Transcriptomic profiling and discovery of key genes involved in adventitious root formation from green cuttings of highbush blueberry (*Vaccinium corymbosum* L.)

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Abstract

Background Propagation of cuttings was mostly used in various plant species including blueberry, the special root characteristics of blueberry usually resulted in a difficulty in adventitious root (AR) formation. The AR formation was influenced by various factors, of which auxin was considered to play a center role, however little is known of the related regulative mechanisms. In this study, transcriptome analysis using RNA_seq from the stem of green cuttings of southern highbush blueberry 'Biloxi' was performed to discover candidate genes associated with AR formation. **Results** Rooting phenotypes, especially rooting rate, was significantly promoted by exogenous auxin IBA application. The adventitious root primordium initiation (rpi) began to be formed at 14 day (d) after cutting, developed into root primordium (rp) at 21d, finally the rp further developed to mature AR at 28d. Higher IAA and lower ABA and zeatin might facilitate the AR formation and development. A time series transcriptome analysis indentified 14970 differentially expressed genes (DEGs) during AR formation, of which there were 7467 up-regulated and 7503 down-regulated genes, respectively. Of these, about 35 candidate DEGs involved in auxin-induced pathway and AR formation were further identified, including 10 auxin respective genes ARFs and SAURs , 13 transcription factors LOB domain-containing protein (LBD s), 6 auxin transporter AUX22 , LAX3/5 and PIN-like 6s (PIL6s) and 6 rooting-associated genes root meristem growth factor 9 (RGF9), lateral root primordium 1 (LRP1s), dormancy-associated protein homolog 3 (DRMH3). All these identified DEGs were highly up-regulated in certain AR developed stage, indicating their potential roles in blueberry AR formation. **Conclusions** The transcriptome profiling indicated candidate genes or major regulative factors that influence adventitious root formation in blueberry, and provided a comprehensive understanding of rooting mechanism of the auxin-induced AR formation from blueberry green cuttings.

Background

Adventitious root (AR) formation is a developmental process which reflects the plasticity of plants to adjust to stressful conditions and to regenerate plant tissues from the same individuals independent of sexual reproduction [1-4]. The ARs are usually generated spontaneously or in response to certain stimuli from stems, leaves, or non-pericycle tissues of older roots [5, 6]. It could be divided into several stages based on their physiological and metabolic processes: a) dedifferentiation; b) cell division and c) adventitious root primordia initiation, development and outgrowth [7]. Plant hormones, such as auxin, abscisic acid, cytokinin and ethylene, were proven to play vital roles in enhancement of AR formation, of which auxin was considered as a central player [8, 9]. Plant growth regulator, mainly indole-butyric acid (IBA), is often exogenously applied to promote ARs emergence from cuttings of difficult-to-root plant species including blueberry [10]. By assessing the rooting abilities of blueberry hardwood cuttings under IBA concentrations, 1000 - 2000 ppm IBA was suggested to be more effective to induce rooting quality [11, 12]. However, knowledge about auxin signaling cascade and auxin-induced gene transcriptional information during the onset of ARs initiation and thus formation in rooting cues of blueberry cuttings is very little to known.

In *Arabidopsis*, the role of auxin in ARs formation was proven to be regulated directly through the changes of auxin-related gene expression [13, 14]. Transcriptional regulators *AUXIN RESPONSE FACTOR* (*ARFs*) and *AUXIN/INDOLE-3-ACETIC ACIDS* (*AUX/IAAs*) genes were demonstrated to be involved in regulating ARs formation, for which *ARFs* would activate or repress early responsive genes via binding with auxin response elements in the promoter region of these genes [15]. To date, total 29 members of *ARFs* had been isolated and identified from *Arabidopsis*, of which *ARF7* and *ARF19* were suggested to participate in AR formation by positively activating the transcription of their downstream genes of *LATERAL ORGAN BOUNDSARIS-DOMAIN* (*LBDs*) [16-18]. It was indicated that the over-expression of *ARF7* and *ARF19* could enhance ARs emergence, while the loss-of-function of *arf7*, *arf19* and *arf7/arf19* double mutants showed a severe defection in ARs formation [16]. The *LBD16* played a pivotal role in AR initiation, and the activation of *LBD16* could finally result in establishment of adventitious root-primordium identity, *lbd18* mutant exhibited a reduced number of roots and the *lbd16lbd18* double mutants showed a dramatic reduction in roots in comparison with *lbd16* or *lbd18*. However, over-expression of *LBD18* rescued the root formation in *lbd18* and *lbd16lbd18* mutant and also could stimulate root formation in *arf7arf19* mutants [19, 20]. In contrary, *Aux/IAA* negatively regulated the transcription of auxin-induced gene as a repressor through combining with the *ARFs* transcription activators [21, 22]. But, to date, functions of the auxin induced or responsive genes in ARs formation in blueberry stem cuttings is also not clear.

