Transcriptome analysis of hormone-induced gene expression in *Brachypodium distachyon*

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*Brachypodium distachyon* is a new model plant closely related to wheat and other cereals. In this study, we performed a comprehensive analysis of hormone-regulated genes in *Brachypodium distachyon* using RNA sequencing technology. *Brachypodium distachyon* seedlings were treated with eight phytohormones (auxin, cytokinin, brassinosteroid, gibberellin, abscisic acid, ethylene, jasmonate and salicylic acid) and two inhibitors, Brz220 (brassinosteroid biosynthesis inhibitor) and prohexadione (gibberelline biosynthesis inhibitor). The expressions of 1,807 genes were regulated in a phytohormone-dependent manner. We compared the data with the phytohormone responses that have reported in rice. Transcriptional responses to hormones are conserved between *Brachypodium* and rice. Transcriptional regulation by brassinosteroid, gibberellin and ethylene was relatively weaker than those by other hormones. This is consistent with the data obtained from comprehensive analysis of hormone responses reported in *Arabidopsis*. *Brachypodium* and *Arabidopsis* also shared some common transcriptional responses to phytohormones. Alternatively, unique transcriptional responses to phytohormones were observed in *Brachypodium*. For example, the expressions of ACC synthase genes were up-regulated by auxin treatment in rice and *Arabidopsis*, but no orthologous ACC synthase gene was up-regulated in *Brachypodium*. Our results provide information useful to understand the diversity and similarity of hormone-regulated transcriptional responses between eudicots and monocots.

Human diets greatly depend on grasses, which have applications as energy crops and sustainable energy sources. Rice (*Oryza sativa*) and maize (*Zea mays*) are commonly used as model plants for monocots, as their genome sequence and extensive genetic resources are available¹. Draper et al. proposed *Brachypodium* (*Brachypodium distachyon*) as a novel model plant in 2001 because it has advantages such as a small genome size, short life cycle, small plant size, and the ability to self-pollinate². Furthermore, *Brachypodium* is more closely related to wheat [*Triticum aestivum*] and rye [*Secale cereale*] than rice and maize are, which makes it convenient for analysis of different grass groups using model systems¹. The whole genome sequence of *Brachypodium* inbred line Bd21 was released in 2010 as the first genome sequence available for the Pooidae subfamily³. Recently, a comprehensive collection of full-length cDNAs was reported by Mochida et al.⁴. Therefore, fundamental resources for *Brachypodium* molecular biology are being rapidly developed. Recently, Priest et al. reported an analysis of global gene expression in *Brachypodium* in response to abiotic stress⁵. Large-scale gene expression data are essential resources for model systems.

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Arabidopsis, The Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org) and Arabidopsis eFP browser6 integrate the public transcriptome data with other information to provide information useful for analysis of gene functions. Many tools for biological data mining, such as co-expression analysis (e.g., ATTED-II), transcriptome network analysis (AtCAST10), and prediction of transcriptome dynamics in natural conditions10, depend on the availability of large-scale transcriptome data.

Plant growth and development depend on phytohormone-mediated regulation of gene expression. Auxins, cytokinins (CKs), gibberellins (GAs) and brassinosteroids (BRs) generally promote plant growth and greatly influence plant stature and organ size. In contrast, ethylene, abscisic acid (ABA), salicylic acid (SA) and jasmonate (JA) regulate stress-related responses and/or growth retardation. The comprehensive response to a hormone was first reported in the eudicot model plant Arabidopsis thaliana by the AtGenExpress project (http://atpsmd.yokohama-cu.ac.jp)11. Hundreds of genes have been identified as being regulated by phytohormones. These comprehensive transcriptome data have been utilized in hundreds of studies of large-scale or gene-specific regulation of transcripts. Therefore, analyses of phytohormone-regulated transcriptomes in Brachypodium are essential for transcriptomic and genetic studies of this plant. Brachypodium shares about 80% of its genes with rice as homologs12, and is more closely related to rice than to Arabidopsis and other model plants. Analyses of phytohormone-regulated transcriptomes were performed in rice for auxin, CK, SA, ABA, JA, and ethylene13 and for auxin, CK, GA, BR, ABA, and JA14. Comparisons of the responses in Brachypodium and these other model plants will reveal both responses common to all plants and those specific to Pooidae.

GA and BR treatment generates almost no change in gene expression in wild-type Arabidopsis seedlings15; indeed, the endogenous GA and BR levels are too high (i.e., saturated) for exogenous hormones will reveal both responses common to all plants and those specific to Pooidae.

In this study, we performed a comprehensive RNA-seq analysis of the transcriptional responses in Brachypodium to eight phytohormones; auxin, BR, CK, GA, SA, ABA, JA, and ethylene. The compounds assayed included indole-3-acetic acid (IAA; auxin), trans-zeatin (tZ; CK), BL, Brz220 (Bz; inhibitor of BR synthesis), GA₄, prohexadione-calcium (Phx: inhibitor of GA synthesis), SA, ABA, methyl jasmonate (MJ; JA) and 1-amino-cyclopropane-1-carboxylic acid (ACC; ethylene precursor). In addition, we applied combination treatments of BL and Brz220 (a BR-biosynthesis inhibitor) and GA4 and Phx (GA-biosynthesis inhibitor) to Brachypodium. Transcriptional regulation of genes in Brachypodium was compared to that in Rice and Arabidopsis thaliana.

