Cover Page

Hormone Distribution And Transcriptome Profiles In Bamboo Shoots Provide Insights On Bamboo Stem Emergence And Growth

Short title: Hormone and transcriptome analysis of bamboo shoots

Subject areas: growth and development, regulation of gene expression

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No. of black and white figures: 0

No. of color figures: 4

Supporting Information: 2 supplemental figures, 4 supplemental tables, 1 movie

Title page

Hormone Distribution And Transcriptome Profiles In Bamboo Shoots Provide Insights On Bamboo Stem Emergence And Growth

Short title: Hormone and transcriptome analysis of bamboo shoots

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Abbreviations: ABA abscisic acid, cpm counts per million, GA gibberellic acid, GLM generalized linear model, JA jasmonic acid, MIN mature internode, MNO mature node, PC principal component, SA salicylic acid, SAM shoot apical meristem, YIN young internode, YNO young node

Footnotes: The nucleotide sequences reported in this paper have been submitted to the short read archive (SRA) under BioProject PRJNA342231.

Abstract

Growth and development are tightly coordinated events in the lifetime of living organisms. In temperate bamboo plants, spring is the season when environmental conditions are suitable for the emergence of new shoots. Previous studies demonstrated that bamboo plants undergo an energy-consuming ‘fast stem growth’ phase. However, the events during the initiation of stem elongation in bamboo are poorly understood. To understand the onset of bamboo stem growth, we performed hormone and transcriptome profiling of tissue regions in newly elongating shoots of the Moso bamboo *Phyllostachys edulis*. The growth hormones auxins, cytokinins, and gibberellins (GAs) accumulated in the shoot apex while the stress hormones abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) are found in the lower part of the stem. The mature basal part of the stem showed enrichment of transcripts associated with cell wall metabolism and biosynthesis of phenylpropanoid metabolites, like lignin. In the young upper stem region, expression of cell formation- and DNA synthesis-related genes was enriched. Moreover, the apical region showed enhanced expression of genes involved in meristem maintenance, leaf differentiation and development, abaxial/adaxial polarity, and flowering. Our findings integrate the spatial regulation of hormones and transcriptome programs during the initiation of bamboo stem growth.

Keywords

bamboo, flowering-related genes, hormone profiling, *Phyllostachys edulis*, stem elongation, transcriptomics

Introduction

Plants have evolved morphological adaptation mechanisms to thrive in natural ecological conditions. One structural trait that is regulated according to a specific environment is the height of the shoot axis. For instance, the dwarfism in natural *Arabidopsis* accessions in the Swiss Alps is an ecological adaptation to increasing altitude (Luo *et al*., 2015). Another example are deepwater rice varieties, which survive slow-rising floods of up to five meters for several months because of their ability to increase their height through stem elongation (Kende *et al*., 1998; Hattori *et al*., 2009). A further illustration of height regulation in a particular environment is the grass species bamboo. Some bamboo species have acquired the stem elongation ability as an adaptation strategy to compete for light access with tall trees in the forests (Buckingham *et al*., 2014).

The temperate woody bamboo *Phyllostachys edulis*, also known as Moso bamboo, belongs to the tribe Arundinarieae under the subfamily Bambusoideae of the grass family Poaceae (Kelchner & Bamboo Phylogeny Group, 2013). Bamboo undergoes a 'fast stem growth' phase elongating as high as ~100 cm in a span of 24 hours (Movie S1) (Ueda, 1960; Fu, 2001). It is commonly known that bamboo prefers to grow in moist conditions (Fu, 2001). At our sampling site, we observed over the course of three years that the shoot emergence starts during the spring season, approximately in the first week of April and continues until the end of May. In 2015 and 2016 the appearance of shoots coincided with increasing amounts of rain, increase solar irradiation, and higher temperatures (Figs. 1A, B, C). The growth of a bamboo stem is attributed to sequential elongation from the basal to the top internode (Figs. 1D, E) (Cui *et al*., 2012).

Previous studies suggested that the developing stems exhibit active cellular metabolism favoring pathways for energy production essential for cell structural components and growth (Zhou *et al*., 2011; Cui *et al*., 2012; He *et al*., 2013; Peng *et al*., 2013a). The soluble sugars and starch from leaves, branches, trunks, and rhizomes of mature bamboo are the sources of carbon which is then fixed mainly as hemicellulose and cellulose to meet the requirements for cell wall components necessary for cell elongation (Xu *et al*., 2011; Song *et al*., 2016). Those reports focused on the 'fast stem growth' phase of bamboo. However, an equally important developmental stage, the onset of bamboo stem elongation, is still uncharacterized. To this end, we performed transcriptome and hormone profiling of defined tissue regions of shoots [shoot apical meristem region (SAM), young internode (YIN), young

node (YNO), basal mature internode (MIN) and mature node (MNO)] with obvious initiation of internode elongation (Fig. 1F).

Results

Whole transcriptome analyses show that our dataset is suitable for dissection of expression profiles in tissue regions

To gain understanding of the cellular processes in specific regions of emerging bamboo shoots, we performed RNA-Seq transcriptome profiling. We first analyzed global expression differences and similarities among the five tissues using principal component analysis (PCA). Through this, we found systematic differences among the four sampled bamboo shoots when counts per million (cpm) values of single replicates was used as the input data (Fig. S1). To account for this unexpected effect, we analyzed the gene expression data using a generalized linear model (GLM) which resulted in a clearer separation of samples along principal components (PCs) 1 and 2 (Fig. 2A). PC 1 separated the samples according to their distance from the SAM and could thus be seen as a proxy for a developmental axis along the bamboo shoot whereas PC 2 separated the internode and node tissues, with the SAM in between both. The first two PCs indicated that our sampling strategy would allow us to isolate transcripts specific from each tissue region in the stem.

Our goal was to identify predominant cellular processes in a specific tissue region of the young stem. For this we performed *k*-means clustering of the detected 27,254 transcripts with $k = 16$ to obtain an overview of the global expression patterns (Fig. 2B; Table S1). In selected clusters, we then focused on enriched functional categories from the MAPMAN ontology (Figs. 2C to H; complete data in Table S2).