Numerous studies in *Arabidopsis* and other species have indicated that auxin polar transport (APT) system, mediated mainly by influx and efflux carriers, is essential for AR initiation and subsequent development, because APT system is strictly directional to establish auxin asymmetric localization and thus induces ARs initiation and emergence [23-25]. Large numbers of auxin influx carriers, e.g. *AUX1* and *AUX1-LIKE* (*LAX*) families, and efflux carriers, e.g. *PIN-FORMED* (*PINs*) members and their homologues *PIN-LIKES* (*PILs*), have been successfully indentified [26-28]. It was found that the *pin1* mutant fails to establish auxin gradient and show developmental disorders in root formation (Benkova et al. 2003). The AR development were significantly inhibited in *OsPIN1* RNA interference (RNAi) transgenic plants, and exogenously-applied NAA could rescue the rooting phenotypes occurring in *RNAi-OsPIN1* plants [29]. Besides, ARs formation might be controlled by different genes in different developmental stages, *lateral root primordial 1* (*LRP1*) acts as a transcriptional activator in auxin downstream of *AUX/IAA* and was expressed mainly in very early stages of adventitious root primordium emergence [30-32]. In blueberry, however, how these carriers and rooting-related genes were regulated by auxin is still open.

Blueberries (*Vaccinium corymbosum* L.), a member of the Ericaceae family, are commercially important small fruit crops for their healthy and flavorful bioactive compounds, blueberry acreage was continuously expanded year-by-year worldwide especially in China [33]. It could be propagated by methods of seeds, grafting, tissue culture, cuttings, and others, of which cuttings was mostly used because it could ensure the characteristic of mother plants, increase plant uniformity, shorten juvenile phase and etc. [34-37]. Whereas, due to the special root architecture which was composed mainly by fine roots, its cultivation requires certain environmental conditions e.g. soil moisture, permeability and pH, which usually leading to a lower rooting percentage [10]. Till now, difficulty of blueberry propagation using cuttings is still a main

factor limiting its expansion [11, 38]. It is necessary to reveal the mechanisms, whatever environmental or genetic, that control blueberry AR formation from cuttings. With the rapid development of biological information technology, Illumina sequencing technology (RNA-seq) provides a new gateway to identify the gene expression patterns, regulatory networks and even SNPs variants that involve in complex biological process of plants [39-42]. RNA-seq technology is a highly efficient, widely used and conventional molecular biology method to obtain transcriptomic information and has been successfully applied in blueberry to indentified candidate genes involving agronomic traits, such as the putative genes related to antioxidants [39, 43], fruit development and ripening [44, 45] and genes involved in chilling-mediated flowering pathway [46]. However, no sequence transcriptional information was available for ARs formation from cuttings of blueberry. The object of this study was to explore the gene regulative networks that control blueberry AR formation from cuttings by assessing rooting phenotypes, microscopic observation of AR development and further a time-caused transcriptional information analysis during ARs formation. The results would provide database insight to explain the rooting cues of cuttings, and also would facilitate the fast propagation of blueberry.

Results

Phenotypic analysis

The rooting phenotype of blueberry green cuttings varied significantly between control and IBA treatment (Figure 1a, 1b). The rooting percentage and average root number per cutting were both significantly promoted by exogenously-applied IBA (Figure 1c, 1d). There was no significant difference in average root length between these two treatments (Figure 1e), suggesting that exogenous auxin IBA could enhance the adventitious root formation without influencing their average lengths.

Microstructure observation of adventitious root formation

To clear and definite the AR developmental process against the stem of blueberry green cuttings, the microstructure was observed. The results showed that callus (c) tissues started to form at 7 days after cutting (DAC) in IBA treatment, then adventitious root primordium initiation (rpi) was produced at 14 DAC, and developed into adventitious root primordium (rp) at 21 DAC, the rp was finally developed to adventitious root (r) and outgrowth from the stem (Figure 2), suggesting the blueberry AR formation was initiated from non-root pericycle cells as a type of auxin-induction.

Analysis of plant hormones in blueberry green cuttings during ARs formation

Changes of plant hormone during blueberry AR formation were analyzed. The level of IAA exhibited a significantly increase at 21 day after cutting (DAC) under IBA treatment (Figure 3a, 3b), which might indicating that the higher IAA might contribute to initiate adventitious root primordium (rpi). No significant difference in GA3 content was observed in green cuttings between control and IBA treatment (Figure 3c). The content of ABA and zeatin under IBA treatment was kept at a lower level during the whole trail, and a decreasing tendency of ABA and zeatin was recorded in control treatment and got a similar level as IBA

treatment after 21 DAC (Figure 3d), suggesting that a lower level of ABA and zeatin may facilitate the ARs formation.

