論　文（Original article）

Changes in the *Cryptomeria japonica* shoot transcriptome after short-term treatments with different concentrations of CO_2

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Abstract

A transcriptome analysis was conducted to identify and characterize the differentially expressed genes (DEGs) in *Cryptomeria japonica*, a coniferous species endemic to Japan, after short-term treatments with different concentrations of CO_2. The *de novo* assembly of the obtained RNA reads resulted in 35,211 tentative transcripts. The expression levels of 113 and 30 genes were increased in response to elevated (800 ppm) and lowered (200 ppm) CO_2 concentrations, respectively. The deduced functions of these genes indicated that different molecular pathways were activated in response to the two different CO_2 treatments. The expression levels of the gene transcripts involved in the photosynthesis and photorespiration pathways were not affected by the CO_2 concentration, except for a homolog of a chloroplast RNA polymerase subunit that is involved in the transcription of chloroplast coding genes. Together with the enrichment of genes acting in chloroplasts among detected DEGs, adjusting the transcription of genes related to chloroplast functions may be one of the earliest responses to change CO_2 at the transcriptional level.

Key words : CO_2, *Cryptomeria japonica*, transcriptome

Introduction

The atmospheric CO_2 concentration has increased due to industrial activities and is expected to continue to increase in the future (Prentice et al. 2001). CO_2 is one of the substrates of photosynthesis; therefore, changes in the atmospheric CO_2 concentration are expected to have a large impact on plant growth. Plant species respond differently to CO_2 changes through a complex network of proteins and other molecules. To understand plant responses to CO_2 fluctuations at a molecular level, we must identify the genes involved in such molecular networks.

The effects of elevated atmospheric CO_2 concentration on plants vary by species (Ainsworth and Long 2005, Wang et al. 2012). Therefore, it is crucial that we understand how CO_2 concentration affects each species. *Cryptomeria japonica* is a coniferous species endemic to Japan. It is also widely planted, constituting approximately 40% of the country's artificial forests. A free-air CO_2 enrichment experiment was conducted to evaluate the physiological and growth changes in *C. japonica* (Hiraoka et al. 2017). A two-year elevated CO_2 (eCO_2) treatment (550 ppm) was found to have positive effects on the photosynthetic rate and promoted dry mass growth for the whole plant and all organs but had negative effects on stomatal conductance and the maximum carboxylation rate. These observations suggest that carbon metabolism pathways in *C. japonica* are adjusted in response to eCO_2, but the genes involved are largely unknown.

Transcriptome analyses have been conducted in many plant species to identify the genes that respond to changes in CO_2. As the atmospheric CO_2 concentration (aCO_2) is currently increasing, most studies have focused on the effects of eCO_2 on plants. In these studies, the differentially expressed genes (DEGs) between eCO_2 and aCO_2 conditions were intensively surveyed. Microarray technologies were used in the early stages of these studies. However, these analyses require genomic information of the target species, and this has led to the *Populus* species becoming a very well-studied woody species. After exposure for 3 and 6 years to eCO_2 (550 ppm), 8 and 28 DEGs were detected in the cDNA arrays of *Populus* × *euramericana* carrying 38,223 genes, respectively (Taylor et al. 2005). After exposure for 12 years to eCO_2 (560 ppm), *Populus tremuloides* microarrays carrying 61,252 genes were used to identify 539 DEGs (Wei et al. 2013). While 5,127 out of 56,000 genes were differentially expressed when comparing the transcriptomes of the leaves of triploid white poplar ((*Populus tomentosa* × *P. bolleana*) × *P. tomentosa*) after three months of treatment with three different CO_2 concentrations (385, 550, and 720 ppm, Liu et al. 2014), the number of DEGs detected under eCO_2 in the *Populus* species was relatively small.

In more recent years, high-throughput RNA sequencing technologies (RNA-Seq) have become increasingly popular

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for detecting DEGs. RNA-Seq is advantageous compared to microarray analysis, as it can be used to analyze a larger number of genes. RNA-Seq analysis was conducted for poplar seedlings grown under eCO_2 (560 ppm and 720 ppm) for 16 weeks in open-top chambers (Kim et al. 2021). Seedlings of two clones (*Populus alba* × *Populus glandulosa* hybrid "Clivus" and *Populus euramericana* "I-476") were tested, and only 26 and 15 genes were identified as likely to respond to the eCO_2 for Clivus and I-476, respectively. RNA-Seq can provide tentative reference gene sets for target species; consequently, DEG screening is more easily applicable in non-model plant species, where there is no prior knowledge of the gene transcripts. DEG analysis was therefore selected for use with the coniferous species *Abies koreana*. By analyzing the needles of three-year-old trees treated with eCO_2 for 21 days (Hwang et al. 2019), 3,165 differentially expressed transcripts were detected from 334,898 contigs.