Results

Determination of hormone treatment conditions and identification of hormone-regulated genes. We selected phytohormone-responsive marker genes in Brachypodium using the following procedure. Twenty-eight phytohormone-responsive genes from rice13 and Arabidopsis thaliana11,15 were selected and their sequences were subjected to a BLAST search for the most similar sequences in Brachypodium genes in the AtCAST database (http://atpsmd.yokohama-cu.ac.jp/atcast/html/genelist/A0036_A0081.html). These findings suggest that the application of hormone biosynthesis inhibitors (Brz220 for BR and Phx for GA) to generate hormone-deficient conditions is a practical means of identifying BR- or GA-regulated genes in Brachypodium.

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control treatments (Table 5). Two hundred and twenty-four DEGs were detected in response to GA, 474
DEGs in response to BR, and 52 DEGs in response to ethylene. We designated these analyses as low
stringency; the DEGs are listed in Table 4, Data S9–S16.

| Hormone treatment | Chemicals | Concentration | Time |
|-------------------|-----------|---------------|------|
| Mock              | —         | —             | 3 h  |
| Auxin             | IAA       | 10μM          | 3 h  |
| Cytokinin         | tZ        | 1μM           | 3 h  |
| Gibberellin inhibitor | Phx   | 100 μM       | 5 h  |
| Inhibitor and Gibberellin | Phx+GA4 | 100 μM+3μM | 2 + 3 h |
| Brassinosteroi... | Brz220    | 100μM         | 5 h  |
| Salicylic acid    | SA        | 100μM         | 3 h  |
| Mock              | —         | —             | 1 h  |
| Abscisic acid     | ABA       | 10μM          | 1 h  |
| Jasmonate         | MJ        | 30μM          | 1 h  |
| Ethylene          | ACC       | 100μM         | 1 h  |

Table 2.  Hormone treatment conditions in *Brachypodium*.

| Hormone | Arabidopsis (Rice) gene | Brachypodium gene | Brachypodium qPCR |
|---------|-------------------------|-------------------|-------------------|
| Auxin   | Aux/IAA16               | Bradi4g02660.1    | No response       |
| Auxin   | Aux/IAA9                | Bradi2g31820.1    | Responded         |
| Auxin   | Aux/IAA18               | Bradi2g33417.1    | Responded         |
| Auxin   | (LOC_Os02g57250)        | Bradi3g55410.1    | Responded         |
| CK      | AtARR1                  | Bradi1g69480.1    | No response       |
| CK      | AtARR9                  | Bradi2g61000.1    | Responded         |
| CK      | (OsRR10)                | Bradi4g43090.2    | Responded         |
| BR      | BR6ox2                  | Bradi1g15030.1    | Responded         |
| BR      | dwf4                    | Bradi1g69040.1    | Responded         |
| BR      | CPD                     | Bradi4g43110.1    | No response       |
| GA      | GAI                     | Bradi1g11090.1    | Responded         |
| GA      | SCL3                    | Bradi2g60750.1    | No response       |
| GA      | AtEXP1                  | Bradi2g22290.1    | Responded         |
| ABA     | AT1G79520               | Bradi2g02050.1    | No response       |
| ABA     | Hs/AtB                  | Bradi3g26920.1    | Responded         |
| ABA     | GLTP                    | Bradi1g11280.1    | Responded         |
| ABA     | (LOC_Os09g21120)        | Bradi4g29360.1    | Responded         |
| Ethylene| ERS2                    | Bradi3g55730.1    | No response       |
| Ethylene| EFE,ACO4                | Bradi3g57620.1    | No response       |
| Ethylene| DL4170C                 | Bradi2g27140.1    | No response       |
| Ethylene| (OsETR2)                | Bradi5g00700.1    | Responded         |
| Ethylene| (LOC_Os01g73200)        | Bradi4g27580.1    | Responded         |
| JA      | JMT                     | Bradi2g47550.1    | No response       |
| JA      | OPR3                    | Bradi3g37650.1    | Responded         |
| JA      | (OsMYC2)                | Bradi3g34200.1    | Responded         |
| SA      | PR-1                    | Bradi1g57590.1    | Responded         |
| SA      | WRY70                   | Bradi2g44270.1    | Responded         |
| SA      | AtMES1                  | Bradi2g52110.1    | Responded         |