***De novo* sequencing, Assembly, and Gene Annotation in RNA-seq of blueberry stem**

The stem from base of cuttings were sampled every 7 days after insertion, and ten samples (CK7, CK14, CK21, CK28, CK35, T7, T14, T21, T28 and T35) were subjected to total RNA extraction and RNA-seq analysis. High-throughput sequencing generated 41.45 - 44.46 million (M) pairs of 150 bp raw reads from each library. After a stringent quality filtering process, 212 million clean reads (99.54% of the raw data) remained, with a Q30 percentage (an error probability lower than 0.1%) ranging from 92.53% to 94.00%. The number of clean reads per library ranged from 41.27 to 44.25 M (Table 1). The total clean reads were *de novo* assembled into transcripts by trinity, and 672606 transcripts and 308719 unigenes were assembled with an average length of 735 bp (N50=1082) and 617 bp (N50=844), respectively. All unique sequences were further annotated based on Blastx searches with a cut-off *E*-value of 10^{-5} against five databases, total unigenes of 107111 (34.70%), 53859 (17.45%), 6696 (2.17%), 101729 (32.95%) and 89910 (29.12) were respectively annotated by NCBI non-redundant (NR) database, gene ontology (GO) database, kyoto encyclopedia of genes and genome (KEGG) database, evolutionart genealogy of genes: Non-supervised orthologous groups (eggNOG) database and Swiss-Prot database. Based on the NR annotations, 25.33% of the annotated sequences had strong homology (*E*-value $< 10^{-60}$), 21.80% of the annotated sequences showed strong homology ($10^{-60} < E\text{-value} < 10^{-30}$), and an additional 82.87% of the annotated sequences showed homology ($10^{-30} < E\text{-value} < 10^{-5}$) to available plant sequences (Figure 4a). The similarity distribution was comparable, with 26.41% of the sequences having similarities higher than 80%, while 73.5% of the hits had similarities of 0-80% (Figure 4b). With respect to species, 72.99% of the unique genes showed high matches with sequences form other species, followed by *Vitis vinifera* (9.3%), *Oryza sativa Japonica Group* (5.74%) and *Coffea canephora* (2.83%) (Figure 4c).

Ten DEGs libraries were further analyzed based on RSEM quantitative software, and the FPKM (fragments per kb per million fragments, FPKM) of all unigenes were calculated at each sampled period in blueberry between control (CK) and IBA treatment (T). Differences in gene expression was examined using the threshold of $|\log_2\text{FoldChange}| > 1$ at *P*-value ≤ 0.05 . The DEGs were identified by pairwise comparisons of ten libraries, i.e. CK7 vs. T7, CK14 vs. T14, CK21 vs. T21, CK28 vs. T28, and CK35 vs. T35 (Figure 5a). A total of 14970 DEGs were detected between CK and T libraries (Figure 5b), of which there were 7467 up-regulated and 7503 down-regulated genes. For each sampled period, 3252 DEGs was detected between CK7 and T7 libraries, these unigenes were directly affected by IBA treatment and might be associated with callus tissue formation. In CK14 vs. T14, there are 3999 DEGs were indentified, which might be related with rpi formation. In CK21 vs. T21, total 1488 DEGs was determined, which were contributed to rp formation. In CK28 vs. T28, 2648 DEGs were indentified, which were contributed to AR outgrowth. And, in CK35 vs. T35, total 3583 DEGs were detected, which might be contributed to AR development (Figure 5b, Figure 2). However, there were no commonly up-regulated or down-regulated DEGs at all sampled periods, as illustrated in the Venn diagram (Figure 5c), suggesting DEGs might play special roles during AR formation, outgrowth and development progress.

DEGs enriched in the auxin signaling pathway

Auxin was proved to play key roles in promoting AR formation, therefore the DEGs in auxin-signaling pathway were annotated and further analyzed. A total of 29 auxin-related DEGS were mapped (Figure 6). Of these DEGs, there were ten unigenes belongs to auxin-responsive protein including four *ARFs* and six *SAURs* family. Six auxin transporter-like genes were also identified including three influx carriers *AUX22*, *AUX-LIKE 3* (*LAX3*), *LAX5* and three efflux carriers *PIN-LIKE 6* (*PIL6s*). All these DEGs were up-regulated during AR development in some extent (Figure 6). To verify the result of comparative transcription analysis, two auxin responsive factor *ARFs* family (i.e. *ARF7* and *ARF9*) and six auxin transporter genes (i.e. *AUX22*, *LAX3*, *LAX5* and *PIL6a-6c*) were selected to identify the DEGs expression profiles by qRT-PCR (Figure 7, 8, 9). RNA-seq showed that *ARF7* and *ARF9* exhibited a significant transcript peak at 28 d in IBA treatment, while both these two *ARFs* showed a decrease trend in control (Figure 7). RNA-seq data revealed that *AUX22* was kept at a lower expression in control, but it showed a significant up-regulation at 7d – 21d in IBA treatment (Figure 8). Its homologous genes *LAX3* and *LAX5* showed a decrease tendency in control, it was significantly un-regulated at 14 d and 28 d in IBA treatment (Figure 8), suggesting these genes might play an important role in auxin transporting and contributed to auxin asymmetrical distribution which were facilitated to induce the adventitious root primordium initiation at early stage of AR formation. As for three auxin efflux carriers *PIL6a-6c*, in control treatment these three genes was low expression at 7 – 21 DAC, and they was up-regulated until 28 DAC, whereas when applying IBA, *PIL6a* was significantly up-regulated at 14 DAC, *PIL6b* and *PIL6c* showed a remarkable increase from 7 DAC and peaked at 35d (Figure 9), suggesting auxin enhance expression of *PIL6s* and thus promote the AR formation. qRT-PCR data indicated a similarity with transcript information (Figure 7, 8, 9).

