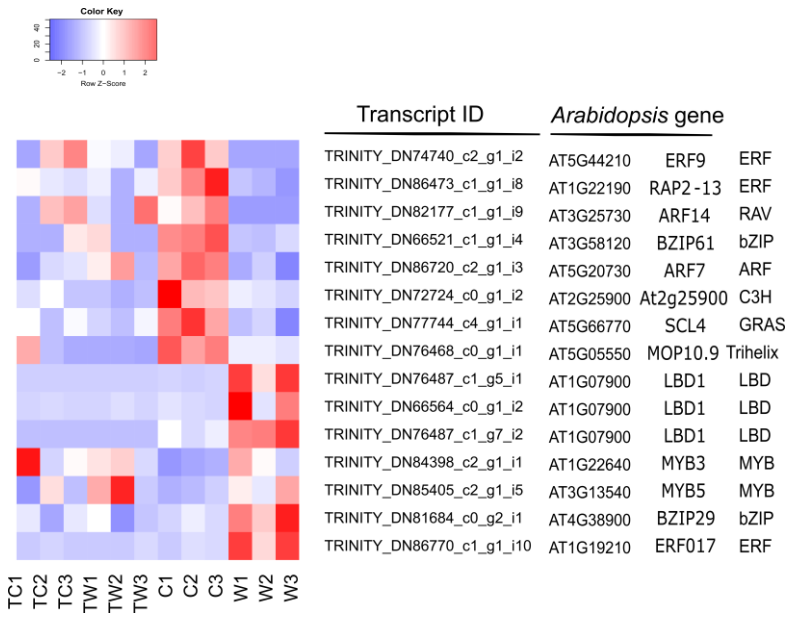

Fig 3.2 Heatmap of expression of transcription factor (TF) genes in comparisons in waterlogging experiment. Heatmap colors indicate the Z-scores of TMM normalized TPM values. The red and blue color indicates higher and lower expression, respectively.

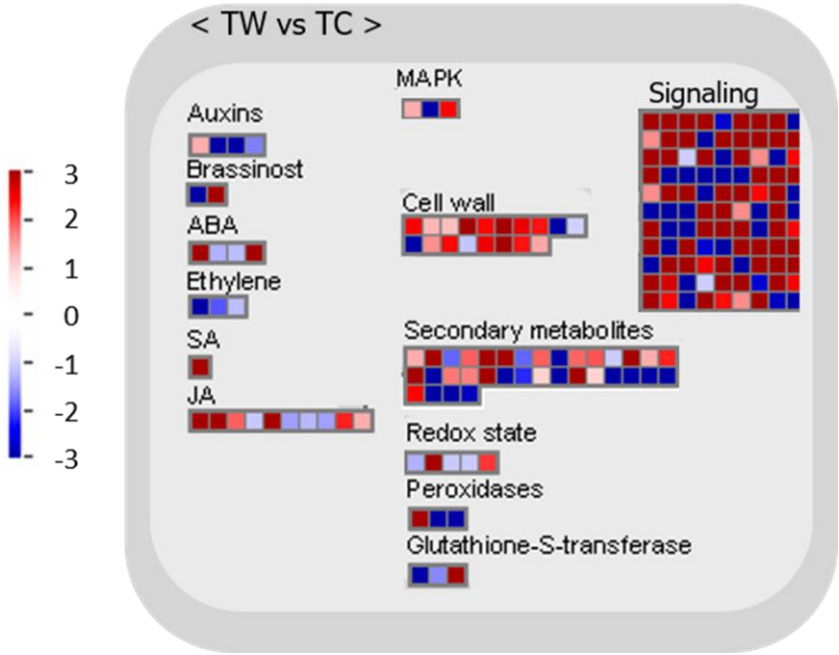

Fig 3.3 MapMan analysis of stress-related DEGs of *P. densiflora* genes compared to TW and TC. The different colors represent the log2 TPM values of the gene expression. Red indicates upregulated and blue indicates down regulated genes. ABA, abscisic acid; brassinost., brassinosteroid; JA, jasmonic acid; SA, salicylic acid.

3.3.4 Validation of RNA-seq expression of waterlogging hardened trees in transplant stress

To confirm the accuracy of RNA-seq expression, we compared the qRT-PCR expression with nine genes in TW versus TC. Log_2 fold change values of RNA-seq expression showed significant correlation with log_2 fold change values of and qRT-PCR (R^2 = 0.914, Fig 3.4).

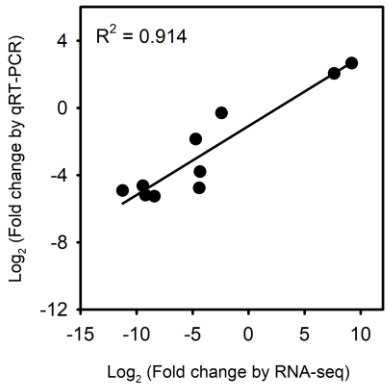

Fig 3.4 Validation of RNA-Sequencing results using quantitative real-time PCR (qRT-PCR). Correlation of $\log_2$ FC value analyzed by RNA-Seq (x aixs) with data obtained using quantitative real-time PCR (y axis) in trees grown waterlogging hardening versus control in transplant stress.

3.4. Discussion

3.4.1 Genes regulated by transplant stress

Transplant stress was defined as negative effects on growth and mortality after transplanting into different environmental condition (Rietveld, 1989) and a process of recovery (Struve, 2009). To response to root pruning, genes related to response to wounding, biotic stimulus, oxidative and osmotic stresses were upregulated and downregulated photosynthesis in this study (Table S9). Most of transplanted seedlings experience downregulation of photosynthesis because seedlings were exposed to different environmental condition such as light, water and nutrient (Close *et al.*, 2005). Besides, root loss by pruning is also a cause of transplant stress by reducing the uptake of water and nutrient, so transplanted tree experienced similar stress with drought (Close *et al.*, 2005).

To recovery injury, cell wall modification and secondary metabolites genes upregulated to recovery wounded tissue and defense to transplant stress including lignin, cellulose, flavonoid and phenylpropanoid biosynthetic genes (Table S9). Cell walls were stiffened by ROS in stress condition and it cause polymer cleavage (Tenhaken, 2015). Therefore, cell wall modification and loosening is important for defense of pathogens and abiotic stress tolerances (Carpita and Gibeaut, 1993; Reiter, 2002; Sasidharan *et al.*, 2011; Le Gall *et al.*, 2015; Tenhaken, 2015). Phenylpropanoid compounds included lignin, flavonoids and phenolic molecules associated with recovery of wounded tissue and defense processes. (Dixon and Paiva, 1995; Barku, 2019). In this study, main cause of transplant stress was wounding stress caused by root pruning and transplanted trees showed general mechanism response to stress such as cell wall modification and formation of secondary metabolites to defense aganist ROS.

3.4.2 Waterlogging hardening effect after transplanting

To identify the trascriptional response in third waterlogging experimental year, we compared transcriptome of W and C before transplanting but there were no categorized biological process both upregulated and downregulated genes in W compared to C. Similar to transcriptome data, in same site, stomatal conductance and photosynthetic rate decreased and midday leaf water potential increased in first and second year, while there were no differences between W and C in third year (Bhusal *et al.*, 2022). The total precipitation of third year was higher than first and second year, so additional irrigation was lower than other year (Bhusal *et al.*, 2022).

After three years waterlogging exposure, we compared TW and TC to identify the hardening effect in transplant stress in transcriptional level. In biological process on TW, oxylipins biosynthetic and metabolic processes were upregulated compared to TC (Fig 3.1A). Oxylipin and phenylpropanoid metabolic process contribute metabolic process in plant immunity (La Camera *et al.*, 2004). In *Arabidopsis thaliana*, waterlogging induced accumulation of oxylipins and increased oxylipins involved stress tolerance including increases of biomass, PSII activity and anthocyanin by regulated primary and secondary metabolism (Savchenko *et al.*, 2019).

The upregulated phenylpropanoid biosynthesis at TW related to tolerance of transplant stress by upregulation of genes related to flavonoid and cell wall compared to TC (Figs 3.1A, 3.3). To cell wall lignification, lignins are transported to cell wall and plasma membrane by only ABCG29 in ABC transporter (Alejandro *et al.*, 2012). In TW, the up-regulated GO term were corresponded with cell wall lignification process such as up-regulation of cell wall, ABCG29 in ABC transporter activity, PAL4 and UCT72B1 in phenlypropanoid biosynthesis (Figs 3.1A, 3.3). Overexpression of PAL enhanced lignin and stress tolerance, while downregulation of PAL increased susceptibility to virus infection due to decrease of SA (Huang *et al.*, 2010; Gho *et al.*, 2020; Pan *et al.*, 2021). UGT72 family play a role in the homeostasis of monolignols including lignin and flavonoids (Speeckaert

et al., 2022). UGT72B1 was crucial for normal cell wall lignification and expressed in young xylem tissue (Lin *et al.*, 2016). Among the up-regulated TFs at TW, MYB3, MYB5 and LBD1 were involved in secondary formation of cell wall and lignin biosynthesis (Fig 3.2) (Ajengui *et al.*, 2018; Chen *et al.*, 2021; Xiao *et al.*, 2021; Kim *et al.*, 2022). Besides, the degradation of leaf NSC at TW also is important for recovery of damaged tissue by carbon allocation into starch to cell wall and export to root (Table 3.2) (Thalmann *et al.*, 2016).

In the upregulated molecular function, quercetin 7-O-glucosyltransferase, quercetin 3-O-glucosyltransferase and UDP-glucosyltransferase involved in flavonoid biosynthesis (Fig 3.1A) (Yin *et al.*, 2017; Dong *et al.*, 2020; Xu *et al.*, 2021). Among them, overexpression of UDP-Glucosyltransferase increased shoot branching and survival in *Arabidopsis* (Tognetti *et al.*, 2010). In stress pathway, redox thioredoxin, peroxidase and glutathione-S-transferase were expressed highly by accumulation of flavonoid, but some genes related to antioxidant were downregulated ($\log_2$FC >7, Fig 3.3). These result indicated that phenlypropanoid pathway reprogrammed by more upregulating the lignin to resistance wounding (La Camera *et al.*, 2004).

JA and SA induced by phenylpropanoid pathway were involved in abiotic stress such as flooding stress, wounding and pathogen and defense system has been reported in many previous study (Kamal and Komatsu, 2016; Zhang *et al.*, 2020; Kim *et al.*, 2021; Raza *et al.*, 2021). In this study, genes related to JA, SA and phenylpropanoid were upregulated in TW than TC (Fig 3.3). Defense system induced by JA was activation of wound-inducible proteinase inhibitor and wounding signaling (Ryan, 2000; León *et al.*, 2001). JA pathway is essential for tolerance of necrotrophic microbes which damage to cells and get energy from dead cells (Brenya *et al.*, 2020). SA also play a crucial role for defense system as the main regulator of biotrophic microbes which derive energy from living cells (Thomma *et al.*, 2001). The upregulated MAPK at TW was activated by JA and SA and was involved in plant defense signaling response to wounding, infection and abiotic stresses (Fig 3.3) (Zhang and Klessig, 2001).

After transplanting, waterlogging hardening enhanced recovery ability by inducing cell wall formation and some antioxidants, while it could be negative effect to height and root collar diameter growth due to degradation of auxin (Fig 3.3, Table 3.1).

Chapter 4. Conclusion

The aim of this study was to identify the hardening effect of different water availiability on transplant stress tolerance in *P. densiflora*. This study focused on the physiological and transcriptional responses of different drought hardening intensity after transplanting (growing season and cold stress), and waterlogging hardening effect on transplant stress.

In second chapter, this study showed that moderate drought hardening improved stress tolerance ability of the plants after transplanting than non- and severe drought hardening, and upregulation of secondary metabolites involved in improvement of stress tolerance. Before transplanting, *P. densiflora* showed no reduction in growth and photosynthesis under droght stress, which was related to upregulation of genes related to drought tolerance. Secondary metabolites genes induced by phytohormone genes such as JA and ethylene under two drought condtions. However, TFs belonging to WRKY, NAC, MYC, and ERF family were highly upregulated under severe drought hardening than moderate drought hardening. Genes related to secondary metabolites, heat shock proteins, MAPKs, and phytohormones were upregulated, which related to the drought tolerance of severe drought hardened plants.

To identify the cross-stress response, we compared drought and transplant stress. Contrary to what was expected, drought and transplant stress had common nine pathways and wounding due to root cutting was main cause in transplant stress rather than water stress. Despite a few genes were commonly regulated between drought and transplant stress, after transplanting, phenlypropanoid and terpenoid metabolism, secondary metabolites and cell wall organization genes were upregulated under moderate drought hardening after transplanting, which mediate wounding and biotic stress caused by root cutting in transplant procedure. In physiological study, leaf water potential was higher in moderate drought hardening after transplanting, which showed moderate drought hardening conserved water

and increased stomatal conductance (non-significant) to promote the photosynthesis.

In cold stress after transplanting, moderate drought hardening remarkably improved cold stress tolerance compared to non- and severe drought hardened seedlings. Compared to growing season, many genes were commonly expressed between drought and cold stress. Upregulation of secondary metabolites, cryoprotectant (proline and NSC), and phytohormone genes were highly upregulated under moderate drought hardening. With upregulation of proline biosynthesis, leaf proline content of moderate drought hardening was also higher than non-hardened plants by inhibition of ice formation. As an upregulation of defese response in growing and cold season, moderate drought hardening improved root collar diameter growth by promotion of photosynthetis rate after transplanting.

In third chapter, this study revealed that waterlogging hardening can improve plant's ability to withstand transplant stress. After transplanting, genes participating in oxylipin and secondary metabolites biosynthesis were upregulated under waterlogging hardening, which led to repair damaged root and resist bacterium and fungi infection. On the other hand, waterlogging hardening could negatively affect the height and root collar growth due to downregulation of auxin and ethylene.