Cell formation- and DNA synthesis-related genes are highly expressed in young tissues of bamboo stems

First, we analyzed cluster 5, which contained 965 genes most of which showed higher expression in the young tissues (SAM, YIN, YNO) compared to mature tissues (MIN, MNO) (Fig. 2C). According to cluster 5, the young tissues showed an enrichment of genes associated with synthesis of DNA (i.e., replication proteins, histones) and DNA precursors (i.e., ribonucleoside-diphosphate reductases), the

cell cycle (i.e., cyclins), cell division (i.e., replication proteins, retinoblastoma-related), and cell organization (i.e., kinesins, microtubules) (Fig. 2C; Table S1).

Transcripts associated with phenylpropanoid biosynthesis are significantly enriched in the shoot mature tissues

In the mature tissues MIN and MNO (represented by cluster 3) (Fig. 2D), the transcripts involved in amino acid and phenylpropanoid metabolism were significantly enriched. In particular, the prephenate dehydratase and tryptophan synthase genes, involved in the biosynthesis of phenylalanine and tryptophan, respectively, via the shikimate pathway (Rippert *et al*., 2009) were highly expressed (Table S1). It is likely that phenylalanine is the main product of this pathway as it was found at higher concentrations than other amino acids in bamboo shoots (Sun *et al*., 2016). The elevated expression of phenylalanine ammonia lyase (*PAL)*, cytochrome P450s, and 4-coumaroyl CoA-ligase genes suggests that phenylalanine enters the phenylpropanoid pathway (Vogt, 2010; Alber & Ehlting, 2012).

Phenylalanine may then be channeled into the synthesis of monolignols as the transcript for caffeoyl-CoA O-methyltransferase (CCoAOMT) which catalyzes a key step in generating two types of monolignols, coniferyl and sinapyl alcohols (Zhao & Dixon, 2011), was detected in mature tissues (Table S1). Likewise, cinnamoyl-CoA reductase (*CCR*) and cinnamoyl alcohol dehydrogenase (*CAD*) genes for the two successive committed steps common in the formation of p-coumaryl, coniferyl, and sinapyl monolignols (Li & Chapple, 2010) were highly expressed (Table S1). These monolignols are precursors of lignin (Davin & Lewis, 2005). The expression of peroxidases and significant transcript enrichment of plastocyanin-like domain-containing proteins (Fig. 2D; Table S1) indicates their involvement in the oxidation of monolignols which is the last step in lignin polymerization (Zhao & Dixon, 2011; Zhao *et al*., 2013; Printz *et al*., 2016). The expression of a stress-related gene encoding a dirigent protein (Fig. 2D; Table S1) engaged in polymerization of oxidized monolignols to form lignins (Davin & Lewis, 2005) further supports that lignin biosynthesis is active in mature tissues (Itoh 1990; Jolly *et al*.,. 2000; Chang *et al*.,. 2013). This is also in agreement with the expression of *PAL* genes and increased activity of the *PAL* enzyme in the basal part of bamboo shoot (Xu *et al*., 2004). In addition to lignin, the transcript accumulation of UDP-glucosyl and glucoronyl transferases (Fig. 2D;

Table S1) implies that the phenylpropanoid intermediates undergo chemical modifications, resulting in diverse phenypropanoid glucose esters (Lim *et al*., 2001; Xia *et al*., 2009; Vogt 2010).

Notably, there are also a number of genes from the glutathione S-transferase family significantly enriched in cluster 3 (Fig. 2D; Table S1). Glutathione S-transferases are detoxification enzymes involved in the attachment of reduced glutathione (GSH) into oxidized compounds that contain free radicals in the cell (Sheehan *et al*., 2001). Based on the enrichment analysis of identified transcripts in the mature tissues, there are at least two principal metabolic pathways that generate extensive free radicals, the biosynthesis of lignin (i.e., monolignol oxidation activity of peroxidase/plastocyanin-like proteins) (Fig. 2D, Table S1) and the biosynthesis of JA (i.e., LOX-mediated oxidative degradation of lipids) (Figs. 2D, 3F 4F; Table S1). Correspondingly, production of the amino acid cysteine, which is the source of sulfur in GSH was also enhanced due to high expression of genes encoding serine acetyltransferase (SAT) and O-acetylserine(thiol)lyase (OASTL) (Fig. 2D; Table S1) which are essential in the sequential two-step enzymatic reactions for cysteine biosynthesis (Lopez-Martin *et al*., 2008).

Cluster 8, representing the MNO exclusively, contained 546 genes and showed enrichment of similar functional categories as was observed in cluster 3 (MIN, MNO), suggesting that the phenylpropanoid pathway leading to formation of lignin is a favored route in the MNO region. In comparison with cluster 3, different sets of genes encoding stress-related proteins (i.e., dirigent), cytochrome P450s, peroxidases, plastocyanin-like proteins and UDP-glucosyl and glucoronyl transferases were expressed in the MNO region (Fig. 2E; Table S1). Examination of transcripts in the node region (cluster 6) showed that cytochrome P450s were also enriched (Fig. 2F; Table S1), likely catalyzing the reactions in the phenylpropanoid pathway as was described previously in clusters 3 and 8 (Fig. 2D, E).