Transcription factor *LATERAL ORGAN BOUNDARIES DOMAIN/LOB* domain-containing proteins (*LBDs*) were suggested to regulate AR formation as downstream target genes of *ARFs* family [19, 20]. In present work, thirteen homolog genes of *LBDs* were identified based on NR annotation (Figure 6), RNA-seq data revealed that all these *LBDs* were up- or down-regulated at different stage in a certain some extent, especially at 28 DAC. And, 4 out of these 13 *LBDs* (i.e. *LBD16*, 23, 29 and 37) were representatively selected to confirm their expression profiles by qRT-PCR, it indicated that *LBD16* and *LBD37* showed a good reproducibility with RNA-seq data, while qRT-PCR indicated *LBD23* exhibited another up-regulated at 14 DAC in IBA treatment, *LBD29* showed a continuous up-regulation during 21d -35d in IBA treatment and were also up-regulated at 14d and 35d in control (Figure 10).

DEGs involved in root primordium formation

Based on the NR annotation, six rooting-related DEGs were obtained including four homolog genes of *lateral root primrdium 1* (*LRP1*), one putative *root meristem growth factor 9* (*RGF9*), and one *dormancy-associated protein homolog 3* (*DRMH3*). RNA-seq data showed that *LRP1* was significantly up-regulated at 28 d, *LRP1-like*, *LRP-type1*, *LRP-like2* exhibited a continuous up-regulation after 14d and peaked at 28 d in IBA treatment (Figure 11). An obvious up-regulation of *RGF9* was observed at 7d, 14d and 28d (Figure

12), while *DRMH3* showed a significantly up-regulation at 28 d (Figure 12). The qRT-PCR analysis of *RGF9* and *DRMH3* indicated a good consistency with RNA-seq data (Figure 11, 12).

Discussion

Auxin is proven to play an important role in promoting the initiation and outgrowth of adventitious roots [47, 48], however the mechanism especially the gene regulative networks about how auxin-signaling controls the rooting cues from blueberry cuttings is remained unclear. Therefore, in present work, the rooting phenotype under IBA treatment in comparing with control was assessed to evaluate the rooting ability of blueberry green cuttings, anatomical structure of IBA-treated stem was observed to monitor the emergence of adventitious root primordium, the level of endogenous plant hormones during AR formation were determined, and the transcriptomic information were also analyzed to discover candidate genes involved in blueberry AR formation. To our knowledge, this study is the first report to identify gene networks via RNA-seq techniques involved in auxin-signaling pathway in blueberry AR formation. The results of this present study would be helpful to clone and discover key rooting-related genes, which would also help to understand the rooting cues of blueberry.

It was widely accepted that auxin is an effective inducer of AR formation, exogenous plant growth regulators, mainly indolebutyric acid (IBA), were often applied to improve rooting capacity of difficult-to-root plant species including blueberry [14, 38]. It was previously indicated that the rooting abilities of blueberry cuttings differed significantly among cultivars, the interaction of cultivar $\times$ IBA contributed straightforward to produce better rooting phenotype, ~1000 ppm IBA was suggested to be the most effective for rooting of blueberry cuttings [11, 12]. In this work, the rooting ability was significantly enhanced after IBA application in comparison with control, especially in rooting percentage and average root number (Figure 1a - 1f). The analysis of anatomical structure showed a large of callus began to be formed at 7 days after cutting (DAC), adventitious root primordium initiation (rpi) appeared at 14 DAC and morphed to root primordium (rp) at 21 DAC, the rp further developed to adventitious root (AR) at 28 DAC (Figure 2), these data indicated a greater induction of IBA on AR formation. Determination of endogenous hormones revealed that the level of IAA under IBA treatment increased significantly at 21 DAC (Figure 3a). This is probably resulted from the absorbed IBA accelerate the basal transport of IAA, or a small proportion of IBA was converted into IAA [49]. The accumulation of IAA in rooting zone would finally act as a trigger to initiate changes in auxin transport, signaling and function in particular responding cells, which would then initiate self-regulatory auxin canalization and maximization, and thereby starting the program of AR formation [50]. Generally, ABA was suggested to be an inhibitor and the effect of ABA on regulating AR formation was via the balance between IAA and higher ratio of IAA/ABA was conducive to induce AR emergence [51]. In present work, however, ABA content was kept at a lower level in comparison to control (Figure 3b), it was speculated that lower level of ABA might contribute to AR formation. There were no significant difference in GA3 between control and IBA treatment (Figure 3c), but the level of zeatin in IBA treatment was lower throughout the whole trial (Figure 3d), which indicated GA3 might have a very little effect on blueberry AR formation, but a lower level of zeatin seemed contribute notably to the AR formation in cuttings. Cytokinins were proved to have a