In summary, cross-stress hardening was successful on transplant stress tolerance in moderate drought hardening. Moderate drought and waterlogging hardening improve the cell wall organization and defense related to biotic infection caused by wounded tissues after transplanting procedure. Moderate drought hardening showed greater stress tolerance to cold stress than transplant stress under growing season because drought stress has more common stress signaling pathways in cold stress than transplant stress. With upregulation of defense response, moderate drought hardening led to enhancement of root collar growth and photosynthesis, while waterlogging hardening showed negative effect on growth.

In addition to this, there is an uncertainty that hardening effect contributes to improve the productivity. This study investigated limited species for two year after

transplanting. The physiological factors measured in our study could not fully explain growth response by hardening effect. Therefore, further studies on various species are needed to successful adaptation after transplanting.

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부 록

Table S1 Primer suquences used to validate RNA-Sequencing results of trees grown moderate drought versus control after transplanting in growing season and winter

	Gene	Forward primer	Reverse primer
Transplant stress in growing season	TRINITY_DN232563_c2_g1_i14	CGTTCCCGGTAACCTTTCGA	GCTGCAAATGCGAAAGGGAA
	TRINITY_DN240243_c2_g1_i4	GCTCAGAGCGGCTTTCAAAC	CTTTCGGTCCACTGGAGTCC
	TRINITY_DN240187_c5_g6_i2	CGAAGTTATTTGTGCCCCGC	CCCAGGAACAGATTGTCCCC
	TRINITY_DN232612_c5_g2_i8	ATCCTGCACTAATCGCGCTG	GGTGGCACACGCTCAGAATA
	TRINITY_DN231459_c2_g3_i6	CTGTCAGGTCGTTGAGTCCC	GGTTTGGAACGACAAAACCGTC
	TRINITY_DN241898_c1_g1_i5	CGACAGACCTTGCACCTAGG	CACCCCTGGATGTGGCTATG
	TRINITY_DN237318_c0_g2_i1	GGTCCAAATTTGCCCAGCG	GATGCGTTGAGGGTTTCAAGGT
	TRINITY_DN235169_c1_g1_i5	CACTGCAGCCTCTGGTCTAC	GTTGAGGGTTTGTGGTGGGA
	TRINITY_DN217023_c4_g4_i2	GCAGAATCCCCATGGTGAGG	GGGCTTACTGTTGTTGAGGCA
	TRINITY_DN240539_c2_g1_i3	GAGCTCAGCAAGACGGAGAAA	GCATTGTCTGGCCACACAAC
	TRINITY_DN246179_c2_g1_i7	CACTGCAGCCTCTGGTCTAC	GTTGAGGGTTTGTGGTGGGA
	TRINITY_DN246179_c2_g1_i7	CACTGCAGCCTCTGGTCTAC	GTTGAGGGTTTGTGGTGGGA
	AT1G70500	GTGTCCCAACCGCACTTGTG	CGGACTGTCACCAGGAGCAA
	PER52	GATTTGTCCGCTGGTGCCAG	AGCAGCTGTTTAATGGCGGC
	UGT72B1	GAGCAGAACAGTCGCACAGC	CGCTGGTTAATTCGTCGGCC
	PAL4	ACGCGTTGTCAAAGCCCAAG	AGGTGTCCCCTGGAAGTTGC
	F-box	TATTATTGTTGCAGGTGGGTT	AGAATGTTGAAGTTCGGCTAT
Cold stress in winter season	TRINITY_DN200892_c0_g1_i3	CCGAGACACGCTCAGCTATG	TCCAACGTGAGAATGGGTTG
	TRINITY_DN191765_c1_g2_i4	TCCAACTCGATCAGGCGACC	CGGCAATTGGAACCTGGGAG
	TRINITY_DN186114_c0_g2_i6	GGGAATGAGGATGCTTGTGGG	CCCACTTAGGCCAAGAACGGA
	TRINITY_DN182041_c0_g1_i2	GCAGTCGGAAAAGCGGCAAA	GAGAGTGAGCCGCAAAGCAC
	TRINITY_DN205381_c2_g1_i4	GAGGGGCAGTTGCAACCTTG	CCCAATTCTCACGACCAACTGC
	TRINITY_DN199128_c0_g3_i1	TGCTCGTATAGTGGCTGCCC	TACTCGTCTTGGCCAGCGAG
	TRINITY_DN199107_c1_g1_i2	GACCGGGTAGCTGTTTCCAG	GCTCTGAGATGCAGCCATTG
	TRINITY_DN195324_c0_g1_i5	CATGTACGCAACGCCATTCT	CTCCAACATGCTGCTTGTCC
	TRINITY_DN202131_c1_g2_i13	ACGCGTGGATTCAAGTTTCC	CCTGGGATTGGGAGAGACAC
	TRINITY_DN199960_c6_g1_i5	GCCCTTGGCTGCATCTTCAG	CCTGGCACTGTTTCTGACGG
	UGT72B1	GAGCAGAACAGTCGCACAGC	CGCTGGTTAATTCGTCGGCC
	PER52	GATTTGTCCGCTGGTGCCAG	AGCAGCTGTTTAATGGCGGC
	AT1G70500	GTGTCCCAACCGCACTTGTG	CGGACTGTCACCAGGAGCAA
	PAL4	ACGCGTTGTCAAAGCCCAAG	AGGTGTCCCCTGGAAGTTGC
	TPS02	ACTGAGGCTGTGAGAAGATGGA	TTCACGCCCCTGTTCCTTCT
	MYB3	GATGGGTCGTGCTCCATGCT	CGCAGTCTGCAGCTCTTTCC
	ALDH2C4	GATTGTCATGGTGGCCGCTG	CGTGTGGGCAAGATTCACGG
	LDOX	GCAAATGGGTCACCGCCAAA	GGCCGCTCTTGAATTTCCCG
	CAD9	GCCATGTGTCCCAAACCACC	CCCAGAGAACCTGCCCTTTGA
	DTX41	TGGGATTTGGCCTTCTGGGG	GCCAGTCCAAGTGTGTCTGC
	Actin	TGCTCCCAGTAGCATGAAAA	GGTCTTGGCAATCCACATCT

Table S2 Gene ontology (GO) analysis of commonly regulated genes in drought stress

	GO biological process complete	Gene	FDR	Fold enrichment (%)
Up	cellular process (GO:0009987)	107	1.42E-05	65.2
	response to stimulus (GO:0050896)	82	1.53E-13	50.0
	biological regulation (GO:0065007)	70	6.82E-07	42.7
	regulation of biological process (GO:0050789)	69	2.68E-08	42.1
	regulation of cellular process (GO:0050794)	62	1.35E-07	37.8
	organonitrogen compound metabolic process (GO:1901564)	53	2.35E-04	32.3
	response to stress (GO:0006950)	49	1.02E-07	29.9
	cellular macromolecule metabolic process (GO:0044260)	49	1.99E-03	29.9
	cellular response to stimulus (GO:0051716)	48	7.02E-10	29.3
	cellular protein metabolic process (GO:0044267)	45	4.07E-05	27.4
	protein metabolic process (GO:0019538)	45	1.30E-04	27.4
	protein modification process (GO:0036211)	44	1.40E-08	26.8
	cellular protein modification process (GO:0006464)	44	1.32E-08	26.8
	macromolecule modification (GO:0043412)	44	4.01E-07	26.8
	cell communication (GO:0007154)	41	2.74E-11	25.0
	response to chemical (GO:0042221)	41	8.88E-07	25.0
	signal transduction (GO:0007165)	38	1.57E-11	23.2
	signaling (GO:0023052)	38	2.86E-11	23.2
	phosphate-containing compound metabolic process (GO:0006796)	38	1.93E-09	23.2
	phosphorus metabolic process (GO:0006793)	38	3.77E-09	23.2
	response to external stimulus (GO:0009605)	36	9.60E-10	22.0
	phosphorylation (GO:0016310)	35	2.44E-12	21.3
	response to organic substance (GO:0010033)	35	1.91E-08	21.3
	protein phosphorylation (GO:0006468)	34	9.32E-13	20.7
	response to external biotic stimulus (GO:0043207)	33	2.83E-11	20.1
	response to other organism (GO:0051707)	33	2.47E-11	20.1
	response to biotic stimulus (GO:0009607)	33	2.31E-11	20.1
	biological process involved in interspecies interaction between organisms (GO:0044419)	33	2.72E-11	20.1
	developmental process (GO:0032502)	33	3.98E-02	20.1
	anatomical structure development (GO:0048856)	32	4.29E-02	19.5
	defense response (GO:0006952)	30	3.59E-10	18.3
	cellular response to chemical stimulus (GO:0070887)	27	2.37E-07	16.5
	response to oxygen-containing compound (GO:1901700)	27	2.25E-05	16.5
	response to abiotic stimulus (GO:0009628)	27	1.32E-02	16.5
	response to endogenous stimulus (GO:0009719)	26	1.00E-05	15.9
	defense response to other organism (GO:0098542)	24	3.60E-08	14.6
	response to hormone (GO:0009725)	24	7.30E-05	14.6
	cellular response to organic substance (GO:0071310)	22	9.04E-08	13.4
	cellular response to endogenous stimulus (GO:0071495)	19	3.27E-07	11.6
	cellular response to hormone stimulus (GO:0032870)	17	4.34E-06	10.4
	hormone-mediated signaling pathway (GO:0009755)	16	3.94E-06	9.8
	response to bacterium (GO:0009617)	16	1.15E-05	9.8
	cellular response to oxygen-containing compound (GO:1901701)	16	2.13E-05	9.8
	response to lipid (GO:0033993)	16	3.15E-03	9.8
	regulation of developmental process (GO:0050793)	16	3.98E-03	9.8
	plant organ development (GO:0099402)	16	4.00E-02	9.8
	tissue development (GO:0009888)	15	1.55E-03	9.1
	response to wounding (GO:0009611)	12	7.58E-06	7.3
	defense response to bacterium (GO:0042742)	12	4.69E-04	7.3
	ion transport (GO:0006811)	11	3.97E-02	6.7
	innate immune response (GO:0045087)	10	1.29E-05	6.1
	immune response (GO:0006955)	10	1.78E-05	6.1
	immune system process (GO:0002376)	10	6.31E-05	6.1
	cellular response to lipid (GO:0071396)	10	3.56E-03	6.1
	response to fungus (GO:0009620)	10	7.71E-03	6.1
	regulation of response to stress (GO:0080134)	10	1.50E-02	6.1
	plant organ morphogenesis (GO:1905392)	10	2.75E-02	6.1
	regulation of defense response (GO:0031347)	9	5.23E-03	5.5
	response to organic cyclic compound (GO:0014070)	9	7.60E-03	5.5
	regulation of growth (GO:0040008)	8	1.48E-02	4.9
	response to hypoxia (GO:0001666)	8	1.72E-02	4.9
	response to decreased oxygen levels (GO:0036293)	8	1.83E-02	4.9

	response to oxygen levels (GO:0070482)	8	1.88E-02	4.9
	response to jasmonic acid (GO:0009753)	7	1.09E-02	4.3
	response to fatty acid (GO:0070542)	7	1.21E-02	4.3
	regulation of developmental growth (GO:0048638)	7	1.32E-02	4.3
	anion transport (GO:0006820)	7	3.55E-02	4.3
	cation homeostasis (GO:0055080)	7	4.89E-02	4.3
	meristem development (GO:0048507)	7	4.89E-02	4.3
	regulation of root development (GO:2000280)	6	1.27E-03	3.7
	response to oomycetes (GO:0002239)	6	4.06E-03	3.7
	phloem or xylem histogenesis (GO:0010087)	6	1.38E-02	3.7
	cellular response to organic cyclic compound (GO:0071407)	6	1.49E-02	3.7
	export from cell (GO:0140352)	6	1.95E-02	3.7
	regulation of anatomical structure morphogenesis (GO:0022603)	6	4.50E-02	3.7
	meristem maintenance (GO:0010073)	6	4.82E-02	3.7
	exocytosis (GO:0006887)	5	3.53E-03	3.0
	regulation of meristem growth (GO:0010075)	5	6.76E-03	3.0
	pollen-pistil interaction (GO:0009875)	5	8.37E-03	3.0
	secretion by cell (GO:0032940)	5	1.04E-02	3.0
	defense response to oomycetes (GO:0002229)	5	1.09E-02	3.0
	monovalent inorganic cation homeostasis (GO:0055067)	5	1.34E-02	3.0
	programmed cell death (GO:0012501)	5	1.39E-02	3.0
	secretion (GO:0046903)	5	1.45E-02	3.0
	positive regulation of response to biotic stimulus (GO:0002833)	5	1.80E-02	3.0
	positive regulation of response to external stimulus (GO:0032103)	5	1.97E-02	3.0
	positive regulation of defense response (GO:0031349)	5	2.51E-02	3.0
	cell death (GO:0008219)	5	3.11E-02	3.0
	regulation of root meristem growth (GO:0010082)	4	1.05E-02	2.4
	cellular response to organonitrogen compound (GO:0071417)	4	1.18E-02	2.4
	regulation of ion transport (GO:0043269)	4	1.33E-02	2.4
	activation of innate immune response (GO:0002218)	4	1.63E-02	2.4
	activation of immune response (GO:0002253)	4	1.70E-02	2.4
	plant-type hypersensitive response (GO:0009626)	4	1.67E-02	2.4
	programmed cell death induced by symbiont (GO:0034050)	4	1.76E-02	2.4
	biological process involved in interaction with symbiont (GO:0051702)	4	1.83E-02	2.4
	axis specification (GO:0009798)	4	2.47E-02	2.4
	positive regulation of innate immune response (GO:0045089)	4	2.58E-02	2.4
	diterpenoid biosynthetic process (GO:0016102)	4	2.55E-02	2.4
	positive regulation of immune response (GO:0050778)	4	2.70E-02	2.4
	positive regulation of immune system process (GO:0002684)	4	2.67E-02	2.4
	ethylene-activated signaling pathway (GO:0009873)	4	3.16E-02	2.4
	cellular response to nitrogen compound (GO:1901699)	4	3.13E-02	2.4
	diterpenoid metabolic process (GO:0016101)	4	3.35E-02	2.4
	cellular response to ethylene stimulus (GO:0071369)	4	4.33E-02	2.4
	regulation of anion transport (GO:0044070)	3	1.22E-02	1.8
	potassium ion homeostasis (GO:0055075)	3	1.96E-02	1.8
	inflorescence development (GO:0010229)	3	3.82E-02	1.8
	monocarboxylic acid transport (GO:0015718)	3	4.00E-02	1.8
	water transport (GO:0006833)	3	4.90E-02	1.8
	fluid transport (GO:0042044)	3	4.86E-02	1.8
	glucose-6-phosphate transport (GO:0015760)	2	1.63E-02	1.2
	hexose phosphate transport (GO:0015712)	2	1.60E-02	1.2
	triose phosphate transmembrane transport (GO:0035436)	2	3.09E-02	1.2
	triose phosphate transport (GO:0015717)	2	3.06E-02	1.2
	phosphoenolpyruvate transport (GO:0015714)	2	3.03E-02	1.2
	detection of bacterium (GO:0016045)	2	3.96E-02	1.2
	inflorescence morphogenesis (GO:0048281)	2	3.92E-02	1.2
	aldonate transmembrane transport (GO:0042873)	2	4.93E-02	1.2
	phosphoglycerate transmembrane transport (GO:0015713)	2	4.89E-02	1.2
	detection of other organism (GO:0098543)	2	4.85E-02	1.2
	transpiration (GO:0010148)	2	4.81E-02	1.2
Down	cellular process (GO:0009987)	57	4.03E-03	69.5
	metabolic process (GO:0008152)	47	9.22E-04	57.3
	cellular metabolic process (GO:0044237)	45	4.48E-04	54.9
	response to stimulus (GO:0050896)	34	6.93E-03	41.5
	response to stress (GO:0006950)	23	1.61E-02	28.0
	phosphate-containing compound metabolic process (GO:0006796)	17	7.68E-03	20.7
	phosphorus metabolic process (GO:0006793)	17	9.08E-03	20.7
	generation of precursor metabolites and energy (GO:0006091)	12	4.81E-06	14.6
	photosynthesis (GO:0015979)	11	1.49E-07	13.4