Common and specific processes of cell wall metabolism operate in different regions of the bamboo shoot

Comparison of enriched functional categories between young (SAM, YIN, YNO) and mature tissues (MIN, MNO) showed that cell wall modification-associated transcripts (i.e., expansin and glycosyl hydrolase genes) (Figs. 2C, D) were enriched in both young and mature tissues. In the mature part of the stem, this enrichment was largely due to expansins which are cell wall loosening factors (Table S1) (Lipchinsky, 2013). There were more expressed genes coding for cellulose synthases, which catalyze the formation of the cellulose component in cell walls, in the mature tissues than in the young tissues (Table S1). In addition, in the MNO, a distinct set of cellulose synthase and arabinogalactan protein genes were expressed (Fig. 2E; Table S1) (Seifert & Roberts, 2007). The cell wall modification genes that code for glycosyl hydrolases (Naumoff, 2011; Ao *et al*., 2016) were uniquely enriched in the MNO (Fig. 2E; Table S1). Polygalacturonase genes encoding the enzymes that hydrolyze the galacturonic acid in cell walls (Babu & Bayer, 2014) were predominantly expressed in the node region (YNO, MNO) (Fig. 2F; Table S1). Furthermore, the expression of arabinogalactan protein genes (Fig. 2F; Table S1) suggests that they contribute in the generation of cell surface proteins (Seifert & Roberts, 2007) that are commonly abundant in YNO and MNO. Overall, the transcriptomic data suggest that the whole emerging shoots are actively engaging in diverse cell wall metabolic processes such as the synthesis of cellulose and cell surface proteins, and modification and remodeling of cell wall components.

Genes associated with transport and photosynthesis are highly expressed in the nodes and mature internodes, respectively

In cluster 6 (containing 622 genes expressed preferentially in node tissues), the phosphatase genes were enriched (Fig. 2F; Table S1) suggesting the involvement of such enzymes in various biochemical pathways likely related with cell wall and membrane metabolism. For example, the expression of purple acid phosphatase genes may be associated with the regulation of the cell wall pH (Crasnier *et al*., 1982) and Ser/Thr phosphatases may be involved in the regulation of signaling proteins including the cell wall enzymes (Shi, 2009; Pancholi *et al*., 2010). The transcripts encoding

phosphoethanolamine/phosphocholine phosphatases may indicate the need for phosphate group removal from phospholipids contributing to the intracellular phosphate pool and intercellular supply of phosphate (May *et al*., 2012; Stigter & Plaxton, 2015). Moreover, it appears that transport-related processes in the nodes are highly active. In particular, putative transporters of sulfates, sugars, amino acids, nucleotides and oligopeptides were detected.

Cluster 4 contained 750 genes with high expression in the MIN followed by the YIN and SAM tissues. Enrichment of photosynthesis-related transcripts (i.e., light reaction, photorespiration, Calvin cycle) was observed in this cluster (Fig. 2G; Table S1). A number of other transcripts enriched in cluster 4 also supported the idea that photosynthetic or related processes are active. This included the formation of thiamine, which is a precursor of the active form of the transketolase's cofactor thiamine pyrophosphate (TPP) in the Calvin cycle (Khozaei *et al*., 2015) and the formation of tetrapyrroles involving magnesium chelatases and protochlorophyllide reductases (Fig. 2G; Table S1) (Schlicke *et al*., 2015).

Enrichment of the transcripts that encode laccases (Fig. 2G; Table S1) which non-redundantly catalyze the same reaction as peroxidases in monolignol oxidation (Zhao *et al*., 2013) suggests that lignin polymerization also occurs in the mature internodes. Expression of cytochrome P450 genes may also indicate that the encoded enzymes are involved in the phenylpropanoid pathway (i.e., lignin biosynthesis and other phenylpropanoid metabolites) (Vogt, 2010). Moreover, cytochrome P450s may also be involved in oxygenation reactions in other secondary metabolic pathways such as in the isoprenoid biosynthesis (Fig. 2G; Table S1) (Kirby & Keasling, 2009).

Expression of transcription factor genes is enriched in the apical region of a newly emerging bamboo stem

Cluster 13 contained 385 genes primarily expressed in the SAM and YIN regions. Out of those, 74 genes were expressed almost exclusively in the SAM, defined by at least two-fold higher expression levels ($P \leq 0.05$) in the SAM region compared to the adjacent YIN region. Remarkably, there were at least 98 transcription factors (2.34-fold enriched, $P < 0.001$) from various gene families in cluster 13

(Fig. 2H; Tables S2, S3). A number of genes similar to zinc finger *C2H2*-type transcription factor genes, which encode INDETERMINATE domain (IDD)-containing proteins, were highly expressed in the SAM region. This group includes the flowering time regulator genes *ID1* (Colasanti *et al*., 2006; Matsubara *et al*., 2008) and *IDD14* which regulates lateral organ development and gravitropic response (Cui *et al*., 2013). The *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PCF* (*TCP*) transcription factor gene *TCP5* which controls leaf differentiation (Koyama *et al*., 2007; Koyama *et al*., 2010) showed a similar expression pattern.

The identified members of the *ASYMMETRIC LEAVES2* (*AS2*)/*Lateral Organ Boundaries* (*LOB*) gene family are involved in formation of a symmetric flat leaf lamina and specification of abaxial-adaxial leaf polarity [i.e., *AS2*, *AS2-LIKE5* (*ASL5*)] (Iwakawa *et al*., 2002; Iwakawa *et al*., 2007; Sun *et al*., 2012). Also, the organ abaxial cell fate regulators *YABBY* (Siegfried *et al*., 1999) and *MYB* family transcription factor *KANADI1* (*KAN1*) and *KAN2* genes (Eshed *et al*., 2001) were expressed in the SAM region.

The *HOMEOBOX GENE 1* (*ATH1*), which regulates shoot meristem maintenance and photomorphogenesis via gibberellic acid (GA) biosynthesis (Kamiya & García-Martínez, 1999; Rutjens *et al*., 2009), was also included in cluster 13. The identified homeobox and *START* domain protein family genes (Nakamura *et al*., 2006) were previously reported to be active in the epidermis with various functions such as floral organ development [*PROTODERMAL FACTOR 2* (*PDF2*), *HOMEODOMAIN GLABROUS 5* (*HDG5*)] (Kamata *et al*., 2013), regulation of epidermal cell fate in shoots [*MERISTEM LAYER 1* (*ATML1*), *PDF*] (Sessions *et al*., 1999; Abe *et al*., 2003; Takada *et al*., 2013a; Takada *et al*., 2013b), and stomata differentiation [*HOMEODOMAIN GLABROUS2* (*HDG2*)] (Peterson *et al*., 2013).

