Gibberellins Regulate Lateral Root Development that is Associated with Auxin and Cell Wall Metabolisms in Cucumber

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Abstract

Background: Cucumber is an economically important crop cultivated worldwide. However, it is susceptible to various stresses due to weak and shallow root systems, limiting its productivity. Lateral roots (LRs) are critical for plant stress tolerance and productivity. Increasing evidence showed that gibberellins (GAs) play important roles, positively or negatively, in LR development in many plants. Therefore, it is of great importance for cucumber production to study the role of GAs in LR development.

Results: The application of various concentrations of GA₃ and uniconazole, a GA biosynthesis inhibitor, on germinated cucumber seeds for 5 days showed that GAs regulated cucumber LR development in a concentration-dependent manner. 1 μM, 10 μM, 50 μM and 100 μM GA₃ significantly increased secondary root length, tertiary root number and length. It is worthy note that 50 μM GA₃ treatment showed strong effects in increasing root dry weight and the root/shoot dry weight ratio. Pairwise comparisons between GA₃-treated roots and Control at 2, 3 and 5 days after germination (DAG) identified 417 down-regulated genes enriched for GA metabolism-related processes and 447 up-regulated genes enriched for cell wall metabolism-related processes in GA₃-treated roots. Linear factorial modeling was also performed to systematically identify DEGs and a total of 3523 non-redundant DEGs were identified in our RNA-seq data. Of these, most of the genes involved in auxin and cell wall metabolisms were up-regulated in GA₃-treated roots.

Conclusions: GAs positively regulate LR development in cucumber putatively associated with enhanced IAA and cell wall metabolisms. Our findings not only shed light on LR regulation mediated by GA but also offer an important resource for functional studies of candidate genes putatively involved in the regulation of LR development in cucumber and other crops.

Background

The root system constitutes the essential part of the higher plant body. The plant root system performs various functions, such as anchorage, absorption and as the major interface between the plant and various stress factors in the soil environment [1, 2]. Root system architecture is an integrative result of lateral root (LR) initiation, morphogenesis and growth. Therefore, LRs are fundamental to plant survival and productivity [3–6].

The formation of LRs has been extensively studied, especially in *Arabidopsis* [1, 7, 8]. LR initiation begins with priming of three pairs of xylem pole pericycle (XPP) cells within the root transition zone and elongation zone [9–11], and subsequent specification of founder cells followed by asymmetric cell division, resulting in lateral root primordium (LRP) formation [7, 12, 13]. After the LRs emergence and growth, new lateral roots undergo further reiterative branching, forming building blocks of the root system [12, 14–16].

LRs development is tightly regulated by plant hormones [17–24], among which auxin is a crucial signaling molecule regulating LR formation at various developmental stages [11, 25–28]. Exogenously applied auxin strikingly increases of the LR number [29–32]. Similarly, several gain-of-function or loss-of-function mutants that alter auxin signalling display altered LR development [11, 15]. Evidence from many studies has demonstrated that polar auxin transport plays a vital role in regulating the differentiation of founder cells, the first oriented cell division and the development of LR primordia [6, 9, 33–35].

GAs are a class of phytohormones that regulate a wide range of developmental processes including root development [22, 36]. In contrast with auxin, little is known about the roles of GAs on LR development. Some reports describe the inhibitory effect of GAs on LR formation [22, 37–41]. For example, GA-deficient and GA-insensitive transgenic *Populus* plants exhibited increased LR proliferation and elongation under *in vitro* and greenhouse conditions, and these effects were reversed by exogenous GAs treatment [22]. However, the LR formation was promoted by shoot-applied GAs in Arabidopsis. Therefore, the effects of GAs on LR formation remain elusive, and GAs may play different roles in regulating lateral root formation in different plants.

Cucumber (*Cucumis sativus* L.) is an economically important crop cultivated worldwide and has been used as a model plant for basic research on sex determination and vascular biology [42–45]. Cucumber has been widely used in GAs research, which played important role in sex expression [46], flower development [47, 48] and parthenocarpy [49]. However, the roles of GAs in LR formation is still unclear in cucumber. Moreover, cucumber has shallow root system which limits water and nutrients uptake, resulting in low biotic and abiotic stress tolerance and limiting its production. Therefore, it is of great importance for cucumber production to study the roles of GAs in LR development.

In this study, we applied exogenous GA₃ to the germinated cucumber seeds to study the effects of GA₃ on LR development and found that GA₃ positively regulate LR development in a concentration dependent manner. We compared the RNA-seq data from developing roots at 2, 3 and 5 days after seed germination (DAG) between 50 µM GA₃ treatment and Control. The results showed that GA-mediated LR development in cucumber was associated with enhanced auxin mechanisms and cell wall-related processes. Importantly, we found many differentially expressed genes (DEGs), including transcription factors (TFs), that have been implicated in root development, indicating their important roles in GA-mediated LR development in cucumber. This study not only expands our understanding of LR regulation mediated by GA but also offers an important resource for functional studies of candidate genes putatively involved in the regulation of LR development in cucumber and other crops.