response to light stimulus (GO:0009416)	11	7.35E-03	13.4
response to radiation (GO:0009314)	11	8.11E-03	13.4
photosynthesis, light reaction (GO:0019684)	9	1.05E-06	11.0
photosynthesis, light harvesting in photosystem I (GO:0009768)	5	3.45E-05	6.1
photosynthesis, light harvesting (GO:0009765)	5	3.80E-04	6.1
response to light intensity (GO:0009642)	5	2.56E-02	6.1
response to high light intensity (GO:0009644)	4	1.70E-02	4.9
water homeostasis (GO:0030104)	3	7.65E-03	3.7
response to low light intensity stimulus (GO:0009645)	3	1.49E-02	3.7
response to desiccation (GO:0009269)	3	2.33E-02	3.7

The GO biological process (GOPB) terms associated with Fisher's exact test with FDR corrected P-value < 0.05.

Table S3 Gene ontology (GO) analysis of regulated genes in cold stress according to drought hardening intensity

	GO biological process complete	Gene Number	FDR	Fold enrichment (%)
WM vs WC Up	cellular process (GO:0009987)	801	8.64E-20	70.0
	metabolic process (GO:0008152)	617	8.86E-20	53.9
	response to stimulus (GO:0050896)	615	1.13E-36	53.8
	organic substance metabolic process (GO:0071704)	561	5.29E-17	49.0
	cellular metabolic process (GO:0044237)	515	2.91E-10	45.0
	primary metabolic process (GO:0044238)	418	4.92E-05	36.5
	response to stress (GO:0006950)	415	6.64E-36	36.3
	biological regulation (GO:0065007)	412	1.79E-03	36.0
	response to chemical (GO:0042221)	377	2.69E-26	33.0
	regulation of biological process (GO:0050789)	371	1.60E-02	32.4
	developmental process (GO:0032502)	314	1.14E-04	27.4
	organonitrogen compound metabolic process (GO:1901564)	305	2.25E-06	26.7
	anatomical structure development (GO:0048856)	294	4.48E-05	25.7
	response to external stimulus (GO:0009605)	286	4.78E-36	25.0
	response to organic substance (GO:0010033)	275	1.62E-20	24.0
	multicellular organismal process (GO:0032501)	261	1.61E-04	22.8
	response to abiotic stimulus (GO:0009628)	260	4.13E-09	22.7
	response to external biotic stimulus (GO:0043207)	257	8.86E-36	22.5
	response to other organism (GO:0051707)	257	5.91E-36	22.5
	response to biotic stimulus (GO:0009607)	257	4.88E-36	22.5
	biological process involved in interspecies interaction between organisms (GO:0044419)	257	8.93E-36	22.5
	response to oxygen-containing compound (GO:1901700)	257	2.01E-22	22.5
	cellular response to stimulus (GO:0051716)	251	1.05E-09	21.9
	multicellular organism development (GO:0007275)	241	5.56E-04	21.1
	defense response (GO:0006952)	240	3.61E-29	21.0
	small molecule metabolic process (GO:0044281)	233	1.07E-18	20.4
	biosynthetic process (GO:0009058)	209	3.72E-07	18.3
	defense response to other organism (GO:0098542)	208	4.03E-25	18.2
	system development (GO:0048731)	204	2.59E-03	17.8
	cell communication (GO:0007154)	191	3.03E-09	16.7
	organic substance biosynthetic process (GO:1901576)	188	3.67E-05	16.4
	response to endogenous stimulus (GO:0009719)	187	6.26E-11	16.3
	response to hormone (GO:0009725)	180	1.41E-09	15.7
	cellular biosynthetic process (GO:0044249)	178	1.11E-05	15.6
	signaling (GO:0023052)	177	5.00E-09	15.5
	organic acid metabolic process (GO:0006082)	175	8.22E-14	15.3
	signal transduction (GO:0007165)	173	8.21E-09	15.1
	cellular response to chemical stimulus (GO:0070887)	170	1.22E-13	14.9
	macromolecule modification (GO:0043412)	164	1.87E-02	14.3
	oxoacid metabolic process (GO:0043436)	163	9.80E-13	14.2
	response to lipid (GO:0033993)	161	4.37E-13	14.1
	protein modification process (GO:0036211)	161	1.68E-04	14.1
	cellular protein modification process (GO:0006464)	161	1.67E-04	14.1
	phosphorus metabolic process (GO:0006793)	147	1.08E-10	12.8
	localization (GO:0051179)	147	1.39E-02	12.8
	phosphate-containing compound metabolic process (GO:0006796)	141	7.60E-10	12.3
	response to inorganic substance (GO:0010035)	140	7.31E-05	12.2
	catabolic process (GO:0009056)	140	7.22E-05	12.2
	response to bacterium (GO:0009617)	137	1.41E-19	12.0
	carboxylic acid metabolic process (GO:0019752)	130	1.76E-10	11.4
	transport (GO:0006810)	130	5.69E-03	11.4
	establishment of localization (GO:0051234)	130	1.28E-02	11.4
	cellular response to organic substance (GO:0071310)	124	1.34E-08	10.8
	tissue development (GO:0009888)	119	2.64E-04	10.4
	plant organ development (GO:0099402)	118	7.47E-03	10.3
	organic substance catabolic process (GO:1901575)	117	1.05E-03	10.2
	response to fungus (GO:0009620)	114	4.14E-17	10.0
	anatomical structure morphogenesis (GO:0009653)	112	2.24E-05	9.8
	response to wounding (GO:0009611)	111	1.08E-21	9.7
	defense response to bacterium (GO:0042742)	111	1.17E-14	9.7
	regulation of response to stimulus (GO:0048583)	111	3.62E-09	9.7
	phosphorylation (GO:0016310)	108	2.16E-11	9.4

cellular response to oxygen-containing compound (GO:1901701)	107	7.13E-11	9.4
cellular catabolic process (GO:0044248)	107	7.25E-03	9.4
protein phosphorylation (GO:0006468)	103	4.90E-12	9.0
regulation of macromolecule metabolic process (GO:0060255)	95	6.47E-03	8.3
response to organic cyclic compound (GO:0014070)	92	1.23E-13	8.0
response to alcohol (GO:0097305)	92	1.12E-05	8.0
cellular response to endogenous stimulus (GO:0071495)	91	1.53E-05	8.0
lipid metabolic process (GO:0006629)	91	1.65E-04	8.0
cellular nitrogen compound metabolic process (GO:0034641)	90	2.30E-03	7.9
secondary metabolic process (GO:0019748)	89	8.63E-12	7.8
small molecule biosynthetic process (GO:0044283)	89	4.37E-10	7.8
regulation of response to stress (GO:0080134)	88	2.40E-10	7.7
response to acid chemical (GO:0001101)	87	1.45E-05	7.6
regulation of gene expression (GO:0010468)	85	4.58E-02	7.4
carbohydrate metabolic process (GO:0005975)	84	9.54E-06	7.3
cellular response to hormone stimulus (GO:0032870)	84	2.28E-04	7.3
regulation of defense response (GO:0031347)	81	5.42E-11	7.1
response to water (GO:0009415)	81	1.76E-04	7.1
shoot system development (GO:0048367)	80	3.18E-02	7.0
cell wall organization or biogenesis (GO:0071554)	79	6.78E-06	6.9
response to abscisic acid (GO:0009737)	78	4.17E-04	6.8
regulation of biological quality (GO:0065008)	78	4.54E-03	6.8
defense response to fungus (GO:0050832)	77	1.47E-10	6.7
response to water deprivation (GO:0009414)	75	3.26E-04	6.6
organic acid biosynthetic process (GO:0016053)	72	7.68E-09	6.3
transmembrane transport (GO:0055085)	72	1.34E-06	6.3
response to nitrogen compound (GO:1901698)	72	2.41E-06	6.3
response to osmotic stress (GO:0006970)	70	1.53E-04	6.1
hormone-mediated signaling pathway (GO:0009755)	68	1.25E-03	5.9
response to organonitrogen compound (GO:0010243)	66	2.21E-07	5.8
organic cyclic compound biosynthetic process (GO:1901362)	65	7.78E-03	5.7
carboxylic acid biosynthetic process (GO:0046394)	64	1.40E-08	5.6
response to jasmonic acid (GO:0009753)	62	2.41E-07	5.4
response to fatty acid (GO:0070542)	62	2.87E-07	5.4
plant organ morphogenesis (GO:1905392)	62	1.05E-02	5.4
aromatic compound biosynthetic process (GO:0019438)	61	3.73E-03	5.3
response to oxidative stress (GO:0006979)	60	1.02E-06	5.2
monocarboxylic acid metabolic process (GO:0032787)	59	2.71E-05	5.2
cellular amino acid metabolic process (GO:0006520)	57	1.25E-06	5.0
response to temperature stimulus (GO:0009266)	57	1.47E-02	5.0
response to salicylic acid (GO:0009751)	52	3.37E-08	4.5
ion transport (GO:0006811)	50	1.75E-04	4.4
sulfur compound metabolic process (GO:0006790)	48	1.62E-03	4.2
root morphogenesis (GO:0010015)	47	3.76E-02	4.1
immune system process (GO:0002376)	46	3.52E-05	4.0
response to salt stress (GO:0009651)	46	1.28E-02	4.0
nucleobase-containing compound metabolic process (GO:0006139)	46	9.39E-06	4.0
alpha-amino acid metabolic process (GO:1901605)	44	3.78E-07	3.8
chemical homeostasis (GO:0048878)	42	1.34E-02	3.7
organic hydroxy compound metabolic process (GO:1901615)	41	1.96E-05	3.6
plant-type cell wall organization or biogenesis (GO:0071669)	41	6.93E-03	3.6
polysaccharide metabolic process (GO:0005976)	38	1.95E-02	3.3
regulation of cell communication (GO:0010646)	36	1.17E-02	3.1
cell wall biogenesis (GO:0042546)	36	1.26E-02	3.1
response to hypoxia (GO:0001666)	35	1.13E-04	3.1
response to decreased oxygen levels (GO:0036293)	35	1.61E-04	3.1
response to oxygen levels (GO:0070482)	35	1.70E-04	3.1
regulation of signaling (GO:0023051)	35	1.45E-02	3.1
response to chitin (GO:0010200)	34	1.76E-04	3.0
regulation of signal transduction (GO:0009966)	34	1.83E-02	3.0
organelle organization (GO:0006996)	34	1.37E-06	3.0
cellular response to organic cyclic compound (GO:0071407)	32	3.28E-05	2.8
regulation of hormone levels (GO:0010817)	32	3.65E-04	2.8
phenylpropanoid metabolic process (GO:0009698)	30	3.42E-11	2.6
external encapsulating structure organization (GO:0045229)	30	1.83E-02	2.6
secondary metabolite biosynthetic process (GO:0044550)	29	7.79E-07	2.5
small molecule catabolic process (GO:0044282)	29	9.39E-05	2.5
cation transport (GO:0006812)	29	4.82E-03	2.5
cellular response to hypoxia (GO:0071456)	28	1.92E-04	2.4
cellular response to decreased oxygen levels (GO:0036294)	28	2.20E-04	2.4