Two basic helix-loop-helix (bHLH) transcription factor genes similar to *SPEECHLESS* (*SPCH*), which is an essential regulator of asymmetric divisions for the establishment of stomatal lineage, were expressed in the SAM (MacAlister *et al*., 2007). Another expressed bHLH transcription factor gene was *BEE1* (*BR ENHANCED EXPRESSION1*) which is a brassinosteroid response gene and regulator of hypocotyl length (Friedrichsen *et al*., 2002). In addition, four growth regulating factor (GRF) protein genes involved in the regulation of stem development and leaf growth (van der Knaap *et al*., 2000; Kim *et al*., 2003) were identified along with the gene that encodes the MONOCULM1 (MOC1)-

interacting protein 1 (MIP1) which physically associates with the tiller bud formation regulator MONOCULM1 (Sun *et al*., 2010).

Three genes from the APETALA2/Ethylene-responsive element binding protein (AP2/EREBP) family showed specific differential expression in the SAM region. Those three were most similar to *AP2* (floral development and shoot meristem maintenance) (Jofuku 1994; Licausi *et al*., 2013), *AINTEGUMENTA* (lateral organ primordia initiation and growth) (Mizukami & Fischer, 2006) and *WIND1* (cell dedifferentiation) (Iwase *et al*., 2011).

A homolog of the heat-shock transcription factor gene *ATHSFB4*/*SCHIZORIA* which regulates asymmetric cell division in root stem cells (ten Hove *et al*., 2010) was expressed in the SAM. The two transcripts similar to the *SHORT-ROOT* (*SHR*) involved in root radial patterning, leaf growth, and shoot gravitropism (Fukaki *et al*., 1998; Helariutta *et al*., 2000; Dhondt *et al*., 2010) were also detected. Other transcription factor genes were previously reported to be involved in the flowering pathway such as the defense response *EDM2*, floral homeotic MADS-box gene *AGAMOUS* (*AG*), GATA zinc finger *HANABA TARANU*-like, *ES43*/*EARLY BOLTING IN SHORT DAYS* (*EBS*), zinc finger-homeodomain genes, and a B3 DNA binding domain-encoding gene with sequence similarity to *VRN1* (*VERNALIZATION1*) (Piñeiro *et al*., 2003; Zhao *et al*., 2004; Tan & Irish, 2006; Romanel *et al*., 2009; Tsuchiya & Eulgem, 2010; Dreni *et al*., 2011) (Fig. S2). Moreover, the *MYB LATE MERISTEM IDENTITY2* (*ATMYB17*) has a role in a shift from vegetative growth to flowering (William *et al*., 2004).

Hormone levels correlate with the abundance of hormone biosynthesis transcripts in bamboo shoots

The endogenous concentrations of the phytohormones auxins, GAs, cytokinins, ABA, SA, and JA in the SAM, YIN, YNO, MIN, MNO tissues (Fig. 1F) were quantified as previously described (Kojima *et al*., 2009). The concentration of the active auxin free IAA was high in the SAM and YIN regions, while the conjugated auxin IAAsp significantly accumulated in the SAM region (Fig. 3A), suggesting that there is an excess of free auxin which is stored in a conjugated form (Woodward & Bartel, 2005). The predominant expression of *TAA1;4* and *YUCCA7* genes in the SAM which encode the rate-limiting enzymes transaminase and flavin monooxygenase, respectively, for tryptophan-derived IAA

production (Fig. 3A, 4A) (Zhao, 2014) indicates that the bamboo shoot apex is actively synthesizing auxins. The details of expression levels for hormone metabolism-related and signaling-related genes are shown in Tables S4 and S5, respectively.

Similarly, among all the five analyzed tissues, the SAM region showed a high concentration of total gibberellins which is mainly attributed to the GA precursors GA_{12}, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_{24}, and GA_9 (Fig. 3B). This is in agreement with the coordinated expression of genes encoding for the enzymes involved in early steps of GA biosynthesis such as *ent*-copalyl diphosphate synthase (CPS-A, CPS-C), *ent*-kaurene synthase (KS-A), *ent*-kaurene oxidase (KO2), *ent*-kaurenoic acid oxidase (KAO-B) as well as in the parallel sequential oxidations from $GA_{12/53}$ to $GA_{9/20}$ by GA 20-oxidases (GA20ox1, GA20ox2) (Fig. 4B). GA_9 is synthesized from GA_{12} via the non-C-13 hydroxylation pathway while GA_{20} production is through the C-13 hydroxylation pathway from the GA_{53}, a C-13-hydroxylated derivative of GA_{12}. Previously, it was reported that C-13 hydroxylation is an active route for GA biosynthesis in bamboo shoots (Samsoedin *et al*., 1996). Our results confirmed this and additionally showed that the C-13 hydroxylation pathway is localized and actively operating in the SAM region (Figs. 3B, 4B). In all the tissues examined, the bioactive GA_1 showed lower levels than its precursors also validating an earlier report on the order of GA content magnitude which is GA_{19} > GA_{20} > GA_1 (Fig. 3B) (Yanagisawa *et al*., 1992). On the other hand, members of the non C-13 hydroxylation pathway were not identified before. In this work, we detected low levels of bioactive GA_4 and its precursors GA_9 and GA_{24} suggesting that GA_4 in bamboo is minimally used. Altogether, these results suggest that the shoot apical region is an active site of GA biosynthesis in bamboo.

Measurements of cytokinins showed that the active *trans*-zeatin (tZ) and its derivatives, precursor *trans*-zeatin ribonucleotides (tZRPs) and inactive form glucoside tZ9G, are the most abundant form of cytokinins in the bamboo shoots (Fig. 3C). It appears that the tZ is actively produced in the SAM and that excess amounts are inactivated as tZ9G (Fig. 3C) which is likely a homeostatic mechanism to maintain the level of cytokinins in the SAM (Osugi & Sakakibara, 2015). In contrast, isopentenyladenine (iP), dihydrozeatin (DZ), and the tZ isomer *cis*-zeatin (cZ) were in negligibly low levels in all the quantified tissues (Fig. 3C). An earlier report detected similar levels of both the tZ- and cZ-form of cytokinins in bamboo (McCloskey *et al*., 1979). However, the developmental stage of the bamboo shoots measured in the earlier study was not indicated which may explain the difference with

our results. Also, our quantification of the derivatives of iP, cZ, and DZ such as the ribonucleotides (iPRPs, DZRPs, cZRPs), ribonucleosides (iPRs, DZRs, cZRs), and glucosides (iP9G, DZ9G, cZOG, cZROG, cZRPsOG) showed lower concentration compared to the tZ-type derivatives (Fig. 3C).