inhibitory role in AR formation, and cuttings with higher trans-zeatin level always exhibited a weak rooting capacity [7, 8]. Therefore, the AR formation dependent not only on the changes of IAA level but also on the cross-talk or homeostasis of auxin to other hormones e.g. a higher ratio of auxin vs. cytokinin was often associated with higher rooting capacity.

Transcriptome is the complete set and quantity of transcripts in a sample at specific developmental stage, it was a powerful tool to discover gene expression profiling in many biological processes [52]. To reveal the gene regulative networks in blueberry AR formation, the stem samples from different stages after cutting were successfully sequenced through RNA-seq analysis. More than forty million sequence reads for blueberry were generated and a total of 672606 unigenes were assembled by several complementary approaches (Table 1, Figure 4), which provided a valuable resource for surveying specific processes, functions and pathways during blueberry research. With further annotation by KEGG, GO and other database, a large number of DEGs, which was involved in different pathways was identified during AR development (Figure 5, 6). These data provided an overview that DEG transcription information in blueberry AR formation. It was suggested that both auxin polar transportation and accumulation were required for AR initiation which involved multiple genes to co-regulate the events of AR formation, such as auxin responsive factors (*ARFs*), auxin efflux-/ influx transporters e.g. *LAXs*, *PINs* and *PIN-LIKES* (*PILS*) gene families [48, 53-54]. For example, higher expression of *AtPIN1* could enhance AR formation from non-root tissues, knockdown of *OsPIN1* resulted in obvious inhibition of the emergence and development of adventitious roots [29]. The *pils2pils5* double loss of function mutant had higher auxin levels and presence of lateral roots than in the *PILS5* gain of function phenotype [55-56]. Thus it was important to survey the localized expression patterns of the auxin-related genes, the transcription information of other rooting-related genes, and even the metabolism-related enzymes activity of stem during AR development, induced by exogenous auxin application, which might facilitate to reveal the pathway the control rooting events in blueberry. By analysis of auxin signaling pathway, in present work, numerous auxin-induced DEGs were further identified (Figure 6) and some of these genes were selected to be verified by qRT-PCR (Figure 7, 8, 9). Both transcriptome and qRT-PCR data indicated *ARF7*, *ARF9* and their downstream target genes *LBDs* were obviously up-regulated at 28 d after IBA treatment (Figure 7, 10), auxin-induced protein *AUX22* was detected to up-regulated at 7d, 14d and 21d (Figure 8), auxin transporter-like protein *LAX3*, *LAX5* and *PIN*-like protein 6 (*PIL6a* - *PIL6c*) were also up-regulated in certain stage (Figure 8, 9), suggesting these genes might play important roles in auxin-induced AR formation. Though the functions of these genes have been identified in *Arabidopsis* or other plant species, however their roles and functions during blueberry AR formation need to be further verified.

LRP1, which was one of ten member of *SHI* gene family, is known to be expressed in root primordial of *Arabidopsis*, and transgenic expression of *LRP1* resulted in increased root elongation [57]. In *Arabidopsis*, *LRP1* was expressed at a very early stage of lateral root primordium formation and the expression decreased as the primordium grew older [30]. *LRP1*-like transcripts were at first detected in cotyledon, then rapidly became restricted to the upper zone where cell division and root formation took place [58]. In this work, four *LRP*-like genes were identified by transcriptome data and they were certainly up-regulated after IBA treatment during AR formation, especially at 21 d and 28d, during which the rp/rpi and AR begin