cellular response to oxygen levels (GO:0071453)	28	2.29E-04	2.4
ion transmembrane transport (GO:0034220)	28	1.23E-02	2.4
response to ethylene (GO:0009723)	25	4.45E-03	2.2
monocarboxylic acid biosynthetic process (GO:0072330)	25	7.13E-03	2.2
phenylpropanoid biosynthetic process (GO:0009699)	24	5.29E-09	2.1
isoprenoid metabolic process (GO:0006720)	23	4.81E-02	2.0
response to toxic substance (GO:0009636)	22	9.58E-04	1.9
carbohydrate catabolic process (GO:0016052)	22	1.74E-02	1.9
negative regulation of response to stimulus (GO:0048585)	22	4.25E-02	1.9
flavonoid metabolic process (GO:0009812)	21	1.06E-04	1.8
organic acid catabolic process (GO:0016054)	21	9.88E-04	1.8
alpha-amino acid biosynthetic process (GO:1901607)	21	1.51E-03	1.8
innate immune response (GO:0045087)	21	2.11E-03	1.8
cellular amino acid biosynthetic process (GO:0008652)	21	4.36E-03	1.8
immune response (GO:0006955)	21	7.23E-03	1.8
nucleic acid metabolic process (GO:0090304)	21	1.23E-11	1.8
carboxylic acid catabolic process (GO:0046395)	20	1.36E-03	1.7
isoprenoid biosynthetic process (GO:0008299)	20	6.41E-03	1.7
metal ion transport (GO:0030001)	20	2.17E-02	1.7
detoxification (GO:0098754)	18	7.26E-03	1.6
gene expression (GO:0010467)	18	3.15E-11	1.6
lipid modification (GO:0030258)	17	6.20E-05	1.5
cellular cation homeostasis (GO:0030003)	17	4.69E-02	1.5
RNA metabolic process (GO:0016070)	17	1.00E-07	1.5
jasmonic acid metabolic process (GO:0009694)	16	3.94E-07	1.4
auxin transport (GO:0060918)	16	6.17E-04	1.4
hormone transport (GO:0009914)	16	8.54E-04	1.4
terpenoid biosynthetic process (GO:0016114)	16	1.47E-02	1.4
negative regulation of macromolecule metabolic process (GO:0010605)	16	4.53E-02	1.4
flavonoid biosynthetic process (GO:0009813)	15	9.07E-04	1.3
systemic acquired resistance (GO:0009627)	15	8.77E-03	1.3
response to molecule of bacterial origin (GO:0002237)	15	1.01E-02	1.3
establishment of localization in cell (GO:0051649)	15	3.97E-02	1.3
response to oomycetes (GO:0002239)	14	7.53E-03	1.2
phosphorelay signal transduction system (GO:0000160)	14	1.52E-02	1.2
toxin metabolic process (GO:0009404)	14	2.54E-02	1.2
lignin metabolic process (GO:0009808)	13	1.76E-04	1.1
benzene-containing compound metabolic process (GO:0042537)	13	2.30E-04	1.1
cellular response to ethylene stimulus (GO:0071369)	13	1.45E-02	1.1
organic anion transport (GO:0015711)	13	2.65E-02	1.1
jasmonic acid biosynthetic process (GO:0009695)	12	1.06E-06	1.0
phenol-containing compound metabolic process (GO:0018958)	12	1.76E-04	1.0
lipid oxidation (GO:0034440)	12	3.37E-04	1.0
olefinic compound metabolic process (GO:0120254)	12	3.93E-04	1.0
monocarboxylic acid catabolic process (GO:0072329)	12	1.78E-03	1.0
ethylene-activated signaling pathway (GO:0009873)	12	8.80E-03	1.0
hydrocarbon metabolic process (GO:0120252)	12	1.04E-02	1.0
seed maturation (GO:0010431)	12	1.91E-02	1.0
oxylipin biosynthetic process (GO:0031408)	11	4.48E-07	1.0
oxylipin metabolic process (GO:0031407)	11	1.01E-06	1.0
lignin biosynthetic process (GO:0009809)	11	4.78E-04	1.0
regulation of jasmonic acid mediated signaling pathway (GO:2000022)	11	4.74E-04	1.0
hydrocarbon biosynthetic process (GO:0120251)	11	8.61E-03	1.0
response to nematode (GO:0009624)	11	3.50E-02	1.0
olefinic compound biosynthetic process (GO:0120255)	10	9.57E-04	0.9
cellular response to nitrogen compound (GO:1901699)	10	2.25E-02	0.9
calcium ion transport (GO:0006816)	10	2.45E-02	0.9
auxin polar transport (GO:0009926)	10	3.48E-02	0.9
pollen-pistil interaction (GO:0009875)	10	3.47E-02	0.9
cellular response to organonitrogen compound (GO:0071417)	9	1.04E-02	0.8
regulation of auxin mediated signaling pathway (GO:0010928)	9	1.04E-02	0.8
cell recognition (GO:0008037)	9	1.62E-02	0.8
sulfur amino acid metabolic process (GO:0000096)	9	1.95E-02	0.8
diterpenoid biosynthetic process (GO:0016102)	9	2.44E-02	0.8
diterpenoid metabolic process (GO:0016101)	9	4.24E-02	0.8
cellular response to acid chemical (GO:0071229)	9	4.60E-02	0.8
cellular divalent inorganic cation homeostasis (GO:0072503)	9	4.58E-02	0.8
amide biosynthetic process (GO:0043604)	9	1.96E-02	0.8
L-phenylalanine metabolic process (GO:0006558)	8	2.00E-03	0.7

	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process (GO:1902221)	8	1.99E-03	0.7
	import across plasma membrane (GO:0098739)	8	7.11E-03	0.7
	import into cell (GO:0098657)	8	9.46E-03	0.7
	fatty acid oxidation (GO:0019395)	8	1.96E-02	0.7
	fatty acid catabolic process (GO:0009062)	8	2.72E-02	0.7
	amino acid transport (GO:0006865)	8	3.46E-02	0.7
	recognition of pollen (GO:0048544)	8	4.25E-02	0.7
	plant-type hypersensitive response (GO:0009626)	8	4.23E-02	0.7
	methionine metabolic process (GO:0006555)	7	9.67E-03	0.6
	salicylic acid metabolic process (GO:0009696)	7	2.61E-02	0.6
	fatty acid beta-oxidation (GO:0006635)	7	4.27E-02	0.6
	ncRNA metabolic process (GO:0034660)	7	1.14E-02	0.6
	RNA processing (GO:0006396)	7	1.29E-06	0.6
	regulation of defense response to insect (GO:2000068)	6	2.11E-03	0.5
	reactive nitrogen species metabolic process (GO:2001057)	6	3.98E-03	0.5
	sesquiterpenoid biosynthetic process (GO:0016106)	6	1.74E-02	0.5
	aminoglycan metabolic process (GO:0006022)	6	2.44E-02	0.5
	acetyl-CoA metabolic process (GO:0006084)	6	3.38E-02	0.5
	S-adenosylmethionine metabolic process (GO:0046500)	5	5.85E-03	0.4
	nitrate assimilation (GO:0042128)	5	1.05E-02	0.4
	nitrate metabolic process (GO:0042126)	5	1.04E-02	0.4
	L-phenylalanine biosynthetic process (GO:0009094)	5	1.71E-02	0.4
	erythrose 4-phosphate/phosphoenolpyruvate family amino acid biosynthetic process (GO:1902223)	5	1.70E-02	0.4
	aromatic amino acid family biosynthetic process, prephenate pathway (GO:0009095)	5	2.62E-02	0.4
	nitrogen cycle metabolic process (GO:0071941)	5	2.61E-02	0.4
	glutamine metabolic process (GO:0006541)	5	3.94E-02	0.4
	ncRNA processing (GO:0034470)	5	1.70E-02	0.4
	DNA metabolic process (GO:0006259)	5	2.40E-03	0.4
	translation (GO:0006412)	5	1.36E-03	0.4
	peptide biosynthetic process (GO:0043043)	5	1.36E-03	0.4
	negative regulation of defense response to insect (GO:1900366)	4	9.61E-03	0.3
	proanthocyanidin biosynthetic process (GO:0010023)	4	1.40E-02	0.3
	cellular response to DNA damage stimulus (GO:0006974)	4	4.48E-02	0.3
	ribosome biogenesis (GO:0042254)	4	4.53E-02	0.3
	ribonucleoprotein complex biogenesis (GO:0022613)	4	5.09E-03	0.3
	mRNA metabolic process (GO:0016071)	4	3.96E-03	0.3
	regulation of transcription by RNA polymerase II (GO:0006357)	4	4.32E-04	0.3
	defense response to nematode (GO:0002215)	3	4.83E-02	0.3
	S-adenosylmethionine biosynthetic process (GO:0006556)	3	4.81E-02	0.3
	DNA repair (GO:0006281)	3	4.01E-02	0.3
	RNA modification (GO:0009451)	2	2.44E-02	0.2
WS vs WC Up	regulation of protein stability (GO:0031647)	4	1.97E-02	4.1
	protein O-linked glycosylation via hydroxyproline (GO:0018258)	3	4.17E-02	3.1
	arabinogalactan protein metabolic process (GO:0010405)	3	3.59E-02	3.1
	cell wall hydroxyproline-rich glycoprotein metabolic process (GO:0010404)	3	3.04E-02	3.1
	cell wall proteoglycan metabolic process (GO:0010384)	3	2.28E-02	3.1
	proteoglycan metabolic process (GO:0006029)	3	1.90E-02	3.1
	protein O-linked glycosylation (GO:0006493)	3	1.99E-02	3.1
WM vs WC Down	cellular process (GO:0009987)	373	5.17E-10	71.6
	metabolic process (GO:0008152)	290	6.43E-10	55.7
	cellular metabolic process (GO:0044237)	263	5.92E-10	50.5
	organic substance metabolic process (GO:0071704)	263	1.32E-08	50.5
	response to stimulus (GO:0050896)	241	8.65E-06	46.3
	primary metabolic process (GO:0044238)	208	1.68E-04	39.9
	nitrogen compound metabolic process (GO:0006807)	185	2.32E-04	35.5
	response to chemical (GO:0042221)	144	2.21E-04	27.6
	response to stress (GO:0006950)	137	1.99E-02	26.3
	response to abiotic stimulus (GO:0009628)	130	5.96E-06	25.0
	organic cyclic compound metabolic process (GO:1901360)	104	6.41E-06	20.0
	cellular nitrogen compound metabolic process (GO:0034641)	102	7.17E-05	19.6
	cellular aromatic compound metabolic process (GO:0006725)	99	1.66E-05	19.0
	heterocycle metabolic process (GO:0046483)	95	2.75E-05	18.2
	biosynthetic process (GO:0009058)	94	1.69E-02	18.0
	response to oxygen-containing compound (GO:1901700)	89	1.86E-02	17.1

organic substance biosynthetic process (GO:1901576)	88	2.73E-02	16.9
response to radiation (GO:0009314)	86	3.11E-08	16.5
small molecule metabolic process (GO:0044281)	86	8.70E-03	16.5
response to light stimulus (GO:0009416)	85	3.67E-08	16.3
cellular biosynthetic process (GO:0044249)	81	3.45E-02	15.5
post-embryonic development (GO:0009791)	76	8.75E-04	14.6
developmental process involved in reproduction (GO:0003006)	70	1.65E-02	13.4
nucleobase-containing compound metabolic process (GO:0006139)	67	2.40E-02	12.9
organic acid metabolic process (GO:0006082)	65	3.38E-02	12.5
response to inorganic substance (GO:0010035)	65	4.84E-02	12.5
reproductive structure development (GO:0048608)	64	1.68E-02	12.3
reproductive system development (GO:0061458)	64	1.65E-02	12.3
nucleic acid metabolic process (GO:0090304)	58	2.51E-02	11.1
fruit development (GO:0010154)	47	1.74E-02	9.0
RNA metabolic process (GO:0016070)	46	3.04E-02	8.8
regulation of biological quality (GO:0065008)	45	4.00E-03	8.6
seed development (GO:0048316)	45	2.43E-02	8.6
response to temperature stimulus (GO:0009266)	36	2.17E-03	6.9
response to light intensity (GO:0009642)	27	1.42E-05	5.2
plastid organization (GO:0009657)	24	3.43E-05	4.6
RNA modification (GO:0009451)	23	1.71E-05	4.4
porphyrin-containing compound metabolic process (GO:0006778)	21	6.15E-06	4.0
tetrapyrrole metabolic process (GO:0033013)	21	6.52E-05	4.0
chloroplast organization (GO:0009658)	20	9.54E-05	3.8
response to heat (GO:0009408)	16	2.11E-02	3.1
pigment metabolic process (GO:0042440)	15	1.77E-02	2.9
isoprenoid metabolic process (GO:0006720)	15	3.16E-02	2.9
chlorophyll metabolic process (GO:0015994)	14	1.58E-04	2.7
terpenoid metabolic process (GO:0006721)	14	1.70E-02	2.7
porphyrin-containing compound biosynthetic process (GO:0006779)	9	8.57E-03	1.7
tetrapyrrole biosynthetic process (GO:0033014)	9	1.67E-02	1.7
response to virus (GO:0009615)	9	1.71E-02	1.7
cellular response to nitrogen compound (GO:1901699)	8	1.26E-02	1.5
regulation of tetrapyrrole metabolic process (GO:1901401)	7	4.85E-03	1.3
chlorophyll biosynthetic process (GO:0015995)	7	3.38E-02	1.3
regulation of chlorophyll metabolic process (GO:0090056)	6	8.49E-03	1.2
cellular response to organonitrogen compound (GO:0071417)	6	4.46E-02	1.2
cellular response to light intensity (GO:0071484)	4	2.38E-02	0.8

The GO biological process (GOPB) terms associated with Fisher's exact test with FDR corrected P-value < 0.05.