The expression of isopentenyltransferase 3 (*IPT3*) genes in the SAM (*IPT3A*, *B*, *C* and *D*) and YIN (*IPT3C* and *D*) (Fig. 4C) corresponds to the increased concentrations of iPRPs in the SAM and YIN regions compared to the other tissues. Moreover, high levels of tZ in the SAM region are coordinated with the expression of *LOG-like* genes (i.e., *LOGL1A* and *B*, *LOG2A*, *B* and *C*, *LOGL7A* and *LOGL10B*) (Fig. 4C) which encode the phosphoribohydrolases transforming cytokinin ribonucleosides or ribonucleotides to the active form tZ. Our results suggest that newly emerging bamboo shoots are actively producing tZ-type cytokinins, specifically in the SAM region.

The expression of ABA biosynthesis genes *ZEP* and *NCED*, which code for the enzymes zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid dioxygenase, respectively, were detected. The expression patterns of *ZEP1B*, *ZEP1C*, *NCED3A*, and *NCED4* in bamboo MIN seem to support the significant accumulation of ABA in the MIN compared to the other tissues (Figs. 3D, 4D). Likewise, SA was predominantly contained in the MIN region (Fig. 3E). Biosynthesis of SA occurs in two pathways, the isochorismate (ICS) and the PAL pathways. During plant immunity, the plant defense hormone SA is primarily synthesized via the ICS route (Seyfferth & Tsuda, 2014). In our transcriptomic data, the *PAL* genes are highly expressed in the mature tissues, MIN and MNO (Fig. 4E), suggesting that the PAL pathway largely contributes to SA production in bamboo shoots. The difference in SA content between MIN and MNO tissues may be explained by the expression of *BISAMT* genes (Fig. 4E) encoding the *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferases which conjugate methyl to SA (Xu *et al*., 2006), possibly regulating SA levels in the MNO.

Increased expression of most genes involved in the early steps of JA biosynthesis was observed in the mature tissues (MIN and MNO) compared to the young tissues (SAM, YIN, YNO) (Fig. 4F). This included genes encoding lipoxygenases (LOX) catalyzing the conversion of α-linolenic acids to hydroxyperoxy fatty acids. Also genes associated with the three succeeding committed steps, allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*), and oxophytodienoate reductase 7 (*OPR7*) were primarily expressed in mature tissues (Fig. 4F) (Schaller & Stintzi, 2009). The average JA levels in MIN and MNO reflected the expression pattern of JA biosynthesis genes. However, statistical tests

did not show significant differences in JA concentrations among tissues (Fig. 3F). A possible explanation for this is that, in MIN and MNO tissues, JA is converted to many other possible derivatives such as JA-Ile and JA-Met (Gfeller *et al*., 2010; Yan *et al*., 2013). In support of this, *JAR1* and *JMT* genes, encoding amino acid synthetases conjugating isoleucine to JA and methyltransferases methylating JA to form JA-Met, respectively, were primarily expressed in MIN and MNO (Fig. 4F).

We examined the expression pattern of genes associated with ethylene biosynthesis. From the expression of S-adenosyl methionine (*SAM*) synthases (*SAM1A*, *SAM1B*, *SAM2*, *SAM3A*, *SAM3B*), 1-aminocyclopropane-1-carboxylic acid (*ACC*) synthase (*ACS2*), and *ACC* oxidases (*ACO1A*, *ACO2A*, *ACO3A*, *ACO3B*, *ACO5A*, *ACO5B*, *ACO5C*, *ACO5D*), it appears that ethylene is produced mainly in the mature tissues (Fig. 4G).

Discussion

The data on hormone profiling and expression of hormone biosynthesis genes (Figs. 3, 4) suggest that the newly emerging aerial shoots exhibit tissue-specific accumulation of growth hormones. Auxins, gibberellins, and cytokinins accumulated in the apical region of the shoot (Fig. 3G) which indicates their role in growth regulation of newly emerging stems. Furthermore, the site of biosynthesis of these hormones overlaps with shoot regions characterized by enrichment of transcripts involved in cell formation- and DNA synthesis (Fig. 2C).

Our samples from the SAM region used for profiling of hormones and transcriptomes likely included subdomains in the shoot apex such as the meristem peripheral region, the primordium initiation site, and the meristem center corresponding to the spatial distribution model of auxin, GA, and cytokinin (Wolters & Jürgens, 2009; Besnard *et al*., 2011). The presence of cytokinin (Fig. 3C) may mediate the expression and shoot meristem maintenance function of the detected *AP2* transcription factor gene (Table S3) (Rashotte, 2003; Rashotte *et al*., 2006; Kurakawa *et al*., 2007). The increased amount of auxin and GA in the periphery of the meristem promotes initiation of lateral organs and primordium growth (Heisler *et al*., 2005; Shani *et al*., 2006). The expression of the closest homologue of *IDD14* in the SAM region (Table S3) suggests that it is possibly involved in the regulation of auxin biosynthesis (Fig. 3A) controlling the growth of lateral organs and response to gravity (Cui *et al*., 2013). The expression of the transcription factor gene *TCP5* (Table S3) may indicate that it positively regulates the *AS2* genes for leaf differentiation and auxin response (Koyama *et al*., 2010). It also appears that the *AS2* gene (Table S3) regulates proper auxin localization and thereby leaf differentiation (Vu *et al*., 2008). The transcripts of *AS2*, *YABBY*, and *KAN* in the apical region (Table S3) suggest that bamboo utilizes similar antagonistic interactions between abaxial and adaxial genes, as in *Arabidopsis*, for specification of abaxial-adaxial leaf polarity (Nole-Wilson & Krizek, 2006; Machida *et al*., 2015).