to be formatted (Figure 2, 6, 11), indicating the potential role of *LRP-like* genes in blueberry AR formation. Besides, some other genes such as root meristem growth factor 9 (*RGF9*), dormancy-associated protein homolog 3 (*DRMH3*) were identified in this present study (Figure 6, 12). *RGF*, an important peptide hormone, was suggested to regulate root meriste development through the *PLT1/2* stem cell transcription factor or by regulating of the stability of the receptor *RGFR1* [59, 60]. In present work, both transcriptome and qRT-PCR data indicated that *RGF9* was up-regulated at 7d, 14d and 28d after IBA treatment (Figure 12). *DRM*, one of auxin-repressed super-family genes, was suggested to be highly expressed in both dormant and non-growing tissues. *BrDRM1* was indicated to be negatively associated with root growth, which was reduced more than 50% in *BrDRM1*-overexpressing *Arabidopsis* plant and the reduction was correlated to an increase in *BrDRM1* expression levels [61]. However, in this study, *DRMH3* was detected to be highly regulated with AR formation, especially at 28 d after IBA treatment (Figure 12). This inconstant might result from the function of *DRM* varied with plant species or from the functional differences among each member of *DRM* families. Therefore, the molecular function of *DRMH3* in blueberry AR formation should be further analyzed.

Based on gene networks that were previously reported in root formation in *Arabidopsis* and other plant species, the regulative pathway that controls blueberry AR formation was derived (Figure 13). It was speculated that the exogenously-applied auxin would induced the expression of auxin responsive factors *ARF7/9* to percept auxin signaling, whereafter *ARF7/9* directly or indirectly affect on their downstream target *LBDs* genes families to establish AR founder cells with nuclei migration, and then auxin polar carriers including influx carriers *AUX22* or *LAX3/5* and efflux carriers *PIL6s* would be up-regulated to facilitate the establishment of auxin asymmetric distribution and thus include the AR primordium formation, finally AR primordium transform to AR apical meristem and outgrowth form the cuttings under the effect of *LRP1*, *RGF9*, *DRMH3* and other genes (Figure 13). In summary, AR formation is a complex process and is initiated by the auxin-dependent local activation of pericycle cells at the xylem poles. Delineating the molecular events that lead to adventitious root initiation is a prerequisite for a thorough understanding of how auxin modulates developmental functions. The RNA_seq data will help obtain better information about gene transcription and regulative networks during AR formation, but the functional characteristics and potential molecular mechanism of the key candidate genes still need to be elucidated.

Conclusions

Rooting ability, especially rooting rate, of blueberry green cuttings was significantly improved by IBA application, suggesting the AR formation of blueberry was an auxin-dependent progress. Higher IAA content and lower level of ABA or zeatin would facilitate blueberry AR formation. To our knowledge, this present work was the first report to provide comprehensive transcriptome profiling data for a dynamic view of transcript information during blueberry AR formation by using RNA_seq technology. About 14970 DEGs during blueberry AR formation were successfully identified, the expression profiles of 35 candidate genes or transcription factors involved in auxin-signaling and rooting-related pathway were further analyzed. Although further research for functional characteristics of these candidate genes is required,

our findings offered a new insight into the molecular mechanisms underlying blueberry AR formation and provide a relatively complete molecular platform for future studies on progression of blueberry AR formation.

Materials And Methods

Plant material

One southern highbush blueberry cultivar 'Biloxi' was used as material in this study. The materials of 'Biloxi' were obtained commercially from Hongyue Flower Co. LTD and were then planted in Zhuang-hang Comprehensive Experimental Station of Shanghai Academy of Agricultural Sciences, Shanghai, China. In spring, about 150 green cuttings with a length of 10-15 cm were randomly collected and most of the leaves were removed, retaining top 1-2 leaves. The cuttings were immersed in deionized water (control) and 1000 ppm indole-butyric acid (IBA, 1 g dissolved in 600 mL 75% alcohol and diluted with water to 1 L) for 1 min, respectively, and then inserted into growth medium that was consist of peatmoss : vermiculite : garden soil=3:1:1 (v/v). Water management was previously described detail in An et al. (2019) [11]. About 40 days after trail installation, rooting phenotypes including rooting percentage, average root number and average root length were surveyed. All experiments were performed with triplicates.

Analysis of plant hormones in blueberry cuttings

The stem from the base of cuttings was randomly sampled at a 7-day interval after the trial installation (i.e. stems were sampled at 7d, 14d, 21d, 28d, 35d). About 0.5 g of fresh tissues from each sampled time were grinded in liquid nitrogen, and were digested in 5mL ethyl acetate for 12h at 4 °C, then centrifuged at 10000 rpm at 4 °C for 10 min, the residue composition was further digested in 3mL ethyl acetate and centrifuged at 10000 rpm at 4 °C for 10 min. The supernatant was dried by nitrogen flow at 25 °C, then dissolved by 300 uL chromatographic methanol and then ultrasonic extracted for 10 min. Finally, the solution was filtered with 0.22 um membrane filters and 5 uL was injected for analysis. The level of endogenous plant hormone (including IAA, ABA, GA3 and zeatin) was analyzed by the methods of liquid chromatography-mass spectrometry (LC-MS) according to Niu et al. (2014) [62] with minor modification. The mobile phase was consisted of acetonitrile (solvent A) and 0.02% (v/v) glacial acetic acid in water (solvent B). Samples were purified by C-18 column of liquid chromatography system (ACQUITY I-CLASS, Waters), and finally determined with mass spectrometry (AB SCIEX analyst®QTRAP™ 5500). Standards of IAA (cas:87-51-4, Sigama), ABA (cas:21293-29-8, Sigama), GA3 (cas:77-06-5, Sigama) and zeatin (cas:13114-27-7, Sigama) were used to optimize the mass spectrometric parameters and fragment spectra. The calibration curves of IAA, ABA, GA3 and zeatin standards were obtained using six different concentrations (0, 2.5, 5.0 10, 12.5, 25, 50 and 100 ng/mL). The liner regression of calibration curves was detail in Supplementary Table 1.