Table S4 Gene ontology (GO) analysis of commonly regulated genes in transplant stress

	GO biological process complete	Gene	FDR	Rich Factor (%)
Up	metabolic process (GO:0008152)	55	5.74E-03	50.9
	organic substance metabolic process (GO:0071704)	54	2.03E-03	50.0
	cellular metabolic process (GO:0044237)	52	4.01E-03	48.1
	cellular macromolecule metabolic process (GO:0044260)	35	6.87E-03	32.4
	organonitrogen compound metabolic process (GO:1901564)	33	4.89E-02	30.6
	protein metabolic process (GO:0019538)	29	1.98E-02	26.9
	protein modification process (GO:0036211)	28	2.54E-04	25.9
	cellular protein modification process (GO:0006464)	28	2.17E-04	25.9
	macromolecule modification (GO:0043412)	28	1.56E-03	25.9
	cellular protein metabolic process (GO:0044267)	28	1.70E-02	25.9
	phosphate-containing compound metabolic process (GO:0006796)	26	7.89E-06	24.1
	phosphorus metabolic process (GO:0006793)	26	9.92E-06	24.1
	phosphorylation (GO:0016310)	25	5.38E-09	23.1
	protein phosphorylation (GO:0006468)	24	7.43E-09	22.2
	response to organic substance (GO:0010033)	17	3.22E-02	15.7
	defense response (GO:0006952)	14	9.33E-03	13.0
	response to external biotic stimulus (GO:0043207)	13	5.08E-02	12.0
	response to other organism (GO:0051707)	13	4.98E-02	12.0
	response to biotic stimulus (GO:0009607)	13	4.79E-02	12.0
	tissue development (GO:0009888)	11	1.13E-02	10.2
	defense response to other organism (GO:0098542)	11	3.83E-02	10.2
	response to bacterium (GO:0009617)	9	2.23E-02	8.3
	cellular response to oxygen-containing compound (GO:1901701)	9	3.19E-02	8.3
	plant organ morphogenesis (GO:1905392)	8	4.81E-02	7.4
	phenylpropanoid metabolic process (GO:0009698)	7	3.01E-04	6.5
	response to wounding (GO:0009611)	7	1.02E-02	6.5
	secondary metabolic process (GO:0019748)	7	3.22E-02	6.5
	phenylpropanoid biosynthetic process (GO:0009699)	6	1.15E-03	5.6
	secondary metabolite biosynthetic process (GO:0044550)	6	7.01E-03	5.6
	cellular glucan metabolic process (GO:0006073)	6	2.26E-02	5.6
	glucan metabolic process (GO:0044042)	6	2.65E-02	5.6
	cell division (GO:0051301)	6	3.25E-02	5.6
	cellular carbohydrate biosynthetic process (GO:0034637)	6	3.27E-02	5.6
	plant-type primary cell wall biogenesis (GO:0009833)	5	2.17E-04	4.6
	mitotic cytokinesis (GO:0000281)	5	7.47E-04	4.6
	cellulose biosynthetic process (GO:0030244)	5	1.48E-03	4.6
	flavonoid biosynthetic process (GO:0009813)	5	1.77E-03	4.6
	cytoskeleton-dependent cytokinesis (GO:0061640)	5	1.78E-03	4.6
	cellulose metabolic process (GO:0030243)	5	1.80E-03	4.6
	beta-glucan biosynthetic process (GO:0051274)	5	2.70E-03	4.6
	flavonoid metabolic process (GO:0009812)	5	2.94E-03	4.6
	beta-glucan metabolic process (GO:0051273)	5	3.41E-03	4.6
	cytokinesis (GO:0000910)	5	1.26E-02	4.6
	glucan biosynthetic process (GO:0009250)	5	2.07E-02	4.6
	cellular detoxification (GO:1990748)	4	7.04E-03	3.7
	lignin biosynthetic process (GO:0009809)	4	6.79E-03	3.7
	cellular response to toxic substance (GO:0097237)	4	6.90E-03	3.7
	lignin metabolic process (GO:0009808)	4	1.24E-02	3.7
	regulation of root development (GO:2000280)	4	3.46E-02	3.7
	monovalent inorganic cation homeostasis (GO:0055067)	4	3.83E-02	3.7
	formaldehyde catabolic process (GO:0046294)	3	4.59E-03	2.8
	formaldehyde metabolic process (GO:0046292)	3	4.38E-03	2.8
	cellular detoxification of aldehyde (GO:0110095)	3	4.19E-03	2.8
	cellular response to aldehyde (GO:0110096)	3	5.10E-03	2.8
	aldehyde catabolic process (GO:0046185)	3	1.29E-02	2.8
	negative regulation of defense response to insect (GO:1900366)	2	3.81E-02	1.9
Down	cellular process (GO:0009987)	60	3.95E-02	63.2
	response to stimulus (GO:0050896)	35	3.34E-02	36.8
	response to chemical (GO:0042221)	21	4.44E-02	22.1
	response to organic substance (GO:0010033)	17	1.62E-02	17.9
	response to hormone (GO:0009725)	14	2.41E-02	14.7
	response to endogenous stimulus (GO:0009719)	14	2.66E-02	14.7
	plant organ morphogenesis (GO:1905392)	9	1.11E-02	9.5

secondary metabolic process (GO:0019748)	8	9.97E-03	8.4
phenylpropanoid metabolic process (GO:0009698)	7	3.34E-04	7.4
response to toxic substance (GO:0009636)	7	4.23E-03	7.4
isoprenoid metabolic process (GO:0006720)	7	6.35E-03	7.4
lignin biosynthetic process (GO:0009809)	6	9.29E-05	6.3
lignin metabolic process (GO:0009808)	6	1.65E-04	6.3
phenylpropanoid biosynthetic process (GO:0009699)	6	1.36E-03	6.3
secondary metabolite biosynthetic process (GO:0044550)	6	9.78E-03	6.3
detoxification (GO:0098754)	6	9.81E-03	6.3
terpenoid metabolic process (GO:0006721)	6	1.11E-02	6.3
isoprenoid biosynthetic process (GO:0008299)	6	1.07E-02	6.3
cellular glucan metabolic process (GO:0006073)	6	2.02E-02	6.3
glucan metabolic process (GO:0044042)	6	2.29E-02	6.3
terpenoid biosynthetic process (GO:0016114)	5	2.26E-02	5.3
plant-type primary cell wall biogenesis (GO:0009833)	4	4.38E-03	4.2
mitotic cytokinesis (GO:0000281)	4	1.15E-02	4.2
cellular detoxification (GO:1990748)	4	1.04E-02	4.2
cellular response to toxic substance (GO:0097237)	4	9.64E-03	4.2
cellulose biosynthetic process (GO:0030244)	4	1.48E-02	4.2
flavonoid biosynthetic process (GO:0009813)	4	1.63E-02	4.2
cytoskeleton-dependent cytokinesis (GO:0061640)	4	1.65E-02	4.2
cellulose metabolic process (GO:0030243)	4	1.68E-02	4.2
beta-glucan biosynthetic process (GO:0051274)	4	2.35E-02	4.2
flavonoid metabolic process (GO:0009812)	4	2.33E-02	4.2
beta-glucan metabolic process (GO:0051273)	4	2.55E-02	4.2
regulation of root development (GO:2000280)	4	3.28E-02	4.2
sesquiterpenoid metabolic process (GO:0006714)	3	4.31E-02	3.2
specification of axis polarity (GO:0065001)	3	4.18E-02	3.2
detection of bacterium (GO:0016045)	2	4.13E-02	2.1
strigolactone biosynthetic process (GO:1901601)	2	4.02E-02	2.1
strigolactone metabolic process (GO:1901600)	2	3.91E-02	2.1
detection of other organism (GO:0098543)	2	4.93E-02	2.1

The GO biological process (GOPB) terms associated with Fisher's exact test with FDR corrected P-value < 0.05.

Table S5 Gene ontology (GO) analysis of regulated genes in cold stress

	GO biological process complete	Gene Number	FDR	Fold enrichment (%)
Up	cellular process (GO:0009987)	1584	1.09E-47	71.9
	metabolic process (GO:0008152)	1188	1.25E-36	53.9
	organic substance metabolic process (GO:0071704)	1087	4.80E-33	49.3
	cellular metabolic process (GO:0044237)	1042	2.97E-28	47.3
	response to stimulus (GO:0050896)	988	1.15E-20	44.8
	primary metabolic process (GO:0044238)	898	8.03E-24	40.8
	biological regulation (GO:0065007)	807	7.32E-08	36.6
	nitrogen compound metabolic process (GO:0006807)	748	4.77E-14	34.0
	regulation of biological process (GO:0050789)	721	3.70E-05	32.7
	developmental process (GO:0032502)	615	6.25E-10	27.9
	macromolecule metabolic process (GO:0043170)	612	6.78E-09	27.8
	organonitrogen compound metabolic process (GO:1901564)	602	6.12E-14	27.3
	response to chemical (GO:0042221)	594	6.38E-17	27.0
	response to stress (GO:0006950)	588	3.49E-13	26.7
	anatomical structure development (GO:0048856)	566	2.17E-09	25.7
	regulation of cellular process (GO:0050794)	565	4.65E-02	25.6
	multicellular organismal process (GO:0032501)	508	4.25E-09	23.1
	response to abiotic stimulus (GO:0009628)	493	2.97E-15	22.4
	cellular macromolecule metabolic process (GO:0044260)	485	3.51E-09	22.0
	multicellular organism development (GO:0007275)	475	9.32E-09	21.6
	protein metabolic process (GO:0019538)	415	3.87E-08	18.8
	cellular response to stimulus (GO:0051716)	415	1.16E-06	18.8
	system development (GO:0048731)	403	2.40E-07	18.3
	cellular protein metabolic process (GO:0044267)	390	2.40E-07	17.7
	response to organic substance (GO:0010033)	385	8.43E-07	17.5
	biosynthetic process (GO:0009058)	384	7.36E-10	17.4
	organic substance biosynthetic process (GO:1901576)	367	3.65E-10	16.7
	response to oxygen-containing compound (GO:1901700)	367	3.60E-10	16.7
	cellular component organization or biogenesis (GO:0071840)	361	1.20E-03	16.4
	small molecule metabolic process (GO:0044281)	357	3.91E-12	16.2
	localization (GO:0051179)	354	6.52E-17	16.1
	cellular biosynthetic process (GO:0044249)	336	2.67E-09	15.3
	catabolic process (GO:0009056)	323	1.23E-20	14.7
	reproductive process (GO:0022414)	323	5.42E-10	14.7
	reproduction (GO:0000003)	323	1.56E-09	14.7
	cellular nitrogen compound metabolic process (GO:0034641)	322	1.91E-03	14.6
	macromolecule modification (GO:0043412)	315	2.32E-04	14.3
	organic cyclic compound metabolic process (GO:1901360)	310	2.47E-03	14.1
	cellular aromatic compound metabolic process (GO:0006725)	299	3.20E-03	13.6
	establishment of localization (GO:0051234)	297	1.62E-12	13.5
	post-embryonic development (GO:0009791)	297	6.45E-12	13.5
	response to external stimulus (GO:0009605)	297	7.97E-03	13.5
	developmental process involved in reproduction (GO:0003006)	293	1.65E-11	13.3
	transport (GO:0006810)	291	2.11E-12	13.2
	cellular component organization (GO:0016043)	285	5.27E-03	12.9
	protein modification process (GO:0036211)	284	1.22E-04	12.9
	cellular protein modification process (GO:0006464)	284	1.21E-04	12.9
	organic substance catabolic process (GO:1901575)	277	9.93E-18	12.6
	response to endogenous stimulus (GO:0009719)	275	2.06E-04	12.5
	response to hormone (GO:0009725)	271	2.50E-04	12.3
	organic acid metabolic process (GO:0006082)	270	1.26E-09	12.3
	cellular catabolic process (GO:0044248)	268	4.66E-18	12.2
	response to inorganic substance (GO:0010035)	266	2.55E-08	12.1
	reproductive structure development (GO:0048608)	264	7.19E-11	12.0
	reproductive system development (GO:0061458)	264	7.05E-11	12.0
	oxoacid metabolic process (GO:0043436)	255	2.01E-09	11.6
	response to radiation (GO:0009314)	246	2.17E-06	11.2
	response to light stimulus (GO:0009416)	238	7.66E-06	10.8
	cellular response to chemical stimulus (GO:0070887)	233	8.99E-05	10.6
	negative regulation of biological process (GO:0048519)	231	1.79E-07	10.5
	response to lipid (GO:0033993)	224	3.83E-05	10.2
	carboxylic acid metabolic process (GO:0019752)	220	1.90E-11	10.0
	cellular component biogenesis (GO:0044085)	202	1.60E-02	9.2