Table 1.  Selection of marker genes.
Comparison with rice and Arabidopsis. First, we compared the phytohormone-regulated genes in *Brachypodium* with those in rice. A comparison of our experimental conditions with those used in previous rice studies is presented in Table S1. Garg et al. reported the responses of rice seedlings to six hormones: auxin, CK, SA, ethylene, ABA, and JA. We made a list of predicted orthologs of the *Brachypodium* DEGs in rice and evaluated whether they are regulated in the same manner (Table 6). Since Garg et al. reported phytohormone-regulated DEGs in rice without statistical information for each hormone treatment, we simply checked whether the orthologous genes from *Brachypodium* were included in the list of Garg et al. or not. We predicted 408 orthologs to the *Brachypodium* DEGs in rice. In total, 326 orthologous genes (80%) were regulated by phytohormones in the same manner. The transcriptional response to CK and ABA in *Brachypodium* was consistent with that in rice: 100% of the orthologs of the *Brachypodium* DEGs that were regulated by CK (BAP) were also regulated in rice by CK (BAP), and 98% of the orthologous genes were regulated by ABA in rice. *Brachypodium* and rice shared many auxin- and JA-responsive orthologous genes: 67% of the orthologs were also regulated by IAA in rice. Garg et al. reported that Aux/IAA and GH3 are IAA-responsive gene families. Consistent with this report, many genes in the Aux/IAA family (*Bradi1g55370, Bradi2g04910, Bradi2g34030, Bradi2g16850,*...
Eighty-one genes were identified as SA-responsive genes in the high-stringency analysis (Data S3). Genes with the term “defense response to fungus” were enriched in GOE analysis (Table 7). A homolog of the PR-1 gene (Bradi1g57590) was up-regulated by SA treatment (Fig. 3).

ABA response. Four hundred and forty-five genes were identified as ABA-responsive genes in the high-stringency analysis (Data S4). Genes with the term “response to abscisic acid stimulus” were enriched in the GOE analysis (Table 7). Genes homologous to those encoding transcription factors involved in transcriptional regulation in response to ABA (i.e., ABF, NAC, MYb, bHLH) were detected as DEGs (Bradi3g00730, Bradi1g59982, Bradi3g50220, Bradi4g32090, Bradi5g17170, Bradi2g22660, Bradi3g57960, Bradi3g19010, Bradi3g38200, Bradi4g29380, Bradi1g22180, Bradi1g11800, Bradi2g41530, Bradi1g20360, Bradi4g35910).
JA response. Three hundred and eighty-three genes were identified as JA-responsive genes in the high-stringency analysis (Data S5). Genes with the term “response to wounding” were enriched in the GOE analysis (Table 7). Genes involved in the biosynthesis of jasmonic acid (LOX3 homolog (Bradi5g11590), LOX4 homolog (Bradi1g72690), AOS homolog (Bradi3g08160, Bradi1g07480, Bradi1g69330), AOC3 homolog (Bradi1g15840), OPR3 homolog (Bradi3g37650) and OPCL1 homolog (Bradi1g76280) were up-regulated by MJ treatment (Fig. 4). The JAZ genes in Arabidopsis encode JA receptors19. Two members of the JAZ family (Bradi3g23190, Bradi1g58490) were also up-regulated in Brachypodium.

GA response. No gene was detected as a DEG in the high-stringency analysis (Data S6). This indicates that the GA-inducible gene expression response is relatively weaker than those to other hormones, which is consistent with the GA response in Arabidopsis11. When the effects on gene expression of the GA biosynthesis inhibitor Phx treatment were compared to those of the mock 3-h and Phx+GA treatments (low-stringency analysis), 224 genes were detected as DEGs (Data S14). Of these genes, those with the term “response to nitrate” were the most enriched in the GOE analysis (Table 8). In Arabidopsis, many GA-biosynthesis genes are also GA-responsive, since there is a negative feedback regulation in the expression of GA-biosynthesis genes11. The read counts of many genes involved in gibberellin biosynthesis (homologs of GA2oxs, GA3oxs and GA20oxs; i.e., Bradi1g56200, Bradi1g56210, Bradi1g56220, Bradi2g16727, Bradi4g23540, Bradi2g16750, Bradi3g49390, Bradi2g32577, Bradi2g24980, Bradi5g16040, Bradi1g59570, Bradi2g06670, Bradi2g57027, Bradi2g34837, Bradi2g19900, Bradi2g50280) were low, and none were detected as DEGs (log 2 cpm < 1, Table S4). Genes of Xyloglucan endotransglucosylase/hydrolases TCH4 (Bradi3g18690) and XTH12 (Bradi3g10310) were significantly up-regulated. Many genes in Peroxidase superfamily (Bradi1g17860, Bradi1g17877, Bradi5g27160, Bradi2g38690, Bradi2g38700, Bradi1g44790, Bradi1g59520, Bradi2g0830, Bradi2g38670, Bradi3g33780, Bradi1g61550, Bradi2g11320, Bradi1g23190, Bradi1g58490) were significantly up-regulated.

Table 7. GOE analysis of DEGs obtained under high-stringency conditions.
Bradi3g10470, Bradi5g10070, Bradi2g38685, Bradi1g61530, Bradi2g12180, Bradi1g20020, Bradi2g20840, Bradi1g33730, Bradi1g44800, Bradi2g38680, Bradi3g54010) were also up-regulated.

BR response. Only four genes were detected as DEGs in the Brz220 treatment compared with all other treatments (high-stringency analysis, Data S7). This indicates that the BR-inducible gene expression response is relatively weak compared with those to other hormones, which is consistent with the BR response in Arabidopsis11. When the effects on gene expression of the Brz220 treatment were compared with those of the mock 3-h and Brz220 + BL treatments (low-stringency analysis), 474 genes were detected as DEGs (Data S15). Of these genes, those with the term “RNA elongation” were enriched in the GOE analysis (Table 8). Genes with the term “photosynthesis” were also enriched in the GOE analysis. P450 genes involved in the biosynthesis of brassinosteroids (Bradi1g15030, homolog of BR6ox2 and Bradi5g12990, homolog of DWF4) were up-regulated by Brz220 compared to the mock 3-h and Brz220 + BL treatments (Fig. 5), although the fold change of Bradi5g12990 was less than twice. This is consistent with the BR response in Arabidopsis; BR6ox2 and DWF4 were up-regulated in the det2 mutant of Arabidopsis compared to the wild-type (Fig. 5).