Results

GA₃ regulates LR formation in cucumber

To investigate the impact of different GA₃ levels during LR development, germinated seeds were initially subjected to exogenous GA₃ at concentrations of 10 nM, 50 nM, 100 nM, 1 µM, 10 µM, 50 µM, 100 µM

and 200 μM for five days (Additional file 1). We observed that GA_3 concentrations of 10 nM, 50 nM, 100 nM, 1 μM , 10 μM , 50 μM and 100 μM had little effect on primary root length, respectively (Fig. 1). A significant reduction of primary root length was only observed for GA_3 concentration at 200 μM . However, the number of secondary roots displayed no significant difference among the treatments of the eight GA_3 concentrations and Control. Interestingly, the secondary root length was gradually increased by an increase in GA_3 concentration in the range of 10 nM-100 μM , whereas at high GA_3 concentration (200 μM), the secondary root length did not significantly differ from that of the control seedlings (Fig. 1; Additional file 1). Similarly, the effects of GA_3 on the number and length of tertiary roots was also found to be dose dependent (Fig. 1). The tertiary root number and length were both significantly increased under 1 μM , 10 μM , 50 μM and 100 μM GA_3 treatment, whereas the tertiary root number and length under higher doses of GA_3 (200 μM) treatment didn't show a significant difference compared with control (Fig. 1). We also investigated the effects of various levels of GA_3 on the dry weight of roots and the ratio of root dry weight and shoot dry weight (RDW/SDW). The dry weight of roots was significantly increased by 50 μM GA_3 treatment, which also increased the RDW/SDW (Fig. 1).

We also applied uniconazole (Uni, GA biosynthesis inhibitor of *ent*-Kaurene oxidase) to germinated cucumber seeds for five days. Low concentrations of Uni (10 nM, 50 nM and 100 nM) had no significant effects on the primary root length, secondary root length and number (Additional file 2). However, they were significantly suppressed by Uni and gradually decreased with the increase of Uni concentration from 1 μM to 200 μM (Additional file 2). The promotion and inhibition of LR development by exogenous GA_3 and Uni, respectively, indicated that GA plays important roles in regulating LR development in cucumber and we used the 50 μM GA_3 concentrations for further studies.

Overview of the transcriptome profiles of the roots in response to GA_3 treatment

To better understand how GA regulates LR development and identify genes involved in the pathway in cucumber, we performed RNA-seq analysis using RNA isolated from the LR at three developmental timepoints (2, 3 and 5 DAG) with or without 50 μM GA_3 treatment. All tissues were analyzed in two or three independent biological replicates (17 samples in total). Summary statistics for each of the RNA-seq libraries are shown in Additional file 3. The Spearman correlation coefficient (SCC) analysis showed high reproducibility between the biological replicates, ranging from 0.97 to 0.99 (Fig. 2A). A principal component analysis (PCA) was also performed to obtain a general view of the transcriptomes derived from all 17 tissue samples. The first two principal components (PC1 and PC2) accounted for 70% of the total variance and the samples clustered based on both treatment and developmental timepoints (Fig. 2B). That is, there are different transcript profiles between the GA_3 -treatment and Control dependent on developmental timepoints.

Differentially expressed genes (DEGs) in response to GA₃ treatment

To investigate how transcriptome changes of roots across the three developmental timepoints between GA₃-treatment and Control, DEGs were identified by three pairwise comparisons at each developmental timepoint (Fig. 3). A total of 417 unique DEGs exhibited significant lower expression and 447 unique DEGs exhibited significant higher expression at the three timepoints of root development in GA₃-treated roots as compared to the Control (Fig. 3A; Additional file 4). Notably, the number of DEGs gradually increased with the development of roots and the largest number of genes (724) exhibited differential expression at 5 DAG followed by 3 DAG (367) and 2 DAG (239) between the Control and GA₃-treated roots, indicating more transcriptional changes in gene expression at the later developmental stages.

Various biological process GO terms showing significant enrichment were detected among the genes that were up- and down-regulated in the GA₃-treated roots. Notably, GO terms related to cell wall organization/modification, including 'Cell wall organization', 'Cell wall organization or biogenesis' and 'Cell wall modification', and root cell development/ differentiation, including 'Root hair cell development', 'Root epidermal cell differentiation' and 'Lateral root branching', are significantly enriched in the up-regulated DEGs in the GA₃-treated roots (Fig. 3B). This result indicates that GA₃ promotes LR development might be associated with the up-regulation cell wall-related genes. In addition, the over-represented GO terms in the down-regulated DEGs in the GA₃-treated roots included terpenoid- and gibberellin-related terms, such as 'Terpenoid biosynthetic process', 'Gibberellin mediated signaling pathway' and 'Gibberellin metabolic process', suggesting that exogenous GA₃ might inhibits the synthesis and signaling transduction of endogenous GAs.

To systematically explore the RNA-seq data, linear factorial modeling was also applied to identify DEGs significantly affected by GA₃ treatment (GT) and the interaction of GT and developmental timepoint (GT*D). Genes that consistently showed higher or lower expression in GA₃-treated roots across all developmental timepoints were identified as DEGs significantly affected by GT, while genes responding differently to GT and developmental timepoints were considered as DEGs significantly affected by GT*D. A total of 3397 and 31 genes were identified with significant GT and GT*D effects, respectively (Additional file 5). All DEGs identified with pairwise comparisons and linear factorial modeling were pooled, resulting in 3523 non-redundant DEGs (Additional file 6) that were used for the downstream analyses.