Microstructure observation

After insertion, the base of cuttings (1-2 cm) was sampled at a 7-day interval to observe the formation of adventitious root primordium. The samples were fixed in FAA solution (formaldehyde:acetic acid:70% ethanol = 5:5:90). Before microstructure observation, the samples were softened about 10 days by 4% ethylenediamine and then dehydrated using graded ethanol, vitrification by dimethylbenzene and embedded with paraffin.

Total RNA extraction, sequencing and de novo transcriptome assembly

The stem of the base of cuttings (1-2 cm) was randomly sampled at a 7-day interval, the samples were immediately frozen in liquid nitrogen and stored in -80 °C before being analyzed. Total RNA was isolated using the Trizol Reagent (Invitrogen Life Technologies), after which the concentration, quality and integrity were determined using a NanoDrop spectrophotometer (Thermo Scientific). Three micrograms of RNA were used as input material for the RNA sample preparations. Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in an Illumina proprietary fragmentation buffer. First strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and the enzymes were removed. After adenylation of the 3' ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. To select cDNA fragments of the preferred 200 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 15 cycle PCR reaction. Products were purified (AMPure XP system) and quantified using the Agilent high sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The sequencing library was then sequenced on a HiSeq platform (Illumina) by Shanghai Personal Biotechnology Co. Ltd.

Sequence annotation and identification of significantly different expressed genes (DEGs)

To analyze the most descriptive annotation, unigenes were annotated by similarity searching based on five public databases, including NCBI non-redundant protein (NR) database, Gene ontology (GO) database, Kyoto Encyclopedia of Gene and Genome (KEGG) database, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) database and Swiss-Prot database using BLASTx algorithm with an *E*-value of less than 10^{-5} , and the best-aligning results were used to determine the sequence direction of the unigenes. Based on NR annotation, GO terms annotation of the unigenes was obtained using BLAST2GO, and KEGG pathway annotation was performed against KEGG Automatic Annotation Server (<http://www.genome.jp/tools/kaas/>) [63-66]. The significance of difference of gene expression (DEGs) were determined using DESeq software based on the threshold of $|\log_2\text{FoldChange}| > 1$ at *P*-value ≤ 0.05 .

Gene Expression Analysis by qRT-PCR

First-strand cDNA was synthesized from 1 ug of total RNA using the PrimerScript™ RT reagent Kit with gDNA Eraser (RR047, Takara, Japan), diluted 10 folds with ddH₂O and was then used as templates for qRT-PCR analysis. The mixture (10 ul total volume) contained 5.0 ul SYBR Primer ExTaq™ (RR820), 0.5 ul of each primer (10 uM), 2 ul of cDNA and 2ul of RNAase-free water. The reaction was performed on a LightCycler 480 Real-Time PCR System (Roche, Basal, Switzerland). qRT-PCR was initiated as follows: 95°C for 30s, then repeated 40 cycles at 95 °C for 5s and 60 °C for 20s [67]. Template-less controls for each primer pair were included in each run. *GAPDH* was used as the internal control to normalize the amount of mRNA. Each qRT-PCR reaction was performed with three biological replicates and each sample was analyzed in triplicate (technical replicates). The special primer sequences for qRT-PCR analysis were shown detail in Supplementary Table S2.

Statistical Analysis

Data analysis was performed with one-way analysis of variance (ANOVA) with SPSS 18.0 software (SPSS Inc, Chicago, USA). Graphics were created using GraphPad Prism 6.0 (GraphPad Software, Inc.).