Ethylene response. No gene was detected as a DEG in response to ethylene in the high-stringency analysis (Data S8). To confirm response of seedlings to ACC treatment under our experimental conditions, plants were treated with ACC for a further week. Seedlings showed growth inhibition when grown in the presence of 10 μM ACC (Fig. S3), indicating that plants respond to exogenous ACC. Therefore, gene expression in response to ACC treatment was compared to that in response to the mock 1-h and mock 3-h treatments (low-stringency analysis). Fifty-two genes were detected as DEGs (Data S16). Of these DEGs, those with the term “RNA elongation” were enriched in GOE analysis. A gene homologous to EIN4, which is involved in sensing ethylene (Bradi5g00700) was up-regulated, and a gene homologous to ACS6, which is a member of the ethylene synthesis gene family, (Bradi5g19100) was down-regulated by ethylene treatment compared to the mock 1-h and mock 3-h treatments.

Discussion

The comprehensive transcriptional response of Brachypodium to phytohormones was analyzed in this study. To obtain clear responses from Brachypodium, we optimized the experimental design using the expressions of selected Brachypodium marker genes to validate hormone response. The concentrations of phytohormones used in this study were similar (1–10-fold) to those used in the analysis of the response to hormones in rice and Arabidopsis (Table S1), with the exception of the concentration of BL (100-fold compared to that used for Arabidopsis). We successfully identified BR- and GA-regulated genes (e.g., Bradi1g15030, homolog of BR6ox2 as a BR-responsive gene and Bradi3g18690, homolog of TCH4 as a GA-responsive gene).

Table 8. GOE analysis of DEGs obtained under low-stringency conditions.
| Gene IDs | log<sub>2</sub> ratio | stat | cpm |
|----------|----------------------|------|-----|
| AT3G16500 IAA26 PAP1 | 0.83 | 0.30 | |
| AT1G51950 IAA18 | 0.18 | 0.59 | |
| AT3G25890 IAA28 IAR2 | -0.46 | 0.37 | |
| Bradi2g19367 | -0.73 | 1.00 | 1.29 |
| Bradi2g49420 | -0.18 | 1.00 | 6.65 |
| Bradi2g05650 | -1.07 | 1.00 | 3.02 |
| Bradi2g33417 | 0.48 | 1.00 | 0.96 |
| Bradi5g09667 | -0.62 | 1.00 | 2.31 |
| Bradi5g24380 | -1.68 | 1.00 | 2.59 |
| AT5G7420 IAA33 | -0.19 | 0.54 | |
| Bradi4g22057 | 0.22 | 1.00 | 0.87 |
| Bradi4g22050 | 0.00 | 1.00 | -2.76 |
| AT1G15050 IAA34 | 1.13 | 0.01 | |
| AT2G01200 IAA32 MEE10 | | | |
| AT3G62100 IAA30 | 3.22 | 0.00 | |
| AT2G6990 IAA20 | 0.15 | 0.53 | |
| AT4G36460 IAA11 | 1.69 | 0.24 | |
| AT4G40100 IAA10 | 0.61 | 0.05 | |
| AT3G17600 IAA31 | -0.19 | 0.42 | |
| Bradi2g11120 | 2.71 | 0.00 | 3.34 |
| Bradi3g54610 | 3.99 | 0.00 | 4.07 |
| AT4G32280 IAA29 | 4.07 | 0.00 | |
| Bradi4g35960 | 4.38 | 0.00 | -0.28 |
| Bradi3g55410 | 0.19 | 1.00 | 4.16 |
| AT3G15540 IAA19 MSG2 | 4.02 | 0.08 | |
| AT1G2830 IAA6 SHY1 | 2.36 | 0.00 | |
| AT1G15580 IAA5 | 4.99 | 0.10 | |
| AT4G14560 IAA1 AXR5 | 2.76 | 0.03 | |
| AT3G23030 IAA2 | 2.92 | 0.01 | |
| AT4G29080 IAA27 PAP2 | -0.28 | 0.17 | |
| AT5G43700 IAA4 | 1.10 | 0.00 | |
| AT1G04240 IAA3 SHY2 | 0.86 | 0.18 | |
| Bradi2g07770 | 0.12 | 1.00 | |
| Bradi2g31820 | 1.22 | 0.00 | 9.13 |
| AT1G246420 | 2.04 | 0.00 | 5.15 |
| Bradi2g16850 | 1.44 | 0.00 | 8.01 |
| Bradi1g14230 | 1.35 | 0.41 | |
| Bradi4g02580 | 0.81 | 1.00 | |
| AT4G14550 IAA14 SLR | 1.76 | 0.00 | |
| AT3G23050 IAA7 AXR2 | 1.12 | 0.00 | |
| AT1G04250 IAA17 AXR3 | 0.36 | 0.33 | |
| AT3G04730 IAA16 | 0.46 | 0.03 | |
| Bradi4g02600 | 0.45 | 1.00 | |
| Bradi1g14240 | 1.02 | 1.00 | |
| Bradi1g09990 | 0.90 | 0.54 | |
| Bradi2g30300 | 1.85 | 0.00 | 5.19 |
| Bradi2g04910 | 2.02 | 0.00 | 4.73 |
| Bradi1g36630 | 1.15 | 1.00 | |
| AT2G33310 IAA13 | 2.11 | 0.15 | |
| AT1G04550 IAA12 BDL | 0.51 | 0.35 | |
| Bradi1g55370 | 1.76 | 0.00 | 4.93 |
| AT2G22670 IAA8 | 0.22 | 0.06 | |
| Bradi3g13370 | -0.92 | 1.00 | |
| AT5G65670 IAA9 | 0.78 | 0.00 | |
| Bradi1g05410 | 0.60 | 1.00 | |