The long-term effects of eCO_2 have primarily been investigated in tree species, as the effects are thought to be more important for perennial woody species. For this reason, studies that focused on short-term CO_2 treatments and the effects of low CO_2 concentrations (lCO_2) have been limited. However, the transcriptome from such treatments can also be useful for a better understanding of the molecular pathways involved in the response to CO_2. It was reported that the transcriptional changes in response to eCO_2 start within 2 h in *Arabidopsis* (Higuchi-Takeuchi et al. 2020). Short-term responses of the transcriptome using RNA-Seq have previously been reported in the coniferous species, *Pinus massoniana* (Wu et al. 2019). Genes expressed in the seedlings of *P. massoniana* treated with eCO_2 for three different durations (6, 12, and 24 h) were compared with a control sample (0 h), and a total of 7,088 DEGs were detected among the 140,863 transcripts. Although the DEGs may include circadian oscillation genes as the gene expression was compared against a 0 h sample, the results suggested that there were genes transcriptionally regulated by the short-term eCO_2 treatment in this coniferous species. The genes underlying quick CO_2 responses may be captured by transcriptome analysis after short-term treatment. Understanding the genes that respond quickly to CO_2 and how they act in long-term treatments could provide useful information to help elucidate CO_2 acclimation in tree species. Transcriptomes from lCO_2 conditions are also useful as the lCO_2 can cause contrasting responses to activity in the many molecular pathways compared to eCO_2 (Liu et al. 2016). For example, while photosynthesis activity is promoted, photorespiration is suppressed, and stomatal closure is promoted under eCO_2, contrasting results were reported in the lCO_2 conditions. Therefore, comparing the transcriptomes between lCO_2 and eCO_2 conditions may help to highlight the important genes in response to carbon availability.

In this study, a transcriptome analysis of shoots from two-year-old seedlings under short-term treatments with contrasting CO_2 concentrations (lCO_2: 200 ppm and eCO_2: 800 ppm) was conducted to identify the genes rapidly responding to the CO_2 changes. The DEGs between the eCO_2 and lCO_2 treatments were surveyed and annotated. The results will help us to dissect the complicated molecular network controlling *C. japonica* responses to CO_2.

Materials and methods

Plant materials

In May 2017, two-year-old seedlings that had been grown in a plastic greenhouse were transferred to day-light phytotron chambers (Koitotron K30-1602-G; Koito Industries, Yokohama, Japan). Both the greenhouse and the phytotron are located at the Forestry and Forest Products Research Institute in Tsukuba, Japan. Seedlings were cultured in pots (1/5000-are Wagner pot, one individual per pot) filled with Kanuma pumice and red granular soil (1:1 volume ratio) under natural light conditions. Solid fertilizer (15 g per pot) was applied on the soil surfaces in May 2017 (NexCOTE N-P-K = 16-7-12+Mg+TE, HYPONeX, Osaka, Japan). The plants were watered twice a week until the water drained from the bottom of the pots. The day/night air temperature and relative humidity were set at 25°C/20°C and 60%/70%, respectively. CO_2 concentration in the glasshouse was not controlled and was monitored with a portable CO_2 sensor (TR-76Ui, T&D Corp., Matsumoto, Japan) at 10 min intervals from July to October 2017; the CO_2 concentration ranged from 351–761 ppm, with the average of 467 ± 55 ppm (mean ± SD, n = 15,731). Plants were transferred from the glasshouse to a laboratory the evening prior to the sampling date (Table 1). The shoots were covered with aluminum foil before the CO_2 treatments to prevent gene expression perturbations from the light exposure. The seedlings were half-siblings derived from a single mother

Table 1. Sample abbreviations and treatment conditions

Individual ID	CO_2 treatment	Sample abbreviation[a]	Sampling Date	Sampling Time
A	200 ppm	L_A	2017-10-24	11:00
B	200 ppm	L_B	2017-10-25	11:00
C	200 ppm	L_C	2017-10-26	13:00
D	200 ppm	L_D	2017-10-27	11:00
E	200 ppm	L_E	2017-10-31	11:00
B	800 ppm	E_B	2017-10-25	13:00
C	800 ppm	E_C	2017-10-26	11:00
D	800 ppm	E_D	2017-10-27	NOON
E	800 ppm	E_E1[b]	2017-10-31	NOON
E	800 ppm	E_E2[b]	2017-10-31	13:00

a. Samples were given designations with the prefixes of "E" and "L" for eCO_2 (800 ppm) and lCO_2 (200 ppm), respectively.
b. E_E1 and E_E2 were sampled from different shoots of the same individual.

tree (a cultivar, Nakanojo-2).