To further determine the expression patterns of the 3523 DEGs, the application of the degPatterns function on the dataset partitioned the DEGs into clusters of co-expressed genes that share similar expression dynamics. Of these, 3321 DEGs were clearly grouped into 20 clusters named C1- C20 (Fig. 4; Additional file 7). The GO enrichment analysis was performed for the genes in each cluster to investigate the biological significance of the distinct dynamic expression patterns. While no significant enriched biological processes were identified for the genes in C1, C3, C4, C5, C6, C8, C9, C11, C13, C14, C15 and

C20, the genes in C2, C7, C10, C12, C16, C17, C18 and C19 were enriched for many interesting biological processes GO terms (Additional file 8). Of these, C2, C12, C18 and C19 show genes that are constitutively up-regulated by GA₃ treatment. In C12, DEGs that showed increased expression in GA₃-treated roots throughout the time course were enriched for lignin-related processes and 'phenylpropanoid catabolic process'. The DEGs in C18 and C19 were enriched for hormone-related pathways and cell wall related processes. The opposite expression pattern was found in C16 and C17 showing decreased expression in GA₃-treated roots at the three timepoints. C16 and C17 additionally showed that these genes are increased and decreased as the development of roots in both GA₃-treatment and Control, respectively. The DEGs in C16 enriched for hydrogen peroxide-related processes and four processes related to triterpenoid metabolism, including 'triterpenoid biosynthetic process' and 'pentacyclic triterpenoid biosynthetic process'. The significantly enriched GO terms for C17 included many metabolic processes, such as oxoacids, organic acids and fatty acids. In addition, the genes in C7 and C10 are up-regulated in response to GA₃ treatment at 3 and 5 DAG. Top enriched GO terms in C7 and C10 were related with various biosynthetic/ metabolic processes, including phenylpropanoid, ubiquinone, quinone, lignin, aminoglycan, chitin, amino sugar, glucosamine-containing compounds and cell wall macromolecule.

Differentially expressed transcription factors (TFs)

Since TFs are the main regulators of gene expression, we sought out the differentially expressed TFs from the DEG list. There are 259 TFs, accounting for ~ 7.3% of the total DEGs, which were classified into 38 families (Additional file 9). The most abundant TF families were shown in Fig. 5, including bHLH (28), ERF (25), C2H2 (19), GRAS (17), WRKY (17) and MYB (15). Interestingly, 248 out of 259 (95.8%) TFs were significantly affected by GT (Additional file 6; Additional file 9). Of these, 73 TFs were also identified in pairwise comparisons (Additional file 6), including one ETHYLENE RESPONSE FACTOR (ERF) TF, *Csa3G895680*, significantly affected by GT*D interaction and putatively associated with primary cell wall deposition [50].

Many orthologs of the TFs were known from other studies to participate in LR development. For example, the putative cucumber *CYTOKININ RESPONSE FACTOR 2 (CRF2)* (*Csa4G051360*), encoding an ERF protein, was clustered into C17 and down-regulated in GA₃-treated roots (Fig. 4; Additional file 7; Additional file 9). In Arabidopsis, *CRF2* has been proposed to control LR initiation and LR formation [51]. Nine out of 17 differentially expressed GRASs were down-regulated in GA₃-treated roots (Fig. 4; Additional file 7; Additional file 9), including genes (*Csa1G616330* and *Csa5G623560*) putatively regulating GAs response pathway in root endodermis [52] and *Csa4G061850* putatively involved in endodermal fate in the root meristem [53]. In addition, *Csa6G495620* and *Csa7G372280* is a closest ortholog of Arabidopsis GRAS TF SHORTROOT (SHR, AT4G37650) and SCARECROW (SCR, AT3G54220), respectively, which regulates both stem cell niche specification and radial patterning in the root [54–56].

Notably, we found that some TFs in one gene family were all up-regulated or down-regulated by GA₃-treatment. For example, the seven *AP2* genes grouped in C2, C12, C18 and C19 were up-regulated in

response to GA₃ treatment at 2, 3 and 5 DAG (Fig. 4; Additional file 7; Additional file 9). Recently, AP2 TFs were demonstrated to be involved in LR development in Arabidopsis [57]. Auxin response factors (ARFs) play important roles in auxin signaling pathways that are crucial for root branching and LR formation in plants [6, 58]. The five DEGs belonging to ARF gene family were assigned to C4, C12, C18 and C19 and significantly up-regulated by GA₃-treatment (Fig. 4; Additional file 7; Additional file 9). Of these, Csa6G405890 is the closest ortholog of Arabidopsis ARF16 (AT4G30080), which is indispensable for root cap development [59]. Csa2G068680 and Csa2G315390 are one of the orthologs of Arabidopsis ARF6 (AT1G30330) and ARF17 (AT1G77850), respectively. In Arabidopsis, ARF6 positively regulates adventitious roots formation, whereas ARF17 is an inhibitor of the formation of adventitious roots [60, 61]. In addition, GROWTH-REGULATING FACTORS (GRFs) are well known as positive regulators of cell proliferation and expansion and play important roles in regulating root development [62–64]. In our datasets, all five GRF TFs showed consistently decreased expression in response to GA₃-treatment in C1, C3 and C15 (Fig. 4; Additional file 7; Additional file 9).