organelle organization (GO:0006996)	196	1.54E-02	8.9
gene expression (GO:0010467)	176	2.28E-02	8.0
positive regulation of biological process (GO:0048518)	175	2.22E-04	7.9
cellular response to organic substance (GO:0071310)	170	1.10E-02	7.7
fruit development (GO:0010154)	168	2.32E-05	7.6
lipid metabolic process (GO:0006629)	166	1.93E-06	7.5
shoot system development (GO:0048367)	166	5.63E-06	7.5
anatomical structure morphogenesis (GO:0009653)	166	4.00E-02	7.5
organonitrogen compound biosynthetic process (GO:1901566)	165	4.38E-04	7.5
seed development (GO:0048316)	163	1.94E-05	7.4
organonitrogen compound catabolic process (GO:1901565)	159	4.12E-09	7.2
regulation of developmental process (GO:0050793)	159	2.27E-05	7.2
regulation of response to stimulus (GO:0048583)	155	6.96E-04	7.0
cellular response to stress (GO:0033554)	153	1.53E-05	6.9
cellular localization (GO:0051641)	149	1.92E-06	6.8
response to alcohol (GO:0097305)	149	1.13E-04	6.8
carbohydrate metabolic process (GO:0005975)	147	3.81E-07	6.7
regulation of biological quality (GO:0065008)	147	5.11E-05	6.7
response to acid chemical (GO:0001101)	146	1.64E-05	6.6
response to water (GO:0009415)	142	2.01E-05	6.4
negative regulation of metabolic process (GO:0009892)	142	4.01E-04	6.4
response to osmotic stress (GO:0006970)	141	8.27E-10	6.4
macromolecule catabolic process (GO:0009057)	136	2.33E-05	6.2
cellular response to oxygen-containing compound (GO:1901701)	136	2.02E-03	6.2
macromolecule biosynthetic process (GO:0009059)	135	4.64E-02	6.1
carbohydrate derivative metabolic process (GO:1901135)	134	7.00E-05	6.1
positive regulation of cellular process (GO:0048522)	134	1.91E-02	6.1
response to water deprivation (GO:0009414)	133	3.07E-05	6.0
response to abscisic acid (GO:0009737)	133	3.95E-04	6.0
cellular response to endogenous stimulus (GO:0071495)	130	3.83E-02	5.9
small molecule biosynthetic process (GO:0044283)	129	1.01E-06	5.9
cellular lipid metabolic process (GO:0044255)	129	3.81E-05	5.9
macromolecule localization (GO:0033036)	129	2.94E-03	5.9
transmembrane transport (GO:0055085)	128	4.84E-09	5.8
proteolysis (GO:0006508)	127	5.29E-07	5.8
negative regulation of cellular process (GO:0048523)	127	2.74E-02	5.8
cellular response to hormone stimulus (GO:0032870)	126	4.53E-02	5.7
organic substance transport (GO:0071702)	124	1.18E-03	5.6
monocarboxylic acid metabolic process (GO:0032787)	113	1.64E-09	5.1
cellular macromolecule catabolic process (GO:0044265)	112	9.89E-06	5.1
response to temperature stimulus (GO:0009266)	110	1.54E-04	5.0
cellular macromolecule biosynthetic process (GO:0034645)	107	2.04E-02	4.9
protein localization (GO:0008104)	106	4.98E-04	4.8
embryo development (GO:0009790)	105	1.52E-04	4.8
organic cyclic compound biosynthetic process (GO:1901362)	105	4.88E-02	4.8
response to salt stress (GO:0009651)	104	1.17E-08	4.7
nitrogen compound transport (GO:0071705)	104	1.13E-03	4.7
regulation of response to stress (GO:0080134)	101	3.23E-02	4.6
organic acid biosynthetic process (GO:0016053)	100	4.50E-05	4.5
cellular macromolecule localization (GO:0070727)	100	2.09E-04	4.5
secondary metabolic process (GO:0019748)	98	1.77E-02	4.4
embryo development ending in seed dormancy (GO:0009793)	97	9.42E-05	4.4
protein catabolic process (GO:0030163)	97	1.90E-04	4.4
phyllome development (GO:0048827)	94	8.16E-04	4.3
carboxylic acid biosynthetic process (GO:0046394)	92	5.67E-06	4.2
cellular amide metabolic process (GO:0043603)	92	7.75E-03	4.2
lipid biosynthetic process (GO:0008610)	92	1.06E-02	4.2
cellular protein catabolic process (GO:0044257)	91	1.00E-04	4.1
cellular protein localization (GO:0034613)	91	1.48E-03	4.1
proteolysis involved in cellular protein catabolic process (GO:0051603)	90	1.15E-04	4.1
regulation of multicellular organismal process (GO:0051239)	90	4.40E-04	4.1
establishment of localization in cell (GO:0051649)	88	4.22E-02	4.0
ion transport (GO:0006811)	87	8.55E-06	3.9
modification-dependent macromolecule catabolic process (GO:0043632)	85	5.91E-05	3.9
modification-dependent protein catabolic process (GO:0019941)	83	6.97E-05	3.8
homeostatic process (GO:0042592)	81	2.23E-03	3.7
ubiquitin-dependent protein catabolic process (GO:0006511)	80	1.49E-04	3.6
chemical homeostasis (GO:0048878)	78	5.08E-04	3.5
cellular amino acid metabolic process (GO:0006520)	75	5.37E-03	3.4
regulation of multicellular organismal development (GO:2000026)	73	4.15E-04	3.3

organophosphate metabolic process (GO:0019637)	72	2.32E-02	3.3
negative regulation of cellular metabolic process (GO:0031324)	72	4.09E-02	3.3
response to cold (GO:0009409)	71	9.23E-05	3.2
reproductive shoot system development (GO:0090567)	67	1.77E-02	3.0
cellular response to lipid (GO:0071396)	66	2.22E-02	3.0
leaf development (GO:0048366)	65	1.87E-03	3.0
gametophyte development (GO:0048229)	65	1.95E-02	3.0
flower development (GO:0009908)	63	2.15E-02	2.9
regulation of signal transduction (GO:0009966)	62	1.47E-03	2.8
regulation of signaling (GO:0023051)	62	2.44E-03	2.8
regulation of cell communication (GO:0010646)	62	4.03E-03	2.8
regulation of post-embryonic development (GO:0048580)	61	2.04E-03	2.8
organic hydroxy compound metabolic process (GO:1901615)	60	4.89E-04	2.7
cellular carbohydrate metabolic process (GO:0044262)	59	3.11E-02	2.7
chromosome organization (GO:0051276)	59	3.23E-02	2.7
alpha-amino acid metabolic process (GO:1901605)	57	6.95E-04	2.6
ncRNA processing (GO:0034470)	57	3.31E-02	2.6
cation transport (GO:0006812)	56	2.33E-05	2.5
vegetative to reproductive phase transition of meristem (GO:0010228)	54	2.33E-05	2.5
ion transmembrane transport (GO:0034220)	54	1.04E-04	2.5
response to light intensity (GO:0009642)	53	1.91E-02	2.4
peptidyl-amino acid modification (GO:0018193)	53	2.67E-02	2.4
generation of precursor metabolites and energy (GO:0006091)	53	2.89E-02	2.4
proteasomal protein catabolic process (GO:0010498)	52	1.20E-04	2.4
ion homeostasis (GO:0050801)	52	1.75E-02	2.4
small molecule catabolic process (GO:0044282)	51	1.32E-06	2.3
nucleobase-containing small molecule metabolic process (GO:0055086)	50	4.88E-02	2.3
negative regulation of response to stimulus (GO:0048585)	49	1.53E-05	2.2
proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161)	49	5.24E-05	2.2
inorganic ion homeostasis (GO:0098771)	49	2.01E-02	2.2
regulation of growth (GO:0040008)	47	2.31E-02	2.1
metal ion transport (GO:0030001)	46	1.29E-06	2.1
regulation of hormone levels (GO:0010817)	44	1.78E-02	2.0
monocarboxylic acid biosynthetic process (GO:0072330)	43	1.40E-03	2.0
cation homeostasis (GO:0055080)	43	2.75E-03	2.0
inorganic ion transmembrane transport (GO:0098660)	42	7.73E-04	1.9
cellular homeostasis (GO:0019725)	42	3.59E-03	1.9
cellular response to environmental stimulus (GO:0104004)	41	4.44E-03	1.9
cellular response to abiotic stimulus (GO:0071214)	41	4.41E-03	1.9
organic acid catabolic process (GO:0016054)	39	4.84E-06	1.8
metal ion homeostasis (GO:0055065)	39	3.79E-04	1.8
carbohydrate catabolic process (GO:0016052)	39	2.37E-03	1.8
carboxylic acid catabolic process (GO:0046395)	38	3.75E-06	1.7
cation transmembrane transport (GO:0098655)	38	4.02E-04	1.7
cellular chemical homeostasis (GO:0055082)	38	1.11E-03	1.7
chromatin organization (GO:0006325)	38	4.89E-02	1.7
inorganic cation transmembrane transport (GO:0098662)	37	3.11E-04	1.7
response to ethylene (GO:0009723)	37	2.23E-02	1.7
organic cyclic compound catabolic process (GO:1901361)	37	2.30E-02	1.7
fatty acid metabolic process (GO:0006631)	37	2.35E-02	1.7
positive regulation of response to stimulus (GO:0048584)	37	2.49E-02	1.7
response to metal ion (GO:0010038)	36	4.50E-02	1.6
response to carbohydrate (GO:0009743)	34	7.63E-04	1.5
aromatic compound catabolic process (GO:0019439)	34	4.63E-02	1.5
cellular ion homeostasis (GO:0006873)	32	6.35E-03	1.5
glycosylation (GO:0070085)	30	7.33E-03	1.4
glycoprotein metabolic process (GO:0009100)	30	1.24E-02	1.4
photoperiodism (GO:0009648)	29	1.58E-04	1.3
protein glycosylation (GO:0006486)	29	8.51E-03	1.3
macromolecule glycosylation (GO:0043413)	29	8.46E-03	1.3
glycoprotein biosynthetic process (GO:0009101)	29	8.81E-03	1.3
cellular cation homeostasis (GO:0030003)	29	1.62E-02	1.3
phenylpropanoid metabolic process (GO:0009698)	28	1.81E-04	1.3
negative regulation of developmental process (GO:0051093)	28	4.01E-02	1.3
monosaccharide metabolic process (GO:0005996)	26	2.52E-03	1.2
positive regulation of developmental process (GO:0051094)	26	7.75E-03	1.2
abscisic acid-activated signaling pathway (GO:0009738)	26	3.41E-02	1.2
ATP metabolic process (GO:0046034)	25	4.76E-02	1.1
photoperiodism, flowering (GO:0048573)	24	2.21E-04	1.1

	response to endoplasmic reticulum stress (GO:0034976)	24	1.50E-02	1.1
	cellular metal ion homeostasis (GO:0006875)	24	3.79E-02	1.1
	negative regulation of multicellular organismal process (GO:0051241)	24	4.12E-02	1.1
	lipid catabolic process (GO:0016042)	23	8.57E-03	1.0
	negative regulation of post-embryonic development (GO:0048581)	23	2.17E-02	1.0
	response to UV (GO:0009411)	23	2.25E-02	1.0
	alcohol metabolic process (GO:0006066)	23	3.91E-02	1.0
	regulation of cell growth (GO:0001558)	23	4.02E-02	1.0
	alpha-amino acid catabolic process (GO:1901606)	22	9.27E-04	1.0
	phenylpropanoid biosynthetic process (GO:0009699)	22	1.76E-03	1.0
	pyruvate metabolic process (GO:0006090)	21	5.97E-04	1.0
	cellular amino acid catabolic process (GO:0009063)	21	4.97E-03	1.0
	cellular lipid catabolic process (GO:0044242)	21	1.50E-02	1.0
	cellular response to ethylene stimulus (GO:0071369)	20	1.50E-02	0.9
	transition metal ion homeostasis (GO:0055076)	20	2.24E-02	0.9
	negative regulation of defense response (GO:0031348)	19	2.51E-03	0.9
	nucleoside diphosphate metabolic process (GO:0009132)	19	2.83E-03	0.9
	hexose metabolic process (GO:0019318)	19	1.02E-02	0.9
	protein maturation (GO:0051604)	19	2.62E-02	0.9
	ethylene-activated signaling pathway (GO:0009873)	18	7.76E-03	0.8
	SCF-dependent proteasomal ubiquitin-dependent protein catabolic process (GO:0031146)	18	2.35E-02	0.8
	ribonucleoside diphosphate metabolic process (GO:0009185)	17	2.03E-03	0.8
	carbohydrate transport (GO:0008643)	17	2.70E-03	0.8
	monocarboxylic acid catabolic process (GO:0072329)	17	2.68E-03	0.8
	response to disaccharide (GO:0034285)	17	8.75E-03	0.8
	oligosaccharide metabolic process (GO:0009311)	17	4.03E-02	0.8
	lipid modification (GO:0030258)	17	4.83E-02	0.8
	potassium ion transport (GO:0006813)	16	6.40E-04	0.7
	lignin metabolic process (GO:0009808)	16	2.53E-03	0.7
	nucleotide phosphorylation (GO:0046939)	16	9.96E-03	0.7
	response to sucrose (GO:0009744)	16	1.61E-02	0.7
	transition metal ion transport (GO:0000041)	16	2.37E-02	0.7
	ATP generation from ADP (GO:0006757)	15	5.01E-03	0.7
	glycolytic process (GO:0006096)	15	4.98E-03	0.7
	ADP metabolic process (GO:0046031)	15	6.73E-03	0.7
	purine ribonucleoside diphosphate metabolic process (GO:0009179)	15	6.69E-03	0.7
	purine nucleoside diphosphate metabolic process (GO:0009135)	15	6.65E-03	0.7
	benzene-containing compound metabolic process (GO:0042537)	15	8.67E-03	0.7
	glutamine family amino acid metabolic process (GO:0009064)	15	9.92E-03	0.7
	nucleoside diphosphate phosphorylation (GO:0006165)	15	1.49E-02	0.7
	response to high light intensity (GO:0009644)	15	1.48E-02	0.7
	starch metabolic process (GO:0005982)	15	2.05E-02	0.7
	potassium ion transmembrane transport (GO:0071805)	14	1.29E-03	0.6
	protein processing (GO:0016485)	14	2.54E-02	0.6
	response to glucose (GO:0009749)	14	3.61E-02	0.6
	stomatal movement (GO:0010118)	14	4.02E-02	0.6
	positive regulation of growth (GO:0045927)	13	5.49E-03	0.6
	lignin biosynthetic process (GO:0009809)	13	7.73E-03	0.6
	phenol-containing compound metabolic process (GO:0018958)	13	1.21E-02	0.6
	jasmonic acid metabolic process (GO:0009694)	13	1.61E-02	0.6
	import across plasma membrane (GO:0098739)	12	3.03E-03	0.5
	import into cell (GO:0098657)	12	4.61E-03	0.5
	amino acid transport (GO:0006865)	12	2.54E-02	0.5
	lipid oxidation (GO:0034440)	12	4.72E-02	0.5
	monosaccharide transmembrane transport (GO:0015749)	10	8.84E-03	0.5
	carbohydrate transmembrane transport (GO:0034219)	10	1.90E-02	0.5
	organelle disassembly (GO:1903008)	10	2.22E-02	0.5
	L-phenylalanine metabolic process (GO:0006558)	9	2.23E-02	0.4
	erythrose 4-phosphate/phosphoenolpyruvate family amino acid metabolic process (GO:1902221)	9	2.22E-02	0.4
	ribosomal large subunit assembly (GO:0000027)	9	3.11E-02	0.4
	glutamine family amino acid biosynthetic process (GO:0009084)	9	3.72E-02	0.4
	inorganic ion import across plasma membrane (GO:0099587)	8	2.48E-02	0.4
	positive regulation of cell growth (GO:0030307)	8	3.06E-02	0.4
	potassium ion import across plasma membrane (GO:1990573)	6	1.45E-02	0.3
	positive regulation of developmental growth (GO:0048639)	6	4.50E-02	0.3
Down	cellular process (GO:0009987)	1457	1.90E-37	70.6
	metabolic process (GO:0008152)	1099	3.26E-31	53.3
	response to stimulus (GO:0050896)	984	3.31E-31	47.7