RNA sampling and RNA sequencing (RNA-Seq)

The CO_2 treatments and the sampling were performed on the 24, 25, 26, 27, and 31 of October, 2017. Current-year shoots of the *C. japonica* were enclosed in a portable CO_2/H_2O gas exchange analyzer and exposed to a CO_2 concentration of 800 ppm (eCO_2) or 200 ppm (lCO_2). A cylindrical transparent chamber (Model 6400-05, Li-Cor, Lincoln, NE, USA) was used in the experiment. The leaf temperature and photosynthetically active photon flux density radiated on the needles were controlled at 25°C and 800 μmol m^{-2} s^{-1}, respectively, throughout the treatments. After 90 min of exposure to the respective CO_2 concentration, the shoots in the chamber were harvested with a pair of scissors and immediately frozen with liquid nitrogen. The 90 min CO_2 treatments caused significant changes in the metabolite concentrations in the *C. japonica* shoots (Miyazawa et al. unpublished data). Five samples for each treatment were harvested from five individuals (A, B, C, D, and E, Table 1). The elevated CO_2 treatment was not conducted for individual A, and instead, shoots on two branches of individual E were treated with eCO_2. One, two, and two samples treated with eCO_2 were harvested at approximately 11:00, noon, and approximately 13:00, respectively. All samples treated with lCO_2 were harvested at approximately 11:00, except for the sample of individual C harvested at approximately 13:00. Sunrise at Tsukuba is at approximately 6:00 in late October; thus the sampling was carried out 5–7 h after sunrise.

Total RNAs of the sampled shoots were extracted using Agilent Plant RNA Isolation Mini Kit according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA) and then subjected to DNase digestion using the Turbo DNA free kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA extraction was carried out twice for each sample, and these two replicates were subjected to Illumina sequencing. Samples were given designations with the prefixes of "E" and "L" for eCO_2 and lCO_2, respectively, and suffixed to indicate the replicate number (rp1 or rp2). For example, L_A_rp1 denotes replicate 1 of the lCO_2 treatment for individual A. Library preparation was conducted using the NEBNext Ultra RNA Library Prep Kit (New England Biolabs, MA, USA), and paired-end sequencing of 150 bp fragments was conducted on an Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) by Novogene (Beijing, China). The obtained raw reads were deposited and are available in the DDBJ sequence read archive under the accession number DRA012842.

Data processing and *de novo* assembly

Low-quality reads with quality scores of < 30 were trimmed using prinseq-lite.pl v0.20.4 (Schmieder and Edwards 2011). The remaining adaptor sequences were removed using cutadapt v1.18 (Martin 2011). rRNAs were filtered using SortMeRNA v2.1 (Kopylova et al. 2012), and the reads < 50 nt long were removed using the lengthsort command of SolexaQA++ v3.1.7.1 (Cox et al. 2010). *De novo* assembly of the reads was performed using Trinity v2.11.0, with a minimum contig length of 150 bp and the '--include_supertranscripts' option (Grabherr et al. 2011). The resulting contigs were further combined using cd-hit-est v4.7 (Fu et al. 2012) with a sequence identity threshold of 98% (-c 0.98), alignment coverage for the shorter sequence of 100% (-aS 1.0), and alignment coverage for the longer sequence of 0.5% (-aL 0.005). Potential contamination of other organisms in the obtained contigs was detected by comparing the generated contigs to the nucleotide database (nt) retrieved from The National Center for Biotechnology Information (NCBI, https://ftp.ncbi.nlm.nih.gov/blast/db/, downloaded on August 4, 2020) using BLASTn v.2.10.1, with the option to include or exclude Viridiplantae entries and a cut off value of e^{-20}. When one contig did not match the Viridiplantae entries but matched the entry derived from non-plant species, the corresponding contig was removed as a possible contaminating sequence. To filter contigs with low abundance, reads were mapped to the contigs using BWA-MEM v0.7.17 (Li and Durbin 2009). The count data for each contig was then obtained using featureCounts v2.0.3 (Liao et al. 2014). Contigs were filtered when the total transcripts per million (tpm) value of 20 libraries was ≤ 10. The putative coding regions of the remaining contigs were deduced using a TransDecoder v.5.3.0 with -m 30 (https://github.com/TransDecoder/TransDecoder/wiki). Contigs with predicted coding sequences (CDS) were further clustered based on their predicted peptide sequences using a cd-hit with a sequence identity threshold of 98% (-c 0.98), alignment coverage for the shorter sequence of 100% (-aS 1.0). The representative contigs in each cluster by cd-hit were concatenated with contigs having no CDS to make a tentative reference transcript set. As the noncoding RNAs may be functional and differentially expressed with the CO_2 concentrations, contigs without CDS remained in the reference transcript set. The putative functions of the transcripts were deduced using a BLASTx v.2.10.1+ search against *Arabidopsis* reference sequences (Araport11_genes.201606.pep.fasta) retrieved from The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/index.jsp) and UniProtKB/Swiss-Prot database (uniprot_sprot.fasta) retrieved from the UniProt Knowledgebase (https://www.uniprot.org/downloads, Schneider et al. 2005), with default parameters and a cut off value of e^{-5}. In addition, a total of 174,396 mRNA sequences of *C. japonica* were obtained from the NCBI nucleotide database (https://www.ncbi.nlm.