These results suggest that the differentially expressed TFs identified in our datasets may play a key role in regulating GA-mediated LR development in cucumber. However, further study is required to dissect their exact roles in GA-mediated pathways regulating LR development.

DEGs involved in GAs synthesis and signaling pathway

Since GA₃ promotes LR development in our study, we further investigated the expression of genes involved in GAs synthesis and signaling pathways (Fig. 6). CPS, KS and KO, which were each encoded by a single gene in Arabidopsis, catalyze the early stages of GAs biosynthesis pathway [65, 66]. Similarly, we also identified one gene encoding CPS, KS and KO, respectively, in cucumber genome. Cucumber *CPS* (*CsCPS*) and *KO* (*CsKO*) are DEGs with significant GT effects and down-regulated in GA₃-treated roots (Fig. 6; Additional file 5). Two *KAO* genes, *CsKAO1* and *CsKAO2*, were identified in cucumber. The expression of *CsKAO2* was repressed by exogenous GA₃ treatment, whereas *CsKAO1* showed very low expression in our datasets. GA20ox is the key enzyme of bioactive GA synthesis, and cucumber has five *GA20ox* genes [67]. The transcripts of *CsGA20ox1* and *CsGA20ox2* were detected in our datasets, and both of them were DEGs with significant GT effects, showing lower expression levels in GA₃-treated roots than that of Control. Among the five cucumber *GA3ox* genes [67], only *CsGA3ox1* expression was detected in both conditions, showing a significant decrease after GA₃-treatment throughout the time-course. So far, GA7ox was only identified in pumpkin and cucumber but has not been found in other species, which oxidizes GA12-aldehyde to GA12 and possess mono-oxygenase 7-oxidase activity [68–70]. The expression of the two genes encoding GA7ox also exhibited decreased expression in response to GA₃-treatment. GA2ox can inactivate bioactive GAs through catalyzing 2b-hydroxylation [66]. Cucumber GA2ox is putatively encoded by eight genes (*CsGA2ox1*-*CsGA2ox8*) [67], but only *CsGA2ox1*, *CsGA2ox4* and *CsGA2ox6* expression were detected in our datasets. Of these, *CsGA2ox1* and *CsGA2ox4* were DEGs significantly affected by GA₃-treatment and showed consistently higher expression in GA₃-treated roots

compared with Control, whereas the expression of *CsGA2ox6* was not significantly affected by GA₃-treatment.

We also investigated the expression profiles of genes involved in GA signaling pathways, including genes encoding GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), a positive regulator of GA signaling F-box protein SLEEPY1 (SLY1) as well as GA signaling repressors, including DELLA proteins (DELLAs), and SPINDLY (SPY) [65, 71]. The expression of *CsGID1A*, *CsGID1B* and *CsSLY1* were significantly repressed by GA₃-treatment (Fig. 6). For the GA signaling repressors, two genes (*CsGAI2* and *CsGAI3*) encoding DELLA proteins exhibited up-regulation in response to GA₃-treatment, whereas the expression of *CsGAI1* and *CsSPY* were not affected by GA₃-treatment. Overall, 20 out of 34 genes in GA synthesis and signaling pathways were expressed in cucumber roots. Of these, 15 genes were identified as DEGs with significant GT effects, suggesting their important roles in GA-mediated LR development.

DEGs involved in auxin synthesis and signaling pathway

Considering the central roles of auxin in almost all steps of LR development and the fact that GAs influence auxin biosynthesis and signaling during root development [6, 72], we further investigated the expression of genes involved in auxin metabolisms, including IAA biosynthesis, inactivation, transport and signal transduction.

The TRYPTOPHAN AMINO-TRANSFERASE OF ARABIDOPSIS/ YUCCA (TAA/YUC) pathway has been proposed as the primary endogenous auxin biosynthesis pathway that involves in various biological processes, including root development [73–75]. There are eight YUC family genes in cucumber (Additional file 10). Of these, *Csa3G133910* (*CsYUCCA3*) and *Csa3G619930* (*CsYUCCA5*) were identified as DEGs with significant GT effects. They were clustered in C19 and significantly up-regulated under GA₃-treatment (Fig. 7; Additional file 7). To our surprise, no TAAs were identified in cucumber genome using BLASTP, suggesting that the auxin biosynthesis pathways in cucumber remain elusive. We then checked the expression of the genes involved in IAA inactivation, including genes encoding IAA-amido synthase GRETCHEN HAGEN 3 (GH3), Dioxygenase for Auxin Oxidation (DAO) and IAACARBOXYLMETHYLTRANSFERASE 1 (IAMT1). GH3 is putatively encoded by eight genes in cucumber (Additional file 10), by which IAA can also be conjugated to amino acids [76]. Two out of eight GH3 genes, *Csa3G198490* (*CsGH3.3*) and *Csa3G431430* (*CsGH3.5*), were down-regulated in response to GA₃, whereas only the expression of *Csa3G088930* (*CsGH3.4*) was significantly increased in GA₃-treated roots (Fig. 7). The putative cucumber *IAMT1* (*Csa7G081680*) showed similar expression levels in the roots of GA₃-treatment and Control. In addition, there was not an ortholog of Arabidopsis DAO in cucumber. These results suggested that exogenous GA₃ might suppress the processes converting active IAA to inactive forms.