organic substance metabolic process (GO:0071704)	982	1.72E-23	47.6
cellular metabolic process (GO:0044237)	969	4.54E-25	47.0
primary metabolic process (GO:0044238)	798	6.67E-15	38.7
biological regulation (GO:0065007)	722	5.01E-04	35.0
nitrogen compound metabolic process (GO:0006807)	655	4.35E-07	31.7
regulation of biological process (GO:0050789)	651	7.16E-03	31.6
response to stress (GO:0006950)	566	5.93E-15	27.4
macromolecule metabolic process (GO:0043170)	557	3.06E-06	27.0
regulation of cellular process (GO:0050794)	551	2.52E-03	26.7
response to chemical (GO:0042221)	536	2.11E-12	26.0
organonitrogen compound metabolic process (GO:1901564)	531	3.26E-08	25.7
developmental process (GO:0032502)	517	4.89E-03	25.1
response to abiotic stimulus (GO:0009628)	484	1.11E-18	23.5
anatomical structure development (GO:0048856)	475	6.85E-03	23.0
multicellular organismal process (GO:0032501)	428	4.05E-03	20.7
cellular macromolecule metabolic process (GO:0044260)	427	4.35E-05	20.7
cellular response to stimulus (GO:0051716)	395	6.01E-07	19.1
multicellular organism development (GO:0007275)	394	1.27E-02	19.1
response to organic substance (GO:0010033)	382	1.92E-09	18.5
protein metabolic process (GO:0019538)	362	3.14E-04	17.5
response to oxygen-containing compound (GO:1901700)	361	1.25E-12	17.5
biosynthetic process (GO:0009058)	356	1.23E-08	17.3
cellular protein metabolic process (GO:0044267)	344	3.42E-04	16.7
response to external stimulus (GO:0009605)	335	3.71E-10	16.2
organic substance biosynthetic process (GO:1901576)	326	1.01E-06	15.8
macromolecule modification (GO:0043412)	321	2.20E-07	15.6
small molecule metabolic process (GO:0044281)	305	2.22E-06	14.8
biological process involved in interspecies interaction between organisms (GO:0044419)	302	1.01E-11	14.6
response to biotic stimulus (GO:0009607)	300	1.11E-11	14.5
defense response (GO:0006952)	300	1.05E-11	14.5
response to external biotic stimulus (GO:0043207)	299	1.35E-11	14.5
response to other organism (GO:0051707)	299	1.28E-11	14.5
cellular biosynthetic process (GO:0044249)	288	1.08E-04	14.0
cell communication (GO:0007154)	287	1.61E-05	13.9
organic cyclic compound metabolic process (GO:1901360)	286	1.25E-02	13.9
protein modification process (GO:0036211)	273	4.47E-05	13.2
cellular protein modification process (GO:0006464)	273	4.39E-05	13.2
cellular aromatic compound metabolic process (GO:0006725)	273	2.60E-02	13.2
response to endogenous stimulus (GO:0009719)	269	2.02E-05	13.0
phosphorus metabolic process (GO:0006793)	268	3.42E-19	13.0
phosphate-containing compound metabolic process (GO:0006796)	265	1.37E-19	12.8
signaling (GO:0023052)	264	2.82E-05	12.8
response to hormone (GO:0009725)	263	4.71E-05	12.7
signal transduction (GO:0007165)	261	1.31E-05	12.7
response to radiation (GO:0009314)	259	3.21E-11	12.6
response to light stimulus (GO:0009416)	256	1.96E-11	12.4
defense response to other organism (GO:0098542)	250	3.03E-08	12.1
cellular response to chemical stimulus (GO:0070887)	241	5.65E-08	11.7
response to inorganic substance (GO:0010035)	221	1.51E-03	10.7
response to lipid (GO:0033993)	214	2.65E-05	10.4
organic acid metabolic process (GO:0006082)	204	3.62E-02	9.9
oxoacid metabolic process (GO:0043436)	194	2.74E-02	9.4
regulation of macromolecule metabolic process (GO:0060255)	190	9.10E-03	9.2
phosphorylation (GO:0016310)	189	8.05E-18	9.2
cellular response to organic substance (GO:0071310)	181	1.82E-05	8.8
protein phosphorylation (GO:0006468)	175	3.09E-17	8.5
response to bacterium (GO:0009617)	172	1.00E-09	8.3
regulation of nitrogen compound metabolic process (GO:0051171)	169	4.01E-02	8.2
carboxylic acid metabolic process (GO:0019752)	157	2.34E-02	7.6
regulation of gene expression (GO:0010468)	151	1.06E-03	7.3
cellular response to oxygen-containing compound (GO:1901701)	148	1.05E-06	7.2
lipid metabolic process (GO:0006629)	148	1.32E-04	7.2
shoot system development (GO:0048367)	147	4.85E-04	7.1
regulation of cellular biosynthetic process (GO:0031326)	146	3.02E-02	7.1
defense response to bacterium (GO:0042742)	145	2.14E-08	7.0
response to alcohol (GO:0097305)	139	3.14E-04	6.7
carbohydrate metabolic process (GO:0005975)	136	3.21E-06	6.6
regulation of biological quality (GO:0065008)	131	1.70E-03	6.3
cellular response to endogenous stimulus (GO:0071495)	130	5.43E-03	6.3

regulation of macromolecule biosynthetic process (GO:0010556)	130	1.26E-02	6.3
regulation of nucleobase-containing compound metabolic process (GO:0019219)	130	2.23E-03	6.3
cellular response to hormone stimulus (GO:0032870)	124	1.34E-02	6.0
response to abscisic acid (GO:0009737)	123	1.51E-03	6.0
regulation of RNA metabolic process (GO:0051252)	123	4.28E-03	6.0
response to acid chemical (GO:0001101)	121	1.14E-02	5.9
response to temperature stimulus (GO:0009266)	119	9.85E-08	5.8
cellular lipid metabolic process (GO:0044255)	116	7.10E-04	5.6
response to water (GO:0009415)	116	2.36E-02	5.6
small molecule biosynthetic process (GO:0044283)	113	1.27E-04	5.5
regulation of transcription, DNA-templated (GO:0006355)	112	1.83E-03	5.4
regulation of nucleic acid-templated transcription (GO:1903506)	112	1.81E-03	5.4
regulation of RNA biosynthetic process (GO:2001141)	112	1.60E-03	5.4
response to water deprivation (GO:0009414)	109	2.33E-02	5.3
response to fungus (GO:0009620)	108	3.21E-02	5.2
cell wall organization or biogenesis (GO:0071554)	107	1.03E-02	5.2
organic cyclic compound biosynthetic process (GO:1901362)	106	7.19E-03	5.1
hormone-mediated signaling pathway (GO:0009755)	101	2.71E-02	4.9
lipid biosynthetic process (GO:0008610)	100	5.89E-05	4.8
response to wounding (GO:0009611)	96	6.62E-03	4.7
response to osmotic stress (GO:0006970)	96	4.41E-02	4.7
response to nitrogen compound (GO:1901698)	92	1.71E-02	4.5
secondary metabolic process (GO:0019748)	92	2.73E-02	4.5
aromatic compound biosynthetic process (GO:0019438)	91	4.01E-02	4.4
response to organic cyclic compound (GO:0014070)	90	3.01E-02	4.4
phyllome development (GO:0048827)	86	4.01E-03	4.2
regulation of defense response (GO:0031347)	85	2.57E-02	4.1
response to organonitrogen compound (GO:0010243)	81	7.34E-03	3.9
homeostatic process (GO:0042592)	76	4.02E-03	3.7
response to jasmonic acid (GO:0009753)	73	1.79E-02	3.5
response to fatty acid (GO:0070542)	73	2.36E-02	3.5
organophosphate metabolic process (GO:0019637)	70	1.67E-02	3.4
generation of precursor metabolites and energy (GO:0006091)	68	2.23E-06	3.3
ion transport (GO:0006811)	68	1.74E-02	3.3
cellular carbohydrate metabolic process (GO:0044262)	66	3.02E-04	3.2
photosynthesis (GO:0015979)	65	4.16E-16	3.2
cell division (GO:0051301)	65	1.74E-03	3.2
polysaccharide metabolic process (GO:0005976)	65	2.66E-03	3.2
chemical homeostasis (GO:0048878)	64	4.91E-02	3.1
immune system process (GO:0002376)	62	3.23E-03	3.0
plant-type cell wall organization or biogenesis (GO:0071669)	61	3.82E-02	3.0
response to cold (GO:0009409)	60	5.41E-03	2.9
carbohydrate biosynthetic process (GO:0016051)	59	2.06E-05	2.9
cell wall biogenesis (GO:0042546)	58	8.71E-03	2.8
leaf development (GO:0048366)	56	2.70E-02	2.7
response to light intensity (GO:0009642)	55	2.50E-03	2.7
cellular polysaccharide metabolic process (GO:0044264)	51	4.49E-04	2.5
plastid organization (GO:0009657)	48	4.97E-03	2.3
tetrapyrrole metabolic process (GO:0033013)	45	4.55E-04	2.2
response to heat (GO:0009408)	45	8.99E-04	2.2
RNA modification (GO:0009451)	45	1.96E-03	2.2
isoprenoid metabolic process (GO:0006720)	43	8.94E-04	2.1
response to chitin (GO:0010200)	43	2.70E-02	2.1
photosynthesis, light reaction (GO:0019684)	41	2.77E-09	2.0
porphyrin-containing compound metabolic process (GO:0006778)	41	1.99E-04	2.0
cation transport (GO:0006812)	41	4.11E-02	2.0
cellular glucan metabolic process (GO:0006073)	40	3.74E-05	1.9
glucan metabolic process (GO:0044042)	40	9.03E-05	1.9
immune response (GO:0006955)	38	1.50E-04	1.8
chloroplast organization (GO:0009658)	38	1.20E-02	1.8
pigment metabolic process (GO:0042440)	37	9.03E-03	1.8
plant-type cell wall biogenesis (GO:0009832)	37	4.24E-02	1.8
secondary metabolite biosynthetic process (GO:0044550)	36	1.30E-04	1.7
terpenoid metabolic process (GO:0006721)	36	1.58E-03	1.7
innate immune response (GO:0045087)	35	1.61E-04	1.7
isoprenoid biosynthetic process (GO:0008299)	34	6.17E-04	1.6
cellular carbohydrate biosynthetic process (GO:0034637)	34	3.89E-02	1.6
protein folding (GO:0006457)	33	6.13E-03	1.6
organic hydroxy compound biosynthetic process (GO:1901617)	33	1.69E-02	1.6