nih.gov/nucleotide/, queried by "*Cryptomeria japonica*" and "mRNA," downloaded on August 4, 2020) and compared to the tentative reference transcripts using BLASTn with the default parameters and a cut off value of e^{-20}.

DEG analysis and Gene Ontology (GO) annotation

To check for reproducibility, correlation coefficients between the gene expression levels in two replicates of the same individual were calculated using the cor.test function in the R v4.0.2 package following the spearman method (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/cor.test.html, R Core Team 2021). To calculate the correlation coefficient, tpm was used as the expression value for each gene.

DEGs between eCO_2 and lCO_2 were then investigated using DESeq2 v1.30.1 with a two-factor negative binomial GLM to consider the effects of CO_2 and the individuals (Love et al. 2014). In short, when gene expression was higher in one condition than another, and the tendency was shared between all tested individuals, it was identified as a DEG. One of the two replicates of L_C (L_C_rp2) and two replicates of L_A were excluded from the DEG analysis for the following reasons: the expression profile of L_C_rp2 deviated from the rest of the samples, and the shoots treated by eCO_2 (E_A) were not available from individual A. The difference in the gene expression profiles was large between individuals, as described later. Therefore, both the lCO_2 and eCO_2 samples of the same individual should be included in the DEG analysis to consider the effects of individuals. Otherwise, it would not be possible to determine whether the expression value of a gene observed under one condition in one individual is different from that under another condition in the same individual.

Based on the annotation of the homologous *Arabidopsis* genes, GO enrichment analysis was conducted using the web application, g:Profiler (Raudvere et al. 2019). The GO term was considered to be significantly enriched when the adjusted *p*-value was < 0.05.

Results and discussion

Transcriptome sequencing and *de novo* assembly

The *de novo* assembly of the reads resulted in 35,211 tentative transcripts, with an average length of 1,982.44 bp and N_{50} of 2,783 bp. CDSs were predicted for 34,490 transcripts but were not predicted for 721 genes. Based on the BLAST search results, 25,467 of the contigs (72.3%) showed sequence similarity to *Arabidopsis* genes. An additional 815 contigs had similarity to proteins in the UniProtKB/Swiss-Prot database. Therefore, 26,282 contigs (74.6%) were likely to be conserved genes. These contigs were further compared against the transcript sequences for *C. japonica* that were available in the NCBI nucleotide database. The results showed that 25,682 contigs (72.9%) had similarities to the mRNA of *C. japonica*. The contigs obtained may include new transcripts expressed in shoots that have not previously been sequenced.

Gene expression profiles of the samples under different CO_2 concentrations

First, the correlation between the gene expression profiles of two technical replicates was tested. The correlation coefficient (r) range was 0.78–0.94 (Fig. S1, Table S1). The percentages of rRNA in the total RNA were highly variable between samples (2.1%–38.7%), and this may be one of the reasons for the low correlation between replicates. The L_C replicates showed the lowest r-value between the two technical replicates ($r = 0.78$). The L_C_rp2 likely had a deviated expression profile when compared to the rest of the samples, as the r-values between L_C_rp2 and the other samples were relatively low ($r = 0.66$–0.81).