PINFORMED (PIN) family, AUXIN1/LIKE-AUX (AUX/LAX) family and ATP-binding cassette family B (ABCB) family are well known auxin transport proteins in plants [77, 78]. In cucumber, seven, seven and 20 PIN, AUX/LAX and ABCB family members were identified, respectively (Additional file 10). Of these,

two *PINs* (*CsPIN1* and *CsPIN5*), four *AUX/LAXs* (*CsAUX1*, *CsAUX2*, *CsLAX1* and *CsLAX4*) and five *ABCBs* (*CsABCB4*, *CsABCB5*, *CsABCB10*, *CsABCB11* and *CsABCB18*) were DEGs with significant GT effects (Additional file 5). Notably, only *CsLAX1* and *CsABCB18* were down-regulated by GA₃-treatment. The well-known components of the auxin signal transduction pathway include TRANSPORT INHIBITOR RESPONSE1/AUXIN-RELATED F-BOX (TIR1/AFB) and Aux/IAA proteins and the AUXIN RESPONSE FACTORS (ARFs) [78, 79]. Five, 27 and 16 genes were identified in cucumber TIR1/AFB family, Aux/IAA family and ARF family, respectively (Additional file 10). Notably, a total of 21 genes from the three families were significantly affected by GA₃ treatment and 20 of them were constitutively higher expressed in GA₃-treated roots compared to Control (Fig. 7).

Taken together, a total of 36 DEGs involved in IAA biosynthesis, inactivation, transport and signal transduction were identified in our datasets. Among them, 31 genes were up-regulated in response to exogenous GA₃. To determine if GA₃ treatment enhances IAA biosynthesis, we analyzed IAA levels in 5 DAG roots by LC-MS/MS analysis. As shown in Fig. 7C, IAA level was significantly increased in GA₃-treated roots. These results suggest that the enhanced auxin metabolisms are associated with GA-mediated root development.

DEGs involved in cell wall biosynthesis

Given that the up-regulated DEGs were enriched for various cell wall-related processes, including metabolic processes of lignin, chitin and cell wall macromolecule which are the primary components of plant cell wall [80], we also want to know the transcriptional changes of cell wall biosynthesis genes under GA₃-treatment in cucumber roots. The Arabidopsis cell wall biosynthesis genes include 10 cellulose synthase (*CESA*) genes, 30 cellulose synthase-like (*CSL*) genes (*CSLAs*, *CSLBs*, *CSLCs*, *CSLDs*, *CSLEs* and *CSLGs*) and 12 Glucan Synthase-like (*GSL*) genes [81, 82] (<https://www.arabidopsis.org/browse/genefamily/cellwall.jsp>). A total of 47 genes from *CESA*, *CSL* and *GSL* families were identified as putative cell wall biosynthesis genes in cucumber genome. The 47 genes encode eight *CESA* proteins, 30 *CSL* proteins and 9 *GSL* proteins in eight distinct groups (Fig. 8A).

The expression of four *CESA*, 10 *CSL* and one *GSL* genes were significantly affected by GA₃-treatment and 11 of them showed higher expression in GA₃-treated roots (Fig. 8B), indicating cell wall biosynthesis in roots was likely promoted by GA₃-treatment which is consistent with the phenotype of GA₃-treated roots (Fig. 1; Additional file 1). Some of them are putatively involved in root development based on previous studies. For example, *CsCSLD2* is the closest ortholog of Arabidopsis *CSLD3*, which is essential for the synthesis of cell wall at the root hair tip [83, 84]. In Arabidopsis, loss-of-function of *CSLA9*, the closest ortholog of *CsCSLA5*, had reduced numbers of LR [85].

Discussion

Cucumber is a world-wide cultivated vegetable crop that is of important economic and nutritional value. However, it is particularly susceptible to various stresses, such as low temperature, drought, and salt, due

to shallow-rooting system, limiting its productivity and yield [86]. LRs are critical for determining stress adaptability and crop productivity [6, 87, 88]. Although LR formation has been extensively studied, to our knowledge, none of the key genes responsible for LR formation have been identified in cucumber. Therefore, it is of great importance to identify candidate genes and study the mechanisms underlying LR formation in cucumber.

The roles of GAs have been widely studied in regulating stress tolerance [89, 90], flower and fruit development [49, 91, 92], and plant growth [93, 94] in cucumber. By contrast, very little is known about what roles GAs have in LR formation. While positive roles of GAs in regulating LR formation were found in *Arabidopsis*, the inhibitory effects of GAs on LR formation have been reported in other plants, such as *Medicago truncatula* [40], poplar [22, 38], hybrid aspen [39], tomato [37] and carrot [41]. The application of exogenous GA₃ on cucumber seedlings offers an opportunity to gain insight to the effects of GAs on cucumber LR development. Here, we initially applied various GA₃ concentrations to germinated seeds for five days and found that appropriate GA₃ concentration, especially 50 µM, promotes LRs formation and elongation, whereas excessive GA₃ did not show a positive effect on root development (Fig. 1; Additional file 1). Therefore, exogenous GA₃ regulates LR development in a concentration-dependent manner in cucumber.