Abbreviations

ABA: Absciscic acid; ANOVA: Analysis of variance; APT: Auxin polar transport; AR: Adventitious root; ARF: Auxin response factor; AUX/IAA: AUXIN/INDOLE-3-ACETIC ACIDS; c: callus; DAC: Days after cutting; DEGs: Differentially expressed genes; DRM3: Dormancy-associated protein homolog 3; eggNOG: evolutionary genealogy of genes: Non-supervised Orthologous Groups; FPKM: Fragments per kb per million fragments; GA3: Gibberellin 3; GO: Gene ontology; GSA: Genome Sequence Archive; IAA: Indole-3-acetic acid; IBA: Indole-butyric acid; KEGG: Kyoto Encyclopedia of Gene and Genome; LAX: AUX1-LIKE; LBD: LOB domain-containing protein; LC-MS: Liquid chromatography-mass spectrometry; LRP1: Lateral root primordial 1; NR: Non-redundant protein; PILs: PIN-LIKES; PIN: PIN-FORMED; RGF9: Root meristem growth factor 9; RNAi: RNA interference; rp: root primordium; rpi: root primordium initiation;

Declarations

Ethics approval and consent to participate

Not applicable

Consent for Publication

Not applicable

Availability of data and material

All of the raw data have been deposited in Genome Sequence Archive (GSA) in National Genomics Data Center under project number CRR078491 - CRR078500.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XY, HA organized the entire project, HA, JZ, XF, SJ performed the experiments and data analysis. XY and HA wrote and edited the manuscript. All authors read and approved the final manuscript.

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Table

Table 1 Summary of Illumina transcriptome sequencing for green cuttings of blueberry

Samples	Raw reads (bp)	Clean reads (bp)	Q20 (%)	Q30 (%)	N percentage
CK7	44459682	44246320	97.03	92.88	0.00%
CK14	42676938	42497314	97.16	93.15	0.00%
CK21	43466804	43256880	97.05	92.90	0.00%
CK28	41456090	41271800	97.09	93.01	0.00%
CK35	43865958	43657612	97.06	92.93	0.00%
T7	42921102	42763116	97.28	93.41	0.00%
T14	46198394	45959600	96.85	92.53	0.00%
T21	42930992	42735594	97.10	93.02	0.00%
T28	46227958	46005308	96.93	92.72	0.00%
T35	40733654	40602334	97.56	94.00	0.00%

Supplementary File Legends

Supplementary Table 1 Regression equation of calibration curves for IAA, ABA, GA3 and Zeatin

Supplementary Table 2 Primers for qRT-PCR analysis used in this study

Figures

Figure 1

Rooting phenotypes of stem of blueberry green cuttings of blueberry cv. 'Biloxi' treated with deionized water (control) and 1000 ppm IBA. Note: a-b, rooting phenotype of green cuttings of 'Biloxi' in control and IBA treatment, respectively; c, rooting percentage; d, average root number per cutting; e, average root length. All data were the mean value of three replications, the bar indicates standard error; ** indicates the significance at $P \leq 0.01$.

Figure 2

Microstructure observation of AR formation from stem of blueberry green cuttings Note: c, callus; rpi, root primordium initiation; rp, root primordium; r, root; the bar indicates the scale at 500 μ m

Figure 3

Dynamic of endogenous hormones in blueberry green cuttings during ARs formation. The bar indicates the standard error

Figure 4

Characteristics of the homology search of assembled blueberry unigenes against NR database. Note: a, E-value distribution of the top BLAST hits for each unique sequence with a cut-off E-value of 1.0 E-5; b, Similarity distribution of the top BLAST hits for each unigene; c, Species distribution of all homologous unigenes with an E-value of at least 1.0 E-5

Figure 5

Statistics of differently expressed genes (DEGs). Note: a, significantly up- or down-regulated genes using the threshold of $p\text{-value} \leq 0.001$ and $\log_2\text{Ratio} \geq 1$ in CK28 vs. T28; b, Graphical representation of overall differently expressed genes in IBA treatment; c, Number of unigenes in IBA treatment illustrated using a Venn diagram

Figure 6

Heatmap of the expression of unigenes related to auxin responsive and root primordium formation in stem of blueberry green cuttings.

Figure 7

The expression of auxin responsive factors ARF7 and ARF9 during the AR formation in stem of blueberry green cuttings. The bar indicates standard error.

Figure 8

The expression of auxin influx carriers *AUX22*, *LAX3* and *LAX5* during the AR formation in stem of blueberry green cuttings. The bar indicates standard error.

Figure 9

The expression of auxin efflux carriers PIN-like 6a, 6b, 6c during the AR formation in the stem of blueberry green cuttings. The bar indicates standard error.

Figure 10

The expressions of four LBDs during the ARs formation in blueberry green cutting. The bar indicates standard error.

Figure 11

The expression of four lateral root primordium protein-related genes LRPs during the AR formation in the stem of blueberry green cutting

Figure 12

The expression of RGP9 and DRMH3 during AR formation in stem of blueberry green cutting

Figure 13

The assumed gene networks that regulate AR formation in blueberry green cuttings Note: AR, adventitious root; ARF7/9, Auxin responsive factors 7/9; LBDs, Lateral organ boundaries domain; AUX22, Auxin induced protein 22; PIL6, PIN-LIKE 6; LRP1, Lateral root primordium 1; RGF9, root meristem growth factor 9; DRMH3, Dormancy-associated protein homolog 3

Supplementary Files

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