Comparing the whole transcriptome profile by hierarchical clustering showed that the gene expression profile was more strongly affected by individuals than by the CO_2 treatments (Fig. 1), as the samples appeared to be clustered by individuals but not by CO_2 treatments. The result suggested that both eCO_2 and lCO_2 treatments used in this study did not create a strong stress effect on the seedlings. The differences between the individuals could be due to both chronological and genetic differences. Individual C (E_C and L_C) had a more distinct expression pattern when compared with the other individuals (Fig. 1). This was probably because sampling of the E_C and L_C was done in the reverse order against the others as described in the Materials and methods. Furthermore, as the individuals were half-siblings, there were genetic differences

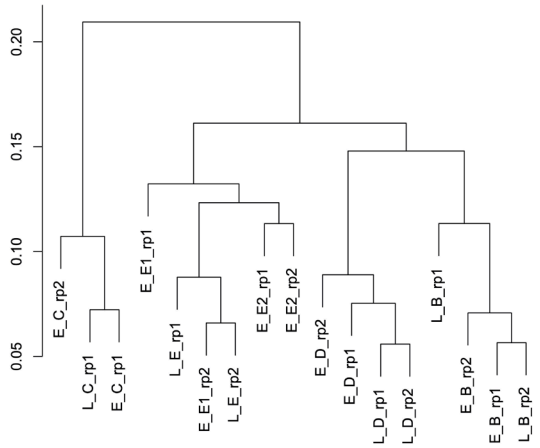

Fig. 1. Hierarchical clustering of samples based on the expression patterns of the transcripts.
Prefix denotes CO_2 conditions, and the suffix indicates the technical replicate number (rp1 or rp2). L_C_rp2 was excluded from the clustering analysis as it had a deviated expression pattern from the rest of the samples.

derived from their pollen parents. Variations caused by their genetic backgrounds could be reduced in future studies by using genetically identical samples, such as cuttings derived from a single individual. Although reducing the differences in the genetic background would allow us to detect DEGs with smaller expression differences, the DEGs identified among genetically different individuals may be more significant and fundamentally important in CO_2 responses in *C. japonica*.

In order to consider the individual effect in DEG detection, comparing samples treated with eCO_2 and lCO_2 within the same individuals was desirable, as mentioned in Materials and methods. L_A was removed from the DEG analysis because the E_A sample (i.e., shoots treated by eCO_2 in individual A) was unavailable. The deviated expression of the L_C_rp2 was supported in the clustering analysis (Fig. S2); thus, L_C_rp2 was also removed from the later analysis.

DEGs under different CO_2 concentrations

As a result of DEG analysis, 143 transcripts were identified as candidate DEGs between eCO_2 and lCO_2. Among them, 113 and 30 genes had increased expression under the eCO_2 and lCO_2 conditions, respectively (Fig. 2). It is of note that the direction of transcriptional regulation could not be addressed in this study, due to the lack of samples under aCO_2. We assumed that the gene expression levels at aCO_2 would be similar between the shoots within an individual, as the condition was thought to be almost the same before CO_2 treatment. When the observed expression of a gene was higher with the lCO_2 treatment than the eCO_2, it could be a result of increased gene expression under the lCO_2 and/or decreased gene expression under eCO_2, and *vice versa*. The gene expression under aCO_2 should be analyzed to confirm the constancy of gene expression under the aCO_2 and to elucidate the direction of transcriptional regulation in the future.

The percentage of the DEGs detected in the analyzed genes was less than 1% in this study (0.4%). The percentages also tended to be small in the analyzed tree species regardless of the duration of CO_2 treatment or the applied CO_2 concentration,