We also sprayed 50 µM GA₃ to soil in 72-well trays for 5 days after the germinated seeds were sown to the soil. A strong positive effect of 50 µM GA₃ on root growth was also observed in this condition (Additional file 11). These results suggested the important roles of GAs in regulating LR development in cucumber. Further evidence supporting this notion came from our previous study, in which the transgenic *Arabidopsis* lines over-expressing *CsGA20ox1* that show a significant increase in primary root length and LR number [67].

To dissect the molecular mechanisms underlying GA-mediated LR formation and identify important candidate genes involved in this pathway, RNA-seq was performed. Down-regulated DEGs identified by pairwise comparisons in GA₃-treated roots were enriched for various GA metabolism pathways (Fig. 3B). We further analyzed the expression changes of genes involved in GA biosynthesis and signal transduction. The genes positively regulated GAs biosynthesis and signal transduction were down-regulated in response to GA₃, including *CsCPS*, *CsKO*, *CsKAO2*, *CsGA20ox1* and *2*, *CsGA3ox1*, *CsGA7ox1* and *2*, *CsGID1A* and *B*, and *CsSLY1* (Fig. 6). Interestingly, the negative regulators of GAs biosynthesis and signal transduction, such as genes encoding GA2oxs and DELLAs, were up-regulated in GA₃-treated roots (Fig. 6). These results indicated the inhibition of endogenous GAs biosynthesis and signal transduction by exogenous GA₃ in cucumber, which might result from the feedback regulation of GA metabolism. Many previous studies also showed similar results in other crops, including Ginkgo [95], lodgepole pine [96], Triploid Loquat [97], *Jatropha curcas* [98] and crape myrtle [99]. However, some other studies indicated the induction of endogenous GAs biosynthesis and signal transduction by exogenous GA in other crops, such as tree peony [100], apple [101] and sugarcane [102]. Therefore, the effects of

exogenous GA₃ on endogenous GA metabolisms vary in different crops and the mechanisms need to be further studied.

It has been reported that LR formation is modulated by Gibberellin-Auxin Crosstalk [22, 103–105]. DEGs involved in IAA biosynthesis, transport and signal transduction were all up-regulated by exogenous GA₃, with the exception of *CsLAX1*, *CsABCB18* and *CsTIR1* (Fig. 7). By contrast, two out of three *GH3* genes converting active IAA to an inactive form were down-regulated, indicating GAs regulated LR development likely through enhancing IAA metabolism. In addition, GAs have been demonstrated to be involved in cell wall organization and modification [22, 106, 107], which are important for root development [22, 108, 109]. Therefore, it is not surprising that we found up-regulated genes in GA₃-treated roots at the three timepoints were enriched for some cell wall-related processes. The increased expression of most of the DEGs involved in cell wall biosynthesis (Fig. 8) and the DEGs in some clusters, such as C7, C10 and C12, significantly enriched for metabolic processes of some components of cell wall (Additional file 8) further support the notion that GAs regulating LR development that is associated with altered cell wall metabolisms.

Conclusions

The promotion and inhibition of LR development by exogenous GA₃ and Uni, respectively, indicated the important roles of GAs in regulating LR development in cucumber. Comparative transcriptomic analysis of developing roots showed that GA modulation of LR development is at least partly associated with enhanced auxin and cell wall metabolisms. Importantly, a number of DEGs, including TFs and genes involved in GA, IAA and cell wall related processes, putatively involved in GA regulation of LR development in cucumber were also identified, providing potential targets for further dissecting the molecular mechanisms underlying GA-mediated LR development in cucumber and other crops.

Methods

Plant materials, treatment and growth conditions

Cucumber (*Cucumis sativus* L.) inbred line ‘Daqingba’ was used in this study. The ‘Daqingba’ was developed and kindly provided by Dr. Chenxing Cao from Shandong Agricultural University. The seeds were sown in petri dishes with MS medium. After germination, the seeds with similar germination status were transferred to MS medium with various concentrations of GA₃ and uniconazole (Sigma) in glass jar. The germinated seeds were also transferred to MS medium in glass jar without any treatment as control. The seeds were cultured in incubator under 24 h dark at 28 °C. After germination, the seedlings were grown in a growth chamber at 25 ± 1 °C with a 16 h light/ 8 h dark photoperiod.

Phenotypic characterization

The roots were carefully washed with water to remove MS medium and then scanned with an Epson Expression 10000G photo scanner (Seiko Epson Corporation, Nagano, Japan). The secondary and tertiary root length was determined by the length of the three longest secondary and tertiary roots, respectively. The root length measurements were performed with Image J. Shoot and root dry weights were determined at 5 DAG. For the dry weight measurements, the roots and shoots were dried at 75 °C for 3 days and weighted. At least 20 seedlings were analyzed per biological replicate and three biological replicates were performed for each treatment.

Sample collection, RNA extraction and data processing of RNA-Seq data

The roots were collected at 2, 3, and 5 DAG. About 0.5 cm root tip of primary, secondary and tertiary roots and the 0.5 cm section from the proximal end of secondary and tertiary roots were also collected. In addition, the whole secondary and tertiary roots were collected if they were not longer than 1.0 cm. Tissues for RNA extraction from 10–20 individual seedlings were pooled as one replicate and two to three biological replicates were performed for each treatment and timepoint combination.