In the mature basal part of the shoot, cell wall metabolism genes (i.e., expansins, cellulose synthases, glycosyl hydrolases, arabinogalactan protein genes) and phenylpropanoid biosynthesis genes leading to lignin formation (Figs. 2D to G; Table S1) were expressed. The transcripts related with photosynthesis in the mature internodes (Fig. 2G) may indicate that the requirement for having an active photosynthetic machinery supporting the phenylpropanoid pathway is satisfied (Vogt 2010). A very recent report suggests that the photosynthesis in bamboo stem plays a key role in stem

elongation (Cui *et al*., 2016). Furthermore, the expression of expansins (Cosgrove, 1997; Cosgrove, 2000; Lipchinsky, 2013) suggest that extension in the walls of cells facilitates cell elongation in the basal internodes. The expressed glycosyl hydrolase genes may be involved in cell wall remodeling which includes simultaneous degradation and reorganization of polysaccharides (Minic & Jouanin, 2006; Minic *et al*., 2007; Minic *et al*., 2009) for the establishment of bamboo cell walls. The identified arabinogalactan protein genes may mediate cell wall signaling (Ellis *et al*., 2010; Tan *et al*., 2012) which is important for stem elongation.

Our transcriptomic data further suggest that cellulose synthase family genes facilitate cellulose synthesis which likely supports the construction of structural component of cell walls at the early stage of bamboo stem elongation (Chen *et al*., 2010; Carpita 2011). This may explain also the high abundance of cellulose (~50%) in several years old bamboo stems (Wahab *et al*., 2013; Li *et al*., 2015). Formation of cellulose may indicate that it contributes in the thickening of cell walls which is usually coupled with lignification (Keegstra, 2001; Lybeer *et al*., 2006). This is in agreement with the enriched expression of genes associated with lignin biosynthesis that is essential for mechanical and structural strength of bamboo stem.

Several studies have shown that lignin plays an important role in defense responses against pathogen invasion (Bhuiyan *et al*., 2009a; Bhuiyan *et al*., 2009b; Wang & Balint-Kurti, 2016; Xu *et al*., 2011; Kawasaki *et al*., 2006). The enriched expression of stress-related genes (i.e. PR-related, NBS-LRR, chitinase, thaumatin, dirigent) in the mature basal part of the stem (Fig. 2D, E, G; Table S1) may be associated with the defense response pathway. Similarly, expression of defense-related genes were previously reported in the basal part of the elongating *Arabidopsis* stem (Hall & Ellis, 2013). The co-localization of the stress hormones ABA, SA, and JA in the basal stem (Fig. 3G) may indicate their role in mediating the expression of defense-related genes (Verma *et al*., 2016). These hormones are mainly implicated in the canonical pathway of stress response. Apart from this, stress hormones are also involved in the regulation of development and growth of plants (Rivas-San Vicente & Plasencia, 2011; Yan *et al*., 2013; Fujii, 2014). The stress hormone-regulated expression of defense-related genes that results in an increase in the strength of cell wall structure is likely being utilized by bamboo during the initiation of stem elongation.

Our profiling data may also suggest that during stem elongation a continuous process is active in the apical region involving cell division (Fig. 2C) and subsequent tissue identity acquisition. The newly formed cells are capable of expansion and cell wall thickening, remodeling and lignification, all of which are essential for generating a rigid stem structure (Fig. 2D, E, G). While there are opposing hormone levels at the apical and basal regions of the shoot at the stage of initiation of stem elongation, it is also possible that there is a change in concentration of hormones as the bamboo shoot elongates its stem. An example of this is the further increase of GA content along the stem axis at a later stage during the 'fast stem growth' phase (Cui *et al*., 2012; He *et al*., 2013) further supporting that endogenous GA regulates stem elongation (Hedden & Thomas, 2012; Hedden & Sponsel, 2015).

A number of genetic factors that regulate the transition from vegetative phase to flowering in response to environmental signals have already been identified in diverse species (Andrés & Coupland, 2012). An exception to this is bamboo, although floral transcriptomes of different accessions have been reported recently (Zhang *et al*.,. 2012; Gao *et al*.,. 2014; Wysocki *et al*.,. 2016). In nature, the shift from vegetative to floral development in most bamboo species lasts for a few to even 120 years, making the experimental observation of reproductive phase transition difficult. Even before the reproductive stage, the 'fast stem growth' phase is preceded by the emergence of new bamboo shoots from the subterranean rhizome-borne buds. How environmental cues stimulate the shift from dormant buds to shoot formation and finally to stem elongation is still unclear. At our sampling site, the appearance of bamboo shoots above the ground coincides with environmental changes during springtime (Figs. 1A, B, C). This suggests that environmental stimuli, like humidity, temperature, or day length may trigger and promote the growth of aerial stems. Unexpectedly, the homologs of flowering regulator genes *VRN1* (encoding a B3 DNA binding domain) and *FT* (encoding the FT-like7 protein) were highly expressed in the shoot apex (Fig. 2H; Fig. S2). In wheat, *VRN1* is expressed in the shoot apex and leaves in response to vernalization (Yan *et al*., 2003) while contradicting reports have shown that *FT* mRNA or FT protein is transported into the shoot apex to promote flowering under short days (Huang 2005; Tamaki *et al*., 2007; Wigge 2011). Additionally, there are other homologs of flowering genes such as the *AP2*, *ID1*, *EDM2*, *AG*, *HANABA TARANU*-like, *ES43*/*EBS*, *LMI2/MYB17*, and zinc finger-homeodomain genes (Jofuku 1994; Piñeiro *et al*., 2003; William *et al*.,

2004; Zhao *et al*., 2004; Colasanti *et al*., 2006; Tan & Irish, 2006; Matsubara *et al*., 2008; Tsuchiya & Eulgem, 2010; Dreni *et al*., 2011) that are highly expressed in the SAM region (Table S3; Fig. S2).