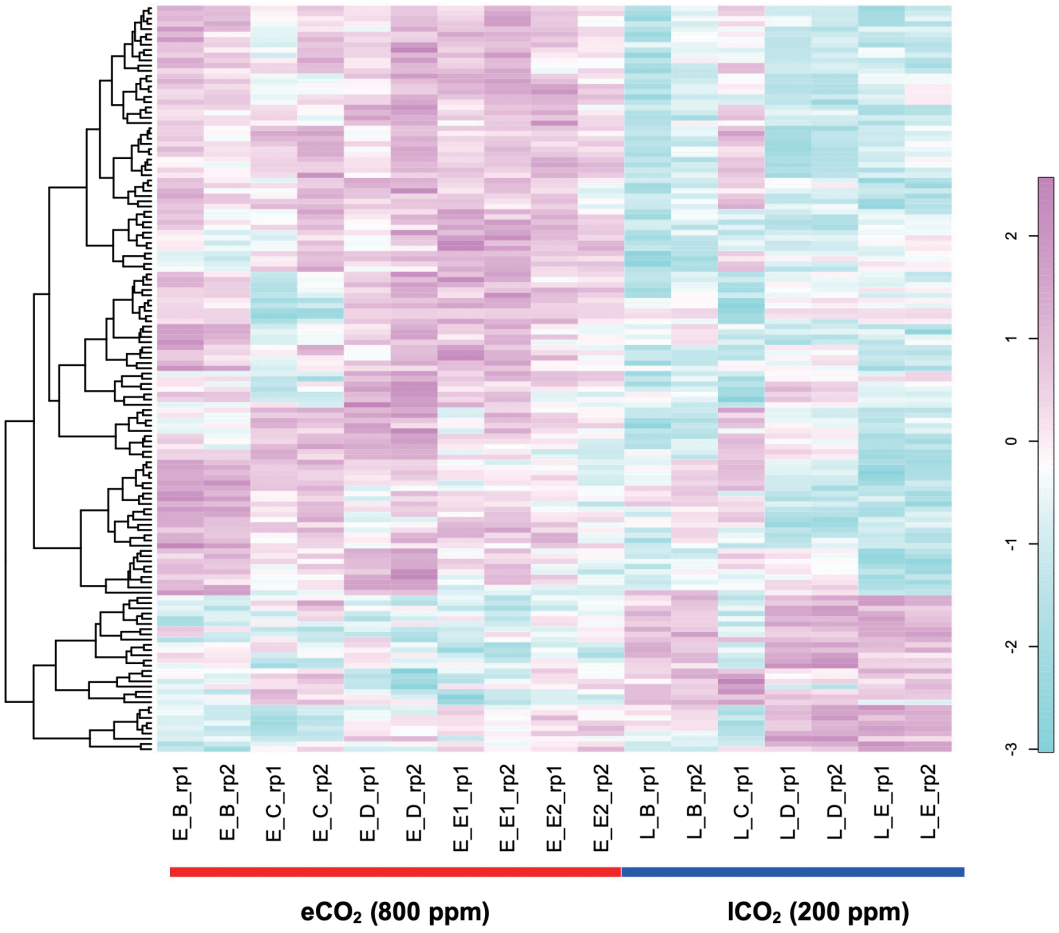

Fig. 2. Expression profiles of DEGs under different CO_2 concentrations.
Hierarchical clustering and heatmap of the relative expression levels of the detected DEGs. The sample names are indicated at the bottom of the heatmap. The abbreviation for each sample is listed in Table 1, and the suffix indicates the replicate number (rp1 or rp2). The color scale represents the relative expression values. Blue represents a low level, and magenta represents a high level of transcript abundance.

as described earlier in this manuscript. One of the exceptions was triploid white poplar, as 9.1% of the analyzed genes were detected as DEGs. The difference in growth temperatures of each CO_2 treatment, however, may also contribute to a higher percentage of DEGs (Wei et al. 2013). Another example is *P. massoniana*, as 7.6% of the analyzed unigenes were detected as DEGs in this species (Wu et al. 2019). It should be noted that the DEGs may include circadian oscillation genes, and thus the percentage might be an overestimate. The effects of CO_2 fluctuation on transcriptional regulation might be small in most of the expressed genes in *C. japonica*, as was reported in other plant species (Kanani et al. 2010, Eisenhut et al. 2017).

Annotation of highly significant DEGs

Based on their homology to the known gene database, 113 of the 143 DEGs (79.0%) were identified as conserved genes, whereas 30 genes did not show homology to the genes in the searched database. All DEGs had a CDS of more than 30 amino acids, except for one DEG. To identify what genes rapidly responded to the changes in CO_2 concentrations at the transcriptional level, the functions of highly significant DEGs were deduced from the homology search (Table 2). All but one of the top 20 significant DEGs was more expressed under eCO_2, and eight DEGs, including the most significant, were unknown genes. One of the key enzymes of the photorespiration pathway in angiosperm, glutamine synthetase 2, is absent in coniferous species (Miyazawa et al. 2018). This indicates that conifers might have specific carbon metabolism regulation and unique genes involved in this pathway. Eleven of the DEGs showed homology to *Arabidopsis* genes, but their function in the CO_2 response was unclear.

Only 1 DEG that was expressed more in the lCO_2 group was listed in the top 20 significant DEGs, and it was a homolog of nine-cis-epoxycarotenoid dioxygenase 4 (NCED4). NCED4 is an important enzyme associated with the synthesis of the plant hormone, abscisic acid (ABA), which is involved in many plant stress responses (Seo and Koshiba 2002). The downregulation of an NCED4 homolog under eCO_2 was also observed in *P. massoniana* (Wu et al. 2019). Thus, the abundance of NCED transcripts under lCO_2 observed in this study might be due to the downregulation of NCED under eCO_2. However, it is also possible that ABA synthesis was upregulated under lCO_2, as ABA participates in the acclimation to low CO_2 conditions (You et al. 2020). Although further experiments are required, ABA might have a common biological function in lCO_2 acclimation