Figure 1. Phylogenetic tree of Aux/IAA genes and their transcriptional responses to auxin in *Brachypodium* and *Arabidopsis*. *Brachypodium* Aux/IAA family members were retrieved using the BLAST software. Protein sequences were aligned with MAFFT and similarities (percentage identity) are shown as a phylogenetic tree. *Brachypodium* genes are shown in red and *Arabidopsis* genes in black. log<sub>2</sub> ratio represents the gene expression ratio between the read count in IAA treatment divided by the average read count from all other treatments. Stat represents the p-value of the microarray experiment of *Arabidopsis* treated with IAA<sup>11</sup> or the FDR of RNA-seq data when the count of IAA treatment was compared with all other treatments. log<sub>2</sub> ratio is hatched in red if genes are up-regulated. cpm represents the average of log<sub>2</sub>-scaled read counts per million reads in all experiments.
In rice, 80% of the orthologs of the *Brachypodium* DEGs were also regulated by CK, ABA, auxin, JA, SA, and ethylene. These data show that major transcriptional responses to these phytohormones are shared in monocots, although fewer orthologous genes were commonly regulated by SA and ethylene.

It was reported that the SA level in rice was the highest among the tested plants. This may be one reason for the different responses to SA at similar concentrations. The difference in ethylene-regulated genes between rice and *Brachypodium* was also reported by Pacheco-Villalobos et al.\(^{21}\). Sato et al.\(^{14}\) also reported the responses of rice seedlings to six hormones: auxin, CK, GA, BR, ABA, and JA. However, it was difficult to compare our data with theirs because they reported their data as part of a large transcriptome dataset and no analysis of hormone-responsive gene expression was performed using the data.

The transcriptional responses to phytohormones and the mechanisms involved in transcriptional regulation in *Arabidopsis* are well characterized. In addition, manually curated GO terms for *Arabidopsis* is very helpful to analyze large transcriptome dataset. Therefore, we compared the transcriptional response to phytohormones in *Brachypodium* to that in *Arabidopsis thaliana* in detail. A comprehensive analysis of phytohormone-regulated genes of *Brachypodium* revealed common transcriptional responses between *Brachypodium* and *Arabidopsis*. To compare the accumulation of GO terms in *Brachypodium* to *Arabidopsis*, we assigned new GO terms to the *Brachypodium* genes using the *Arabidopsis* GO terms.

Some results of the GOE analyses in *Arabidopsis* and *Brachypodium* were common following treatment with the same phytohormones. The GO terms “response to auxin stimulus” had the lowest p-values in auxin-regulated DEGs. Similarly, “cellular response to cytokinin stimulus” in CK-regulated DEGs, “defense response to fungus” in SA-regulated DEGs, "response to abscisic acid stimulus" in ABA-regulated DEGs and “response to wounding” in JA-regulated DEGs had the lowest p-values in *Brachypodium* (Tables 7, 8). These GO terms were also enriched in the hormone-responsive genes in *Arabidopsis* (Table S3). The GO term “response to nitrate” was enriched in DEGs in the Phx treatment (Table 7), which was consistent with the enriched GO of the GA-biosynthesis mutant *ga1-5* when compared to the wild type (Table S5). The GO term “photosynthesis” was enriched in DEGs of Brz-treated *Brachypodium* (Table 7), which was consistent with the enriched GO terms of the BR-biosynthesis mutant *det2* when compared to wild-type *Arabidopsis*. Consistent with the findings in BR-treated *Arabidopsis det2*, genes involved in BR-synthesis were detected as DEGs in Brz-treated *Brachypodium* (Fig. 5). Fewer were detected as DEGs in response to ACC than to other hormones (Tables 4, 5). This was also consistent with transcriptional regulation in ethylene-treated *Arabidopsis*\(^{11,15}\). These results suggest that many molecular mechanisms characteristic of the responses to phytohormones in *Arabidopsis* are also present in *Brachypodium*.