While it is known that bamboo has an unpredictable flowering behavior, one possible explanation for the expression of flowering genes is that bamboo shoots regularly acquire the potential for flowering. However, additional stimuli for reproductive phase transition are necessary. Another possibility is that the expression of flowering-related genes in the SAM region might instead be involved in promoting the outgrowth of buds from rhizomes or stem elongation without directly promoting flowering. In many plant species (including rice and *Arabidopsis*), vegetative to reproductive phase transition is usually accompanied by stem elongation, which is indicative of their shared signaling pathways and a shared evolutionary history. Maybe bamboo acquired the ability for stem elongation through modification of the signaling pathway that is known to control flowering in other species. An analogous situation was reported in potato, where different FT-like paralogues control flowering and tuberization, respectively (Navarro *et al*.,. 2011).

In this study, we show the coordinated expression of hormone biosynthesis genes along with hormone concentrations (Figs. 3, 4) indicating the robustness of our profiling data. The data presented here will be an additional resource for plant biologists interested in studying functional gene analysis of bamboo. Future directions might entail introduction of bamboo genes into closely related grass species with established genetic transformation systems such as rice and *Brachypodium*. It might be noteworthy also to examine the promoters and coding regions of strongly expressed genes such as the cellulose synthase genes with the goal of further understanding and increasing the biomass growth of plants for bioenergy purposes. As we have only used a temperate bamboo species in this study, it might be of importance to conduct hormone and transcriptome profiling using a tropical bamboo species for comparative analysis of bamboo growth dynamics.

Materials and methods

Plant materials

Young shoots (~25 cm above ground height) of Moso bamboo (*Phylostachys edulis*) emerging from the soil surface were harvested in May 2014 at the Togo Field Research Station in the prefecture of Aichi in Japan (coordinates 35°06'41.1"N 137°04'48.6"E). In total, four shoot tips of approximately the same size and developmental stage were collected. Each shoot tip was dissected to isolate the shoot apical meristem (SAM), young internode (YIN), young node (YNO), mature internode (MIN) and mature node (MNO) regions, respectively. The same shoots were used for transcriptome and hormone analyses. For Movie S1, a rapidly elongating bamboo shoot was filmed in 2016 for a total of nine days in a time-lapse recording. A Brinno TLC200 camera was used with a Brinno BCS24-70 objective and one picture was recorded every 30 min.

Hormone quantification in plant tissues

The phytohormones auxins, gibberellins, cytokinins, and ABA, SA, and JA in the SAM, YIN, YNO, MIN, MNO tissues (Fig 1F) were measured using ultra-performance liquid chromatography coupled with a tandem quadrupole mass spectrometer equipped with an electrospray interface (UPLC-ESI-qMS/MS) as described previously (Kojima *et al*., 2009).

RNA extraction and library construction

Total RNA of each sample was isolated from frozen tissues using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with the RNase-Free DNase Set (Qiagen). RNA purity was checked using a NanoDrop spectrophotometer and RNA was quantified using a QuantiFluor RNA system (Promega) and an EnSpire Multimode Plate Reader (PerkinElmer). For cDNA library construction, 2 µg of total RNA was used with the lllumina TruSeq RNA Sample Preparation Kit v2 (Illumina) according to the manufacturer's instructions. Agencourt AMPure XP beads (Beckman Coulter) were used to remove small DNA fragments. Clustering of index-coded samples was performed on a cBot Cluster

Generation System using the TruSeq SR Cluster Kit v2-cBot-GA (Illumina) and the TruSeq SBS Kit v5-GA (Illumina). After cluster generation the library preparations were sequenced on an Illumina HiSeq 2000 platform.

Read alignment and transcript quantification

Unprocessed reads were uploaded to the sequence read archive (SRA) and can be accessed via BioProject PRJNA342231. In total, 374.54 x 10^6 paired-end reads with an average length of 55 bp were generated. Raw reads were quality controlled using fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed using trimmomatic-0.36 (Bolger *et al*., 2014). Trimmed reads were mapped to the masked scaffolds of the P.heterocycla-v1.0 genome build (Peng *et al*., 2013b) obtained from www.bamboogdb.org using tophat 2.1.0 (Kim *et al*., 2013) and bowtie 2.2.8 (86.13 % average mapping rate) (Langmead & Salzberg, 2012). Mapped reads were assigned to genomic features and quantified using the R package Rsubread 1.22.2 (Liao *et al*., 2013) according to the gene models described in the fasta headers found in P_heterocycla_v1.0.genemodel-cds-DNA.fa from www.bamboogdb.org.

Transcriptome data analyses

General data analysis was performed using R. Large-scale differences between samples and replicates were analyzed by PCA using R's prcomp function (with the scales argument set to TRUE). Calculation of cpm values and analysis of differential expression of genes was performed using edgeR 3.14.0 (Robinson *et al*., 2009). Firstly, only the transcripts with at least 0.5 cpm in four or more samples were considered expressed and those that failed to meet the threshold were discarded. Since a systematic replicate effect was found (Fig. S1), gene expression was fitted to a generalized linear model (GLM) with both, replicates and tissues as additive factors (~replicates+tissues). Significant differences in gene expression between tissues (taking into account the variation between replicates) were tested for using likelihood-ratio tests implemented in the glmLRT function from edgeR and P-values were corrected using the Bonferroni-Holm method. Clustering of genes according to

expression levels was performed using the *k*-means algorithm implemented in the MBCluster.seq package with GLM-fitted cpm values (Si *et al*., 2014). To create a plant-specific ontology for our transcriptome dataset we assigned MAPMAN identifiers to each of the expressed bamboo transcripts based on the best BLASTN hit in the *Oryza sativa japonica* cv. Nipponbare reference transcripts (Kawahara *et al*., 2013; Thimm *et al*., 2004). In the next step, enrichment of MAPMAN bins in gene sets was calculated and tested for significance using Fisher's exact test with the Bonferroni-Holm method for P-value correction as implemented in R's fisher.test and p.adjust functions.

Raw data and program code to reproduce the transcriptome analyses and a datamining application (including a GUI) have been made available at https://github.com/StefanReuscher/youngBambooShootsRNAseq.