Table 3. GO terms enriched in the eCO_2 or lCO_2 conditions

Enriched GO term	adjusted *p*-value	Number of genes
enriched under eCO_2		
molecular function		
GO:0004478 methionine adenosyltransferase activity	0.008	2
biological process		
GO:0009644 response to high light intensity	0.015	5
GO:0006556 S-adenosylmethionine biosynthetic process	0.017	2
GO:0046244 salicylic acid catabolic process	0.050	2
cellular component		
GO:0005618 cell wall	0.029	11
GO:0030312 external encapsulating structure	0.035	11
enriched under lCO_2		
biological process		
GO:0009813 flavonoid biosynthetic process	0.049	4
GO:0006949 syncytium formation	0.050	2
cellular component		
GO:0009507 chloroplast	0.024	8
GO:0009536 plastid	0.030	8

Table 2. The top 20 significant DEGs in the eCO_2 and lCO_2 conditions

Contig ID	adjusted *p*-value	$\log_2$FoldChange (lCO_2/eCO_2)	Bestmatch in Arabidopsis reference[a]	e-value	score
CJcdiox014701	1.15E-7	-1.250	no blast hit		
CJcdiox024976[a]	2.57E-7	-0.565	AT3G17611 RHOMBOID-like protein 14	1.00E-8	58.5
CJcdiox033608[a]	2.74E-6	-0.684	AT3G17611 RHOMBOID-like protein 14	10.00E-12	66.2
CJcdiox010378	2.43E-5	-1.481	AT4G27670 heat shock protein 21	1.00E-61	198
CJcdiox033941	6.29E-5	-0.555	AT3G29075 glycine-rich protein	2.00E-10	62
CJcdiox013528	8.68E-5	-0.736	no blast hit		
CJcdiox029450	1.55E-4	-1.282	AT5G63130 Octicosapeptide/Phox/Bem1p family protein	1.00E-24	99.8
CJcdiox018740	2.73E-4	-0.510	AT1G64980 Nucleotide-diphospho-sugar transferases superfamily protein	3.00E-126	376
CJcdiox031766	9.04E-4	-0.665	AT4G15130 phosphorylcholine cytidylyltransferase2	5.00E-136	398
CJcdiox011737	1.15E-3	-1.025	no blast hit		
CJcdiox023401	1.57E-3	-0.826	no blast hit		
CJcdiox023758	1.69E-3	-0.606	AT5G62390 BCL-2-associated athanogene 7	6.00E-17	84.7
CJcdiox009901	1.69E-3	0.760	AT4G19170 nine-cis-epoxycarotenoid dioxygenase 4 (NCED4)	0	603
CJcdiox014248	1.69E-3	-1.141	AT5G24530 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase	2.00E-58	202
CJcdiox032192	1.69E-3	-0.623	AT3G17390 S-adenosylmethionine synthetase family protein	0	729
CJcdiox032675	1.69E-3	-0.752	no blast hit		
CJcdiox030771	1.72E-3	-0.752	no blast hit		
CJcdiox002246	1.72E-3	-0.548	AT1G08200 UDP-D-apiose/UDP-D-xylose synthase 2	1.00E-118	369
CJcdiox004009	1.85E-3	-0.769	AT2G25270 transmembrane protein	1.00E-139	431
CJcdiox014524	1.86E-3	-0.498	AT2G17840 EARLY-RESPONSIVE TO DEHYDRATION 7	1.00E-119	381

a. These two contigs did not share a significant sequence similarity (e-value < e^{-5}).

among seed plants.

GO enrichment analysis of the DEGs responding to CO_2

GO term enrichment analysis was also conducted for the detected DEGs (Table 3). Six GO terms were enriched among the DEGs that were more highly expressed in the eCO_2 conditions, whereas 4 GO terms were enriched among the genes more highly expressed under lCO_2 conditions. The enriched terms were different between the two CO_2 treatment conditions, suggesting that different molecular pathways were activated. Although they were statistically significant, the number of genes annotated with enriched GO terms was limited for biological process or molecular function. In contrast, genes annotated with enriched GO terms as cellular component were more frequent among the detected DEGs.

The functional roles of genes with enriched GO terms in the eCO_2 response are unknown. In long-term eCO_2 treatment, the genes related to cell wall loosening and cell expansion are upregulated in the leaves of angiosperm species and contribute to growth stimulation (Huang and Xu 2015). Genes annotated with "cell wall" were also enriched under eCO_2 conditions in this study, such as xyloglucan endotransglycosylase/hydrolase and pectin esterase. They are involved in cell wall loosening and may respond quickly to eCO_2 at a transcriptional level in *C. japonica* as an angiosperm species. In contrast, among the genes with enriched GO terms under lCO_2, one gene with the GO term "syncytium formation" has roles in the regulation of stomata opening (AT1G69530, Zhang et al. 2011). Stomatal opening is one of the quickest responses in plants to low CO_2 conditions; therefore, increased expression of these genes under lCO_2 conditions is plausible. Genes annotated with "chloroplast" and "plastid" were also enriched among the DEGs that were more highly expressed under the lCO_2 conditions. Genes functioning in chloroplasts might be subjected to regulation at the transcription level by short-term CO_2 treatments.