establishment of localization in cell (GO:0051649)	33	2.47E-02	1.6
intracellular transport (GO:0046907)	30	2.66E-02	1.5
phenylpropanoid metabolic process (GO:0009698)	29	3.69E-05	1.4
pigment biosynthetic process (GO:0046148)	28	1.25E-02	1.4
chlorophyll metabolic process (GO:0015994)	27	7.30E-04	1.3
terpenoid biosynthetic process (GO:0016114)	27	2.06E-03	1.3
phloem or xylem histogenesis (GO:0010087)	27	1.66E-02	1.3
cytokinesis (GO:0000910)	27	3.97E-02	1.3
phenylpropanoid biosynthetic process (GO:0009699)	26	2.35E-05	1.3
response to oomycetes (GO:0002239)	25	1.49E-04	1.2
glucan biosynthetic process (GO:0009250)	23	9.02E-03	1.1
circadian rhythm (GO:0007623)	22	1.70E-02	1.1
rhythmic process (GO:0048511)	22	1.69E-02	1.1
cell death (GO:0008219)	22	3.02E-02	1.1
porphyrin-containing compound biosynthetic process (GO:0006779)	21	3.48E-04	1.0
tetrapyrrole biosynthetic process (GO:0033014)	21	1.56E-03	1.0
seedling development (GO:0090351)	21	4.13E-02	1.0
biological process involved in symbiotic interaction (GO:0044403)	20	1.09E-03	1.0
programmed cell death (GO:0012501)	20	2.81E-03	1.0
seed germination (GO:0009845)	20	9.08E-03	1.0
defense response to oomycetes (GO:0002229)	19	1.84E-03	0.9
regulation of cell death (GO:0010941)	19	4.17E-02	0.9
response to high light intensity (GO:0009644)	18	5.60E-04	0.9
hexose metabolic process (GO:0019318)	18	1.46E-02	0.9
photosynthesis, light harvesting (GO:0009765)	17	4.75E-05	0.8
vesicle-mediated transport (GO:0016192)	17	4.45E-03	0.8
mitotic cytokinesis (GO:0000281)	16	1.28E-04	0.8
glucose metabolic process (GO:0006006)	16	1.26E-04	0.8
cellulose biosynthetic process (GO:0030244)	16	1.29E-03	0.8
cellulose metabolic process (GO:0030243)	16	3.09E-03	0.8
chlorophyll biosynthetic process (GO:0015995)	16	4.11E-03	0.8
cytoskeleton-dependent cytokinesis (GO:0061640)	16	6.95E-03	0.8
beta-glucan biosynthetic process (GO:0051274)	16	6.90E-03	0.8
beta-glucan metabolic process (GO:0051273)	16	1.38E-02	0.8
hydrocarbon metabolic process (GO:0120252)	16	3.07E-02	0.8
monosaccharide biosynthetic process (GO:0046364)	15	1.14E-03	0.7
chaperone-mediated protein folding (GO:0061077)	15	1.66E-02	0.7
cellular metabolic compound salvage (GO:0043094)	15	4.42E-02	0.7
plant-type primary cell wall biogenesis (GO:0009833)	14	5.15E-05	0.7
regulation of photosynthesis (GO:0010109)	14	2.73E-03	0.7
programmed cell death induced by symbiont (GO:0034050)	14	6.08E-03	0.7
biological process involved in interaction with symbiont (GO:0051702)	14	6.91E-03	0.7
gluconeogenesis (GO:0006094)	13	4.63E-05	0.6
hexose biosynthetic process (GO:0019319)	13	1.19E-04	0.6
plant-type hypersensitive response (GO:0009626)	13	1.04E-02	0.6
diterpenoid biosynthetic process (GO:0016102)	13	2.34E-02	0.6
lignin metabolic process (GO:0009808)	13	2.87E-02	0.6
diterpenoid metabolic process (GO:0016101)	13	4.37E-02	0.6
photorespiration (GO:0009853)	12	1.16E-02	0.6
terpene metabolic process (GO:0042214)	12	3.84E-02	0.6
photosynthetic electron transport chain (GO:0009767)	12	4.17E-02	0.6
reductive pentose-phosphate cycle (GO:0019253)	11	3.78E-05	0.5
photosynthesis, dark reaction (GO:0019685)	11	5.33E-05	0.5
carbon fixation (GO:0015977)	11	8.16E-05	0.5
photosystem II assembly (GO:0010207)	11	2.16E-03	0.5
regulation of photosynthesis, light reaction (GO:0042548)	11	2.70E-03	0.5
regulation of generation of precursor metabolites and energy (GO:0043467)	11	5.08E-03	0.5
lignin biosynthetic process (GO:0009809)	11	3.91E-02	0.5
regulation of transcription by RNA polymerase II (GO:0006357)	10	1.46E-05	0.5
photosynthesis, light harvesting in photosystem I (GO:0009768)	9	1.26E-02	0.4
protein repair (GO:0030091)	9	2.73E-02	0.4
photosystem II repair (GO:0010206)	7	1.13E-02	0.3
oxylipin biosynthetic process (GO:0031408)	7	3.06E-02	0.3
stomatal closure (GO:0090332)	7	3.86E-02	0.3
oxylipin metabolic process (GO:0031407)	7	4.61E-02	0.3
fructose 1,6-bisphosphate metabolic process (GO:0030388)	6	3.21E-02	0.3
oxidative photosynthetic carbon pathway (GO:0009854)	5	2.83E-02	0.2
positive regulation of transcription by RNA polymerase II (GO:0045944)	3	4.00E-02	0.1
Golgi vesicle transport (GO:0048193)	3	3.02E-02	0.1

Table S6 Gene ontology (GO) analysis of commonly expressed genes between cold and drought stress

	GO biological process complete	Gene Number	FDR	Fold enrichment (%)
Cold stress and Drought stress Up	response to stimulus (GO:0050896)	37	3.01E-05	75.5
	regulation of biological process (GO:0050789)	29	2.17E-03	59.2
	biological regulation (GO:0065007)	29	7.16E-03	59.2
	regulation of cellular process (GO:0050794)	26	2.34E-03	53.1
	response to chemical (GO:0042221)	24	2.34E-03	49.0
	response to stress (GO:0006950)	24	2.90E-03	49.0
	response to organic substance (GO:0010033)	20	2.32E-03	40.8
	response to external stimulus (GO:0009605)	19	1.07E-03	38.8
	cellular response to stimulus (GO:0051716)	19	5.78E-03	38.8
	response to oxygen-containing compound (GO:1901700)	18	2.35E-03	36.7
	cell communication (GO:0007154)	17	2.71E-03	34.7
	signal transduction (GO:0007165)	16	2.47E-03	32.7
	response to external biotic stimulus (GO:0043207)	16	2.27E-03	32.7
	response to other organism (GO:0051707)	16	2.08E-03	32.7
	response to biotic stimulus (GO:0009607)	16	1.94E-03	32.7
	biological process involved in interspecies interaction between organisms (GO:0044419)	16	1.95E-03	32.7
	signaling (GO:0023052)	16	1.82E-03	32.7
	cellular response to chemical stimulus (GO:0070887)	15	2.52E-03	30.6
	defense response to other organism (GO:0098542)	13	1.75E-02	26.5
	cellular response to organic substance (GO:0071310)	12	5.09E-03	24.5
	cellular response to oxygen-containing compound (GO:1901701)	11	1.99E-03	22.4
	phosphate-containing compound metabolic process (GO:0006796)	11	4.85E-02	22.4
	protein phosphorylation (GO:0006468)	9	1.85E-02	18.4
	defense response to bacterium (GO:0042742)	9	1.93E-02	18.4
	phosphorylation (GO:0016310)	9	3.36E-02	18.4
	regulation of response to stress (GO:0080134)	8	3.19E-02	16.3
	regulation of response to biotic stimulus (GO:0002831)	5	6.48E-03	10.2
	regulation of response to external stimulus (GO:0032101)	5	7.01E-03	10.2
	positive regulation of response to stimulus (GO:0048584)	5	1.90E-02	10.2
	positive regulation of response to biotic stimulus (GO:0002833)	4	4.84E-03	8.2
	positive regulation of response to external stimulus (GO:0032103)	4	5.28E-03	8.2
	positive regulation of defense response (GO:0031349)	4	6.75E-03	8.2
	abscission (GO:0009838)	3	1.23E-02	6.1
	leaf abscission (GO:0060866)	2	2.64E-02	4.1
	induced systemic resistance, jasmonic acid mediated signaling pathway (GO:0009864)	2	3.52E-02	4.1
Cold stress and Drought stress Down	response to stimulus (GO:0050896)	30	2.86E-02	62.5
	response to stress (GO:0006950)	21	3.37E-02	43.8
	response to external biotic stimulus (GO:0043207)	15	1.29E-02	31.3
	response to other organism (GO:0051707)	15	1.13E-02	31.3
	response to biotic stimulus (GO:0009607)	15	1.01E-02	31.3
	biological process involved in interspecies interaction between organisms (GO:0044419)	15	9.79E-03	31.3
	response to external stimulus (GO:0009605)	15	3.38E-02	31.3
	defense response to other organism (GO:0098542)	14	1.57E-02	29.2
	defense response (GO:0006952)	14	2.72E-02	29.2
	protein modification process (GO:0036211)	14	3.43E-02	29.2
	cellular protein modification process (GO:0006464)	14	3.27E-02	29.2
	phosphate-containing compound metabolic process (GO:0006796)	12	2.48E-02	25.0
	phosphorus metabolic process (GO:0006793)	12	2.70E-02	25.0
	protein phosphorylation (GO:0006468)	10	1.73E-02	20.8
	phosphorylation (GO:0016310)	10	1.44E-02	20.8
	photosynthesis (GO:0015979)	7	5.05E-04	14.6
	generation of precursor metabolites and energy (GO:0006091)	7	1.34E-02	14.6
	photosynthesis, light reaction (GO:0019684)	6	5.86E-04	12.5
	photosynthesis, light harvesting in photosystem I (GO:0009768)	3	1.40E-02	6.3
	photosynthesis, light harvesting (GO:0009765)	3	2.65E-02	6.3
	detection of stimulus (GO:0051606)	3	4.70E-02	6.3
	detection of bacterium (GO:0016045)	2	2.83E-02	4.2
	detection of other organism (GO:0098543)	2	2.88E-02	4.2
	glycine decarboxylation via glycine cleavage system (GO:0019464)	2	3.31E-02	4.2
	glycine catabolic process (GO:0006546)	2	3.27E-02	4.2

regulation of anion channel activity (GO:0010359)	2	4.60E-02	4.2
detection of external biotic stimulus (GO:0098581)	2	4.42E-02	4.2
detection of biotic stimulus (GO:0009595)	2	4.26E-02	4.2
regulation of anion transmembrane transport (GO:1903959)	2	4.85E-02	4.2

The GO biological process (GOPB) terms associated with Fisher's exact test with FDR corrected P-value < 0.05.

Table S7 Primer suquences used to validate RNA-Sequencing results of trees grown waterlogging treatment versus control after transplanting

Gene	Forward primer	Reverse primer
TRINITY_DN85415_c0_g2_i1	TCATGGAGAGAAGCCAGGGC	GAACACCCGCGCATATGCTC
TRINITY_DN86343_c3_g1_i11	GCCTGCAAAGAGTGTGTTTCAG	CCTTGGAGGAGCTGTAAGGC
TRINITY_DN81275_c0_g2_i3	ACCCAAAGTACCCGGTTCCG	ATGTGGGTCGGGAGGATTGG
TRINITY_DN89101_c0_g1_i4	ACACACTCATGCCCCTGGTC	CAACTGTGTGAAAGCCCCGC
TRINITY_DN85313_c0_g1_i5	CCCATGGAAAATGCTGGGGC	CCCGAGCCAAAACAGATGCC
TRINITY_DN78260_c0_g1_i5	CTGCATGTGGAGGCTGTTGC	TGGGCAGTCCAGTCTCCAAC
TRINITY_DN82177_c1_g1_i9	TAGAGTGGCCGCGGAGAAAG	GTGGCTCGGCACATTCAACC
TRINITY_DN89267_c1_g1_i6	TTTATGGAAGCCGTGCACGC	CGAGCGTGACGTGATGAAGC
TRINITY_DN79247_c0_g1_i10	TGTCCGAGGTGGTGTGGATG	ATCCGGACGTGGCAGGTTAG
F-box	TATTATTGTTGCAGGTGGGTT	AGAATGTTGAAGTTCGGCTAT

초 록

묘목의 적응성은 양묘환경에 의해서 결정되며, 이식한 묘목은 가뭄, 추위, 과습 같은 다양한 스트레스에 노출된다. 스트레스 환경은 식물의 생장 및 생존을 저해하지만 스트레스에 적응한 식물은 다음 스트레스 환경에서 적응성을 증가시키기도 한다. 스트레스 경화는 1차 스트레스와 다른 스트레스 환경, 즉 교차 스트레스 환경에서도 저항성이 증가할 수 있다. 하지만, 스트레스 경화 연구는 작물 위주로 보고되고 있으며 임목의 이식 후 스트레스에 대한 스트레스 경화 효과는 연구가 부족한 실정이다. 소나무는 우리나라 산림을 구성하는 주요 수종이며, 최근 기후 변화로 인해 집단 고사가 보고되고 있다. 본 연구는 이식 후 소나무의 다양한 스트레스 간의 경화 효과를 확인하고자 하였다. 첫 번째 연구 목적은 가뭄 강도에 따라 가뭄 스트레스 저항성이 증가하는지 알아보고자 하였고, 두 번째 연구 목적은 장기간의 가뭄 경화가 이식 후 겪는 여러가지 스트레스 저항성을 증가시키는지 알아보고자 하였다. 두 번째 연구 목적은 과습 스트레스가 이식 스트레스에 미치는 영향을 확인하는 것이다. 가뭄 경화 연구에서는 3년 생 소나무를 다른 가뭄 강도에서 3년 간 생장시킨 후 다른 연구지로 이식하여 생장기와 겨울에 반응을 확인하였다. 가뭄 경화 효과를 확인하기 위해, 이식 전과 후 생장기간 인 여름과 겨울에 전사체 반응을 가뭄강도에 따라 비교하였으며, 생리적 반응 또한 이식 후 여름과 겨울에 측정하였다. 이식 후 생장 기간에는 약가뭄 스트레스가 이식 과정에서 손상된 조직의 세포벽 구성과 방어 반응을 촉진하였으며, 수분 보존 능력 또한 향상되었다. 이식 후 첫번째 겨울의 경우, 약가뭄 스트레스에서 저온 스트레스 저항성에 관여하는 동결방지물질, 이차대사산물, 식물호르몬 유전자 발현이 강가뭄구와 대조구에 비해 증가하였다. 이러한 방어 반응 관련 유전자 발현량 증가가 저항성에 관여

하여 약가뭄 스트레스에서 이식 후 광합성과 생장이 증가하였다. 과습 스트레스 연구에서는 3년 생 소나무를 대조구와 과습 처리구에서 3년간 생장 시킨 후 다른 지역으로 이식하였다. 전사체 분석은 이식 전과 후에 실시하였으며, 과습 처리구와 대조구를 비교하였다. 이식 후 과습 스트레스처리로 인하여 세포벽 형성과 이차대사산물 관련 유전자 발현량이 증가하였으며, 단근에 의한 상처 스트레스를 완화하였다. 하지만 옥신 유전자 발현량의 감소로 인하여 과습 처리구에서 생장이 감소하는 경향을 보였다. 본 연구결과, 약가뭄 스트레스와 과습 스트레스가 방어 반응 관련 유전자 발현이 증가하였으나, 약가뭄 스트레스에서 생장 및 광합성 증가가 나타나 스트레스 저항성이 증가하였다고 판단하였다. 다양한 수종 및 환경 조건을 대상으로 추가적인 스트레스 경화 연구를 통해 스트레스 저항성을 향상시킬 수 있는 환경 조건을 제시하여 현장 적용 및 활용에 도움이 될 것으로 판단된다.

주요어 : 경화(hardening) 스트레스 저항성, 가뭄 스트레스, 과습 스트레스, 이식 스트레스, 소나무
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