Funding

This research was supported by the CREST, Japan Society and Technology Agency, and MEXT Grant-in-Aid for Scientific Research on Innovative Areas (Grant 16H01464) (M.A.), and a research fellowship from the Japan Society for Promotion of Science (R.G.).

Disclosures

The authors declare no competing interests.

Acknowledgments

We thank the members of the Ashikari Laboratory for the help with field experiments. Prof. Takashi Tsuge is acknowledged for allowing us to conduct the bamboo field experiments. We thank the Japanese National Institute of Genetics for the use of their Super Computer Facilities for read alignments.

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Figure legends

Fig. 1. Weather data and bamboo shoot morphology.

Total rainfall (A), highest (red) and lowest (blue) daily temperatures (B), and total solar radiation (C) at the sampling site from February 2015 until May 2016 were recorded by a nearby weather monitoring station (DavisVantagePro2, coordinates 35°06'40.2"N 137°05'04.2"E). Dashed trendlines in (B) and (C) were calculated using R's "loess" function. Two shaded boxes indicate the seasonal time window of shoot elongation. Intact (D) and half-cut (E) bamboo stems at different developmental stages (1 to 5) showing elongating internodes (In) separated by nodes (No). The approximate height of stems from each stage was: 1, 5 cm; 2, 25 cm; 3, 35 cm; 4, 50 cm; 5: 90 cm. Stage 2 shoots (F), marked by an asterisk in (E) were used for molecular analyses. Sampled tissues marked in (F) included the shoot apical meristem (SAM) region, young internode (YIN), mature internode (MIN), young node (YNO) and mature node (MNO). Collection of samples was conducted in May 2014.

Fig. 2: Gene enrichment in selected k-means clusters.

Principal component analysis showing each tissue (SAM, shoot apical meristem; YIN, young internode; YNO, young node; MIN, mature internode; MNO, mature node) plotted along the first two principal components (PC1 and PC2) with the percentage of variance explained (A). A heatmap representation shows the results of *k*-means clustering of genes (B). Each column represents one tissue and each row represents the average relative gene expression of all genes in that cluster. In (C) to (H), relative gene expression is shown for all genes in the indicated cluster. Gene expression was calculated as the $\log_2$-fold change of reads per kilobase of transcript per million (rpkm) relative to the mean expression of each gene. The total number of genes in each cluster is shown along with the results from gene enrichment analysis. For each cluster significantly enriched MAPMAN level 2 bins are shown. Significance was calculated using Fisher's exact test followed by Bonferroni-Holm correction (*0.05 > $P \geq 0.01$, **0.01 > $P \geq 0.001$, ***0.001 > P).

Fig. 3. Biosynthesis and distribution of phytohormones in the bamboo shoots.

Auxins (A), gibberellins (GAs) (B), cytokinins (C), abscisic acid (ABA) (D), salicylic acid (SA) (E), and jasmonic acid (JA) (F) in specific stem regions (see Fig. 1) were quantified as pmol g^{-1} fresh weight (in y-axis of each graph) and results were summarized in an illustration (G). For each tissue, the number of biological replicates is n = 4 unless stated otherwise. N.D. indicates not detected while N.M. means not measured. Error bars indicate standard deviation. Significant differences in metabolite concentrations among the tissues were analyzed by analysis of variance and Tukey's multiple comparison's test. Different letters indicate significant differences of the means ($P < 0.05$). Means with the same letter or with a common letter are not significantly different from each other. Red font indicates hormone metabolism genes detected in our RNAseq data. The yellow box in (B) indicates the reactions catalyzed by the enzymes GA20ox1 and GA20ox2. The description of gene abbreviations and detailed expression levels are shown in Table S4. The colors in (A to G) indicate the stem regions: blue, SAM region; red, YIN; green, MIN; purple, YNO; orange, MNO.

Fig. 4: Expression of hormone metabolism-related genes.

Relative expression of selected genes responsible for key steps in the metabolism of seven plant hormones is shown as heatmap representations. Panels show auxin (A), GA (B), cytokinin (C), ABA (D), SA (E), JA (F) and ethylene (G) metabolism. Each column represents one tissue (SAM, shoot apical meristem; YIN, young internode; YNO, young node; MIN, mature internode; MNO, mature node) and each row represents one transcript. Transcripts are grouped according to gene families or metabolic functions. Gene expression was calculated as the $\log_2$-fold change of reads per kilobase of transcript per million (rpkm) relative to the mean expression of each gene. For each tissue four replicate samples were used. Green boxes indicate genes that are highlighted in the manuscript text.

Fig. S1: Principal component analysis of expression data with and without fitting to a generalized linear model.

Principal component analysis shows samples from each tissue (SAM, shoot apical meristem; YIN, young internode; YNO, young node; MIN, mature internode; MNO, mature node) and replicates (A to D) plotted along the first two principal components (PC1 and PC2) with the percentage of variance explained. In (A) counts per million (cpm) were used as is. In (B) cpm values fitted to a generalized linear model were used.

Fig. S2: Expression of homologues of flowering genes enriched in the bamboo shoot apex.

The expression of flowering-related genes in cluster 13 and their homologues in the whole dataset are shown. Each column represents one tissue (SAM, shoot apical meristem; YIN, young internode; YNO, young node; MIN, mature internode; MNO, mature node) and each row represents one gene. Genes are grouped according to gene families. Gene expression was calculated as the $\log_2$-fold change of reads per kilobase of transcript per million (rpkm) relative to the mean expression of each gene.

Supporting information

Fig. S1: Principal component analysis of expression data with and without fitting to a generalized linear model.

Fig. S2: Expression of homologues of flowering genes enriched in the bamboo shoot apex.

Table S1: Expression data and annotations for all expressed genes.

Table S2: Enrichment analysis of selected *k*-means cluster.

Table S3: Transcription factor genes expressed in the SAM region of bamboo shoots.

Table S4: Details of expression levels for hormone metabolism-related genes.

Table S5: Details of expression levels for hormone signaling-related genes.

Movie S1: Time-lapse movie of an elongating bamboo shoot.