In contrast, there were some differences in the hormone responses of *Brachypodium* and *Arabidopsis*. In *Arabidopsis*, genes involved in ethylene synthesis and signaling, *ACO1*, *ACO3*, *ACO4/EFE*, *ACS4*, *ACS8*,

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**Figure 2.** Phylogenic tree of ARR genes and their transcriptional responses to CK in *Brachypodium* and *Arabidopsis*. *Brachypodium* ARR family members were retrieved using the BLAST software. Similarities in protein sequences (percentage identity) are shown as a phylogenetic tree. *Brachypodium* genes are shown in red and *Arabidopsis* genes in black. log ratio represents the gene expression ratio between the read count in tZ treatments divided by the average read count from all other treatments. Stat represents the p-value of the microarray experiment in *Arabidopsis* treated with t-zeatin for 1 h\(^{11}\) or FDR of RNA-seq data when the count from tZ treatment was compared with all other treatments. log ratio is hatched in red if genes are up-regulated. cpm represents the average of log2-scaled read counts per million reads from all experiments.
and ERF1, are responsive to auxin treatment. Only one gene from the ACO gene family (Bradi2g41840) was detected as a DEG in auxin-treated Brachypodium (Fig. S2). Genes involved in ethylene signaling, such as ERF2, 8, 11, ERS1, EBF2, ETR2, CTR1 and EIN2, are regulated by ACC treatment in Arabidopsis. However, in Brachypodium, only one gene homologous to EIN4 (Bradi5g00700) was detected as a DEG. These differences in auxin- and ethylene-responsive genes suggest divergent auxin-ethylene relationships between eudicots and monocots. Pacheco-Villalobos et al. reported that the cross-talk between ethylene and auxin in Brachypodium differs from that in Arabidopsis. Further analysis of these differentially expressed genes will improve our understanding of the different functions of these hormones and the related genes in Brachypodium and Arabidopsis.

We encountered some difficulties in the analysis of RNA-seq data. We prepared 13–30 million reads because Liu et al. reported that the increase in number of DEGs drops after 10–15 million reads. However, the transcriptional responses of some genes might not have been detected because of their low read counts. For example, none of the genes involved in GA-biosynthesis was detected as Phx- or GA-responsive in Brachypodium. However, it was reported that the expressions of the GA-biosynthetic genes AtGA2ox1, AtGA2ox2, GA3ox1 and GA20ox1 changed in Arabidopsis GA-deficient mutants following endogenous GA treatment. The read counts of these GA-biosynthetic genes in Brachypodium were low (Table S4). In Arabidopsis and rice, few genes respond to ethylene. In this study, no gene in the expansin family was detected as ethylene-responsive in Brachypodium, while AtEXP8 and AtEXP18 in Arabidopsis and OsEXP1, OsEXP2 in rice are responsive. The expression levels of 27 expansin genes were low (cpm < 1, Table S6). The growth of Brachypodium was reduced by ACC treatment (Fig. S3); therefore, some expansin genes may be regulated by ethylene to control cell elongation. To examine the transcriptional regulation of these poorly expressed genes, further analysis using additional RNA-seq reads with different tissues or different growth stages, and/or complementary methods (such as microarray or quantitative PCR) are required.

| Gene IDs | log₂ ratio | stat | cpm |
|---------|------------|------|-----|
| At1g01310 | 0.00 | 0.85 | |
| Bradi2g62280 | -0.17 | 1.00 | 6.69 |
| Bradi2g14240 | -1.98 | 1.00 | -2.53 |
| At4g25780 | -2.04 | 0.00 | |
| Bradi3g60260 | 1.04 | 1.00 | -1.02 |
| Bradi3g60230 | 0.90 | 1.00 | -2.44 |
| At5g57625 | |
| At4g25790 | -0.70 | 0.21 | |
| At5g02730 | 0.00 | 0.80 | |
| At5g09590 | 0.00 | 0.82 | |
| Bradi1g57540 | -3.60 | 1.00 | -2.05 |
| At3g19690 | 0.00 | 0.42 | |
| At4g07820 | 0.00 | 1.00 | |
| At4g33720 | -2.90 | 0.14 | |
| At5g26130 | -0.67 | 0.31 | |
| At4g33710 | 0.00 | 0.32 | |
| At2g14610-PR1 | 2.51 | 0.04 | |
| At2g14580 | 0.00 | 0.26 | |
| At1g50060 | 0.00 | 0.79 | |
| At5g50050 | 0.00 | 0.34 | |
| Bradi1g57590 | 1.73 | 0.07 | 4.21 |
| Bradi1g57580 | 0.10 | 1.00 | 3.62 |
| Bradi1g12360 | -0.10 | 1.00 | 6.46 |
| At4g30320 | -0.41 | 0.47 | |
| Bradi3g53630 | -1.04 | 1.00 | 1.97 |
| Bradi3g53637 | -4.11 | 1.00 | -1.82 |
| At4g33730 | -0.85 | 0.24 | |
| At2g19990 | -2.27 | 0.16 | |
| At4g1470 | 0.00 | 0.54 | |
| Bradi4g38910 | -4.32 | 1.00 | -1.70 |
| Bradi4g09637 | -0.09 | 1.00 | 1.65 |
| Bradi3g53680 | 0.00 | 1.00 | -2.76 |