Total RNA was extracted from a mixture of root tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Fermentas, Burlington, ON, Canada) according to the manufacturers' instructions. Quality of RNA was checked by determining the RNA integrity using RNase-free agarose gel electrophoresis, and RNA Nano6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The cDNA library preparation and sequencing were conducted by the Allwegene Technology Company in Beijing, China. All libraries were sequenced on the Illumina HiSeq TM4000 platform (Illumina, Inc., San Diego, CA, USA). All raw reads generated in this study have been deposited at the Sequence Read Archive (<https://ncbi.nlm.nih.gov/sra>) under BioProject PRJNA667293.

Raw reads were pre-processed to remove adapter sequences and low-quality reads ((eads with ambiguous bases > 10% and more than 50% bases with $Q \leq 20$) using Trimmomatic (v0.33) [110]. Clean reads were mapped to the Cucumber (Chinese Long) v2 Genome using TopHat2 [42, 111]. The read counts for each gene were generated by the HTSeq.py python package [112]. The gene expression level was normalized by the fragments per kilobase of exon per million mapped reads (FPKM) value using Cuffdiff (<http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/>). Summary statistics for each of the RNA-seq libraries are shown in Additional file 1. Correlation between the biological replicates was determined using Spearman correlation coefficient (SCC). Principal component analysis (PCA) of all replicates was performed using DESeq2 [113] and the PCA plot was generated using the ggplot2 R package.

Differential gene expression analysis

Differential gene expression analysis was performed using the DESeq2 R package [113] with the count data which was generated with HTSeq.py python package [112]. For pairwise comparisons, genes with $|\log_2\text{ratio}| > 2$ and a false discovery rate significance score < 0.05 were determined to be significantly

differentially expressed genes (DEGs) at each developmental timepoint between GA₃-treatment and Control.

A differential expression analysis of RNA-seq data was also performed using linear factorial modeling to further assess the effects of GA treatment (GT) and the interaction between GA treatment and developmental timepoint (GT*D) on the gene expression patterns. The likelihood ratio test was used to assess two separate null hypotheses. Null hypothesis 1 was tested to identify genes with significant GA₃-treatment (GT) effects with full model = ~ treatment + developmental timepoints and reduced model = ~ developmental timepoints; Null hypothesis 2 was tested to identify genes significantly affected by GT*D with full model = ~ treatment + developmental timepoints + treatment : developmental timepoints and reduced model = ~ treatment + developmental timepoints. The p values were corrected using the Benjamini-Hochberg method and the threshold of corrected p value < 0.05 was used for selecting DEGs in the two null hypotheses. A further filtration was performed to eliminate the low expressed genes. Genes with average FPKM > 2 among samples were considered as expressed DEGs.

The degPatterns function from DEGreport R package [114] was used to determine the expression patterns and clustering of the DEGs. The minimum number of genes in each cluster was more than 5 (minc = 5). Gene ontology enrichment analysis of the DEGs was performed using the topGO R package [115, 116]. The significantly enriched GO terms were determined by FDR adjusted p-value < 0.05. The heatmaps of DEGs were generated using the pheatmap R package [117].

Genome-wide identification of GA-, IAA- and cell wall-related genes families in cucumber genome

The corresponding Arabidopsis proteins were used as query sequences and BLASTP was used to search against the Cucumber (ChineseLong) protein v2 (<http://cucurbitgenomics.org/blast>). For phylogenetic analysis, ClustalX 1.81 was used to perform multiple sequence alignments with default parameters and the neighbor-joining (NJ) phylogenetic trees were constructed using MEGA 7.0.

IAA quantification

Five DAG roots were extracted and purified as previously described [118, 119] with some modifications: About 200 mg plant tissue was homogenized and extracted for 24 h in methanol containing 2H₂-IAA as internal standard. Purification was done with Oasis Max solid phase extract cartridge after centrifuged. LC-MS/MS analysis was performed on a UPLC system (Waters) coupled to the 6500 Q-Trap system (AB SCIEX). Sample was injected onto a BEH C18 column (1.7 mm, 2.1 × 150 mm; Waters) with mobile phase 0.05% HAc (A) and 0.05% HAc in ACN (B). The gradient was set with initial 5% B and increased to 70% B within 6 min. The multiple reaction monitoring (MRM) mode was used for quantification and the selected MRM transitions were 176.0 > 132.0 for 2H₂-IAA, 174.0 > 130.0 for IAA. Three biological replicates were analyzed for each treatment

Abbreviations

LR: Lateral root; GA: Gibberellin; DAG: Days after germination; DEG: Differentially expressed gene; XPP: Xylem pole pericycle; LRP: Lateral root primordium; TF: Transcription factor; RDW: Root dry weight; SDW: Shoot dry weight; Uni: Uniconazole; SCC: Spearman correlation coefficient; PCA: Principal component analysis; GT: GA₃ treatment; GT*D: The interaction of GT and developmental timepoint; ARF: Auxin response factor; GRF: GROWTH-REGULATING FACTOR; CRF: CYTOKININ RESPONSE FACTOR; GID: GIBBERELLIN INSENSITIVE DWARF; SLY: SLEEPY; TAA/YUC: TRYPTOPHAN AMINO-TRANSFERASE OF ARABIDOPSIS/ YUCCA; GH3: GRETCHEN HAGEN 3; DAO: Dioxygenase for Auxin Oxidation; IAMT1: IAACARBOXYLMETHYLTRANSFERASE 1; PIN: PINFORMED; AUX/LAX: AUXIN1/LIKE-AUX; ABCB: ATP-binding cassette family B; TIR1/AFB: TRANSPORT INHIBITOR RESPONSE1/AUXIN-RELATED F-BOX; CESA: Cellulose synthase; CSL: Cellulose synthase- like; GSL: Glucan Synthase-like.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

QL and ZR conceived and designed the research. QL, BC and TW performed the bioinformatic analysis. HS, TW, JC and CL conducted the experiments and statistical analysis. QL and BC wrote the draft manuscript with revisions from BC, HS, TW, JC, CL and ZR. All authors approved the manuscript.