DEGs in photosynthesis and photorespiration pathways

As photosynthesis and photorespiration are expected to be regulated in opposite directions under eCO_2 and lCO_2 conditions, the genes involved in these pathways were surveyed among the detected DEGs. In *Arabidopsis*, there are 221 and 40 genes annotated with GO terms related to "photosynthesis" and "photorespiration," respectively. Approximately 70% of those genes were expressed in samples analyzed in this study; however, only one DEG was annotated with the GO term "photosystem stoichiometry adjustment." This suggested that the expression of most photosynthesis- or photorespiration-related genes was not affected by short-term treatments with either CO_2 concentration. The CO_2 concentrations applied in this study are close to the natural range of fluctuation and thus did not exert a strong stress response in the seedlings. Another explanation may be that they are regulated in a post-transcriptional manner to enable a quick response to CO_2 fluctuations, as reported in angiosperm species (Liu et al. 2016).

Only one DEG annotated with the photosynthesis-related term was more highly expressed under lCO_2 conditions ($p = 0.036$). It was homologous to sigma factor 1 (*SIG1*, AT1G64860), a nuclear-encoding subunit of chloroplast RNA polymerase (Shimizu et al. 2010). The transcription level of *SIG1* is related to the transcription of the *psaA*, *psbB*, *psbE*, *rbcL*, and *rpoB* operons encoded in the chloroplast (Macadlo et al. 2020). The expression of these chloroplastic genes was not significantly affected after 90 min of exposure to the CO_2 conditions in the analyzed samples. It may have a role in balancing photosynthesis against CO_2 fluctuations, but careful analysis will be required in the future.

Conclusion

In this study, transcripts that quickly respond to eCO_2 and lCO_2 concentrations in *C. japonica* were identified. The observed differences in the expression levels between the eCO_2 and lCO_2 conditions could arise from the downregulation or upregulation of genes in each condition. Including samples under aCO_2 will give a more precise estimation of the transcriptional regulation of these genes. Furthermore, the magnitude of the difference was not large in most cases and varied among individuals. More samples with diverse genetic backgrounds will be required to validate the observed differences. Nevertheless, the results obtained in this study provide the first view of the rapid response in the transcriptome to CO_2 changes and will contribute to future studies aimed at unraveling the molecular mechanisms of CO_2 adaptation in *C. japonica*.

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Supplementary data

Supplementary data can be found at
https://www.ffpri.affrc.go.jp/pubs/bulletin/463/463toc-en.html

Fig. S1. Correlation between 20 RNA-Seq samples based on a spearman correlation analysis.

The deeper blue represents the higher correlation. The sample names are indicated on the left and top of the heatmap. Each cell shows the correlation between two samples. The correlation coefficients of two technical replicates are highlighted by red squares. The sample abbreviations are listed in Table 1. The suffix indicates the replicate number (rp1 or rp2).

Fig. S2. Hierarchical clustering of all samples based on the expression pattern of the transcripts.

Prefix denotes CO_2 conditions, and the suffix indicates the replicate number (rp1 or rp2).

Table S1. Summary statistics of read processing

a. Percentages against the total number of reads after quality control are shown.

b. Correlation coefficients between two replicates.

異なる二酸化炭素濃度で短時間処理したスギ針葉のトランスクリプトーム比較

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要 旨

二酸化炭素濃度によって発現が変動する遺伝子を明らかにするために、スギ針葉を用いてトランスクリプトーム解析を行った。得られたRNAリードの*de novo*アセンブリにより35,211の遺伝子配列が得られた。そのうち、113遺伝子が高CO_2、30遺伝子が低CO_2で高発現していた。推定された遺伝子の機能から、高CO_2と低CO_2では異なる分子経路の遺伝子発現が活性化されていることが示された。光合成や光呼吸の遺伝子の転写は大きく影響されなかったが、葉緑体にコードされる遺伝子の転写を制御する遺伝子の発現が低CO_2条件下で高くなっていた。検出された変動遺伝子の中に葉緑体に関連する機能を持つ遺伝子が多かったこととあわせ、葉緑体関連の遺伝子の転写調節がCO_2変化に対する初期応答の一つであることが示唆された。

キーワード：二酸化炭素、スギ、トランスクリプトーム

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