Figure 3. Phylogenetic tree of PRI, its orthologs and transcriptional responses to SA treatment in Brachypodium and Arabidopsis. Brachypodium PRI-like genes were retrieved using the BLAST software. Similarities in protein sequences (percentage identity) are shown as a phylogenetic tree. Brachypodium genes are shown in red and Arabidopsis genes in black. log₂ ratio represents the gene expression ratio between read counts from SA treatment divided by the average read counts from all other treatments. Stat represents the p-value of the microarray experiment. Arabidopsis treated with SA for 3 h for Arabidopsis genes or FDR of RNA-seq data when the counts from SA treatment were compared with all other treatments for Brachypodium genes. log₂ ratio is hatched in red if genes are up-regulated and in blue if down-regulated. cpm represents the log₂-scaled read count per million reads of RNA-seq data from all treatments.
Figure 4. Biosynthesis pathway of JA and transcriptional responses in *Brachypodium* and *Arabidopsis*. Homologs of jasmonate biosynthetic genes in *Brachypodium* were retrieved using the BLAST software. *Brachypodium* genes are shown in red and *Arabidopsis* genes in black. Stat represents the p-value of the microarray experiment. *Arabidopsis* treated by MJ for 3 hours and FDR of RNA-seq data when the count of MJ treatment was compared with all other treatments. log₂ ratio represents the gene expression ratio between read counts from the MJ treatment divided by the average read counts from all other treatments. log₂ ratio is hatched in red if genes are up-regulated. cpm represents log₂ scaled read count per million reads of RNA-seq data from all treatments.

### Methods

#### Plant materials

Seedlings of *Brachypodium distachyon* Bd21 were grown on 1/2 MS medium supplemented with 1% sucrose and 0.8% agar 4 days after breaking dormancy at 4 °C in the dark. The plants were pre-incubated for 24 h in liquid culture with shaking (70–80 rpm) and then treated with phytohormones (Table 2). The phytohormones were dissolved in DMSO before addition to the liquid cultures. DMSO (0.1% (v/v)) was used for mock treatments. For the “Inhibitor and GA” and “Inhibitor only” processes, the phytohormones (Table 2) were dissolved in DMSO before addition to the liquid cultures before treatment with the GA inhibitor, prohexadione-calcium (Wako), or BR-inhibitor, brassinazole22, respectively. All growth and treatment processes were performed at 22 °C under continuous light. Phytohormone treatments were conducted twice independently as biological replicates, and the hormone responses were confirmed using the transcriptional responses of marker genes (Table 1). Transcriptional responses of marker genes were checked with RT-PCR using gene specific primers (Table S7) prior to the RNA-seq analysis.

#### RNA extraction

RNA was extracted from each sample using the RNeasy Mini Kit (QIAGEN). The concentration and quality of the RNA samples were determined using a Nanodrop 2000 instrument (Thermo Scientific). RNA extracted from two biological replicates was mixed and used for the RNA-seq analysis.

#### Strand-specific RNA-seq analysis

Strand-specific RNA libraries were prepared using the TruSeq Small RNA Sample Prep Kit (Illumina) and TruSeq RNA sample Preparation Kit v2 (Illumina) following the instructions in the Directional mRNA-seq Library Prep. (Pre-Release Protocol Rev.A (Illumina)). Fragmented Poly(A)-RNA was treated with T4 polynucleotide kinase (TAKARA) for phosphorylation of the 5’ end. An RNA 3’ adapter was ligated to the 3’ end using T4 RNA ligase 2, truncated (NEB), then an RNA 5’ adapter was ligated to the 5’ end using T4 RNA ligase 1 (NEB). Single-strand cDNA was synthesized with a primer for the RNA 3’ adaptor. Amplified cDNA was size-selected using 6% polyacrylamide gel electrophoresis, then used as the sequencing library. The quality of all libraries was assessed using
**Figure 5.** P450 genes involved in BR biosynthesis and transcriptional responses to Brz in *Brachypodium* and *Arabidopsis*. *Brachypodium* BR biosynthetic genes were retrieved using the BLAST software. Similarities in protein sequences (percentage identity) are shown as a phylogenetic tree. *Brachypodium* genes are shown in red and *Arabidopsis* genes in black. log₂ ratio represents the gene expression ratio between read counts from the Brz treatment divided by the average read counts from mock 1-h and Brz+BL treatments. Stat represents the p-value of the microarray experiment, *Arabidopsis det2* compared with wild-type [11] for *Arabidopsis* genes or FDR of RNA-seq data when the counts from the Brz treatment were compared with mock 1-h and Brz+BL treatments for *Brachypodium* genes. log₂ ratio is hatched in red if genes are up-regulated and in blue if down-regulated. cpm represents log₂ scaled read counts per million reads of RNA-seq data from mock 1-h, Brz and Brz+BL treatments.