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Figures

Figure 1

The effects of various GA3 concentrations on root development at five days after germination (DAG) in cucumber. The letters in the boxplots indicate the significant differences among different genotypes evaluated by Tukey HSD test ($\alpha = 0.05$). RDW, Root dry weight; SDW, Shoot dry weight.

Figure 2

Correlation between the transcriptomes of root tissues at the three developmental timepoints in GA3-treatment and Control. (A) Spearman correlation coefficient (SCC) analysis of the RNA-seq data of root tissues at the three developmental timepoints in GA3-treatment and Control. C, Control; G, GA3 treatment; r1, r2 and r3 represent different biological replicates; (B) Principal component analysis (PCA) plot showing clustering of transcriptomes at the three developmental timepoints in GA3-treatment and Control.

Figure 3

Differential gene expression analyses in the developing roots between GA3-treatment and Control. Upper panel, the number of DEGs at different developmental timepoints of roots; Lower panel, biological process GO enrichment of up- and down-regulated DEGs in GA3-treated roots. The size of the bubbles indicates the number of DEGs in the given GO term. The color-coding indicates the Gene Ratio calculated as the number of DEGs in the given GO term divided by the total number of genes in the term. DAG, days after germination.

Figure 4

Clustering of unique DEGs identified by pairwise comparisons and linear factorial modeling. The line represents the median Z-score of gene abundance. The actual spread of the data is represented by jittered dots, so the relative expression of every individual gene is represented on the plot. DAG, days after germination.

Figure 5

Overview of the distribution of TF families that differentially expressed between GA3-treated roots and Control.

Figure 6

Schematic overview of GAs biosynthesis (A) and signal transduction (B) alongside the heatmap showing the expression of related genes (C). Each row represents one gene, columns represent samples from different timepoints, and colors represent gene expression levels as log₂-transformed FPKM. Lower levels of expression are represented in yellow and higher expression in blue. Values, which were shown on the heatmaps, represent the average FPKM value of the biological replicates. The gene ID of DEGs in GA biosynthesis and signal transduction were labeled in red. Black gene ID indicates the genes which were not or barely expressed in our datasets or showed similar expression between GA3-treated roots and Control. 2, 2DAG; 3, 3DAG; 5, 5DAG. CPS, ent-copalyl diphosphate synthase; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO, ent-kaurenoic acid oxidase; GID, GIBBERELLIN INSENSITIVE DWARF 1; SPY, SPINDLY; SLY, SLEEPY1; GAI, GIBBERELIC ACID INSENSITIVE.

Figure 7

The effects of GA₃ treatment on auxin metabolism. (A) A schematic model of auxin biosynthesis, inactivation, transport, and signal transduction in plants; (B) Expression analysis of genes involved in IAA biosynthesis, inactivation, transport and signal transduction. Only differentially expressed genes were shown in the heatmap. Each row represents one gene, columns represent samples from different time points, and colors represent gene expression levels as log₂-transformed FPKM. Lower levels of expression are represented in yellow and higher expression in blue. Values, which were shown on the heatmaps, represent the average FPKM value of the biological replicates; (C) IAA level was significantly increased in GA₃-treated roots at 5 DAG. Significant difference as determined by Student's t-tests. YUC, YUCCA; TAA, TRYPTOPHAN AMINO-TRANSFERASE OF ARABIDOPSIS; GH3, GRETCHEN HAGEN 3; DAO, Dioxygenase for Auxin Oxidation; IAMT1, IAACARBOXYLMETHYLTRANSFERASE 1; PIN, PINFORMED; AUX, AUXIN1; LAX, LIKE-AUX; ABCB, ATP-binding cassette family B; TIR1, TRANSPORT INHIBITOR RESPONSE1; AFB, AUXIN-RELATED F-BOX; ARF, AUXIN RESPONSE FACTOR.

Figure 8

Analyses of cell wall biosynthesis genes in cucumber. (A) Phylogenetic analysis of cucumber and Arabidopsis cell wall biosynthesis proteins by using MEGA neighbor-joining; (B) Heatmap showing putative cucumber cell wall biosynthesis genes differentially expressed between GA3-treated roots and Control. Each row represents one gene, columns represent samples from different time points, and colors represent gene expression levels as log2-transformed FPKM. Lower levels of expression are represented in yellow and higher expression in blue. Values, which were shown on the heatmaps, represent the average FPKM value of the biological replicates. CESA, Cellulose Synthase; CSL, Cellulose Synthase- Like; GSL, Glucan Synthase-Like.

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