**Mapping of RNA-Seq reads, transcript assembly and abundance estimation.** The sequences were aligned to the Phytozone 9.0 *Brachypodium distachyon* reference genome Bdistachyon_192 (Bdistachyon_192_hardmasked.fa.gz) using TopHat v2.0.11 [30], which is integrated with Bowtie v2.2.1 [31]. The Bowtie index was generated from Bdistachyon_192 and counting of the mapped reads was performed using Cufflinks v2.2.1 [32] and genome annotation reference PASA_updates.phytozome.gff3 [4]. Read counts were generated using Cuffdiff [33]. Differentially expressed genes (DEGs) were identified with edgeR 16, as follows. Parameters and function usages in edgeR were determined to maximize the specificity for detecting hormone marker genes (Table 1) as DEGs. Read counts were normalized with the function "calcNormFactors" . The TMM normalization method in edgeR can normalize different amounts of RNA-Seq data without increasing the false-positive rate of detecting DEGs [33]. Dispersions among genes and among replications were estimated by the functions "estimateGLMCommonDisp", "estimateGLMTrendedDisp" and "estimateGLMTagwiseDisp" and p-values were calculated using the likelihood-ratio test with the generalized linear models. Calculated p-values were adjusted using the false discovery rate (FDR) of Benjamini and Hochberg's approach [34]. DEGs were defined as FDR < 0.05 and up- or down-regulated genes were identified in the DEGs as those having a log₂ ratio of cpm > 1 or that of cpm < -1, respectively.

**Phytohormone-regulated genes in Arabidopsis.** Microarray data from *Arabidopsis thaliana* treated with phytohormones were retrieved from the AtGenExpress project [11]. Signals in the microarray data were calculated using R package affy (1.42.3) with the MAS5 method [35]. DEGs were identified as p-value < 0.1 by Student's t-test and up- and down-regulated genes were defined in the DEGs as log₂ ratios of signals > 1 and < -1, respectively. GOE analyses were performed with the DEGs.
Determination of orthologous/homologous genes and generation of the phylogenetic tree. To obtain orthologous gene pairs, Arabidopsis protein sequences (TAIR10) and rice protein sequences (RGAP 7) were applied to BLAST (blastp) to search for homologous sequences in the Brachypodium protein sequence (Bdistachyon_192_peptide.fa from Phytozome 9.0). Brachypodium sequences with top hit were collected and applied to BLAST again to search for the most similar sequences in Arabidopsis and rice. If the results of the second BLAST returned the former Arabidopsis or rice sequence, the corresponding gene pairs were selected as orthologous gene pairs. To obtain genes from each gene family in Brachypodium, protein sequences of members of each family in Arabidopsis were applied to BLAST (blastp with default settings) to search for similar sequences in Brachypodium (Bdistachyon_192_peptide.fa). Brachypodium sequences with E-values less than thresholds were collected and the corresponding Brachypodium genes were selected as family members (Figs 1–5 and Figs S1,S2). To obtain superfamily or functionally related subfamily, optimal E-value for each gene family was selected. For the Aux/IAA gene family, 19 protein sequences of Arabidopsis Aux/IAA1-19 with a threshold E-value < 10^{-10} were used. For the ARR family, 17 protein sequences of Arabidopsis ARR1-17 with a threshold E-value < 10^{-1} were used. For the GH3 gene family, sequences of Arabidopsis genes in Fig. S1 with a threshold E-value < 0.1 were used. For the ACS and ACO gene families, 11 protein sequences (ACS1-11) and 4 proteins (ACO1-4) with a threshold E-value < 10^{-15} were used. To obtain homologous BR-biosynthetic P450 genes from Brachypodium, the protein sequences of BAS1, Chibi2, BR6ox2, BR6ox1, DWF4, CPD, ROT3 and CYP90D1/At3g13730 were applied to BLAST (blastp) to search for similar sequences in the Brachypodium protein sequence. Brachypodium sequences with an E-value < 10^{-10} were collected and the corresponding Brachypodium genes were selected as BR-biosynthetic genes. To identify SA-responsive genes homologous to PRI in Brachypodium, the protein sequence of PRI and its Arabidopsis homologous proteins (BLAST E-value < 10^{-10}) were applied to BLAST (blastp) to search for similar sequences in the Brachypodium protein sequence. Brachypodium sequences with an E-value < 10^{-10} were collected and the corresponding Brachypodium genes were selected as PRI-like genes. Similarly, to identify genes homologous to ERF1, the ERF1 protein sequence was applied to BLAST and those with an E-value < 10^{-15} were selected as ERF1-like genes. To identify genes homologous to JA-biosynthetic genes in Brachypodium, the protein sequences of AtLOX2-4, AOS, AOC3, OP3, OPCL1, ACX1, ACX5, AIM1, KAT2, JMT and JAR were applied to BLAST (blastp) to search for homologous sequences in the Brachypodium protein sequence. Brachypodium sequences with an E-value < 10^{-10} were collected and the corresponding Brachypodium genes were selected as being homologous JA-biosynthetic genes. To create the phylogenetic tree of orthologous genes in Brachypodium and Arabidopsis, translated amino acid sequences were aligned with MAFFT^36 using the “auto” settings. The neighbor-joining tree^37 of the aligned sequences was then generated using percent identity and visualized as a Rectangular Cladogram in Dendroscope^38.

Gene ontology term enrichment (GOE) analysis. GOE analysis was carried out using the following procedure. First, Brachypodium protein sequences were applied to BLAST (blastn) to search for the most similar gene in Arabidopsis thaliana. GO terms of the top hits in Arabidopsis were copied as the customized annotations of corresponding Brachypodium genes. GOE analysis was performed using AgriGO^39 with customized annotations as the query, customized annotated reference and with default settings for statistical analysis (p-value was calculated with Fisher’s exact test and “FDR with Yekutieli”).

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Supplementary information

Manuscript.

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