Plant Molecular Biology (2023) 113:205–217
https://doi.org/10.1007/s11103-023-01383-z

Transcriptome analysis reveals the effect of acidic environment on adventitious root differentiation in *Camellia sinensis*

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Received: 27 June 2023 / Accepted: 26 September 2023 / Published online: 16 November 2023

Abstract

The generation of adventitious roots (ARs) is the key to the success of cuttings. The appropriate environment for AR differentiation in tea plants is acidic. However, the mechanism is unclear. In this study, pH 4.5 was suitable condition for the differentiation of AR in tea plants. At the base of cuttings, the root primordia differentiated ARs more rapidly at pH 4.5 than pH 7.0, and nine AR differentiation-related genes were found to be differentially expressed in 30 days, the result was also validated by qRT-PCR. The promoter regions of these genes contained auxin and brassinosteroid response elements. The expression levels of several genes which were involved in auxin and brassinosteroid synthesis as well as signaling at pH 4.5 compared to pH 7.0 occurred differential expression. Brassinolide (BL) and indole-3-acetic acid (IAA) could affect the differentiation of ARs under pH 4.5 and pH 7.0. By qRT-PCR analysis of genes during ARs generation, BL and IAA inhibited and promoted the expression of *CsIAA14* gene, respectively, to regulate auxin signal transduction. Meanwhile, the expression levels of *CsKNAT4*, *CsNAC2*, *CsNAC100*, *CsWRKY30* and *CsLBD18* genes were up-regulated upon auxin treatment and were positively correlated with ARs differentiation.This study showed that pH 4.5 was the most suitable environment for the root primordia differentiation of AR in tea plant. Proper acidic pH conditions promoted auxin synthesis and signal transduction. The auxin initiated the expression of AR differentiation-related genes, and promoted its differentiated. BL was involved in ARs formation and elongation by regulating auxin signal transduction.

Key message

Acidic environments promote auxin synthesis and regulate root primordia differentiation to form ARs by initiating AR differentiation-related genes expression in *Camellia sinensis.*

Keywords Tea plants · Acid environment · Adventitious root differentiation · Phytohormone

Introduction

*Camellia sinensis (L).*O.Ktze is a plant belonging to the genus Camellia within the family Camelliaceae and is known as one the world's top three beverages, along with coffee and cocoa. The popularity of tea is not only due to its rich taste, but is also associated with many beneficial health effects, such as cancer prevention and weight loss (Katiyar and Mukhtar 1996; Wolfram et al. 2006). With the development of tea industry, the area planted with tea trees has been increasing. According to statistics, the area of tea plantations worldwide has now reached more than 70 million mu (one mu equals to 0.067 ha). Therefore, a large number of high-quality tea seedlings are needed for the construction of new tea plantations and the renovation of old tea plantations. Tea trees are mainly propagated by short-spike cuttings to

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maintain the excellent genetic characteristics of cultivars as well as to accelerate propagation and promotion (Duclercq et al. 2011; Sharma et al. 2018; Shang et al. 2019; Wang et al. 2016). Further, this method depends on the formation of adventitious roots (ARs) (Bellini et al. 2014; Luo et al. 2021).

The formation of cuttings AR reflected the plant response to the site of injury and was controlled by a complex regulation of various signaling pathways (Druege et al. 2016). The formation of plant AR was a complicated physiological, biochemical and morphogenetic process. It was influenced by various environmental and endogenous factors including pH, temperature, moisture, light, carbohydrate, injury and plant hormones (Mauriat et al. 2014; Ribeiro et al. 2016; Wang et al. 2019). Plant hormones played a crucial role in the generation of AR. Both endogenous and exogenous hormones could regulate the growth and development of AR (Cao et al. 2018; Han et al. 2014; Zhang et al. 2015; Jia et al. 2019). Brassinosteroids (BRs), as polyhydroxylated steroid hormones, were important plant root growth regulators. BR deficient or BR insensitive mutants exhibited a wide range of growth defects, including short buds and short roots (Close and Sasse 1998). Auxin (IAA) is another key hormone in plants, which has been shown to rapidly promote AR formation in the 1930s (Cooper. 1936). In addition, exogenous application of IAA could also promote AR formation during the cutting propagation in many plants (Damiano et al. 2008; Ragonezi et al. 2010; Wu 2021). Some research indicated that IAA and BR had synergistic or antagonistic effects during root development. High levels of BR promoted cell elongation in the elongation zone of the root. At the same time, low levels of BR and high levels of IAA could maintain the size of stem cells, meristem in the resting central cell and root apical meristem. The above were essential to maintain root development (Mouchel et al. 2006; Maharjan et al. 2011; Nakamura et al. 2006; Yu et al. 2023; Pacurar et al. 2014; Kim et al. 2006).

The "acid growth theory" has long held that rising concentrations of IAA could trigger extracellular acidification, which activates cell wall–loosening enzyme to degrade the cell wall and allow smooth cell proliferation (Du et al. 2020; Elke et al. 2017; Lin et al. 2021; Matgorzata and Waldemar. 2021; Liu et al. 2022). Plant cells are embedded in the cell wall, providing structural integrity, but also limiting the space available to the cell. Therefore, they must be modified for allowing cell expansion. Previous studies showed that changes in external pH will significantly affect the auxin content in plants (Ida et al. 2010; Ales et al. 2015; Radic et al. 2016). The suitable environment for AR in tea plants was acidic, with pH4.5–6.5 being the most favorable for tea plants rooting (Niu et al. 2013; Tang et al. 2019). In acidic or alkaline environments, the differentiation of AR was inhibited to varying degrees, mainly in terms of its slow or non-differentiation, which in turn led to plant death. However, its molecular mechanism has not been fully elucidated. In this study, the widely planted tea cultivar "Fuding Dabai" was used as the research object. The optimal pH conditions for AR differentiation were explored, transcriptome under appropriate pH conditions were analyzed, and the effect of external pH environment on gene expression at the base of tea cuttings was investigated. The synthesis and signal transduction of BR and IAA under suitable pH conditions as well as the molecular mechanism that promote the differentiation of AR of tea plants were also analyzed.

Materials and methods

Plant materials

Semi-woody branches of the current year from "Fuding Dabai" with a general uniform growth trend were selected and cut into short 2–3 cm spikes of one bud and one leaf. One-third of the mature leaves were cut to reduce water evaporation and then washed five times with distilled water (Wei et al. 2014). All branches were collected from the Meitan Tea Germplasm Resource Nursery of Tea Institute of Guizhou Academy of Agricultural Sciences.

pH treatment conditions

Modified Hoagland nutrient solution was used for hydroponics (Kaiser 2005), that is, KNO_3 6.0×10^{-3}, NH_4NO_3 0.5×10^{-3}, $MgSO_4$ 2.0×10^{-3}, $C_{10}H_{12}N_2NaFe$ 0.153×10^{-3}, KI 0.5×10^{-5}, H_3BO_3 0.1×10^{-3}, $MnSO_4$ 0.148×10^{-3}, $ZnSO_4$ 0.534×10^{-4}, $NaMoO_4$ 0.103×10^{-5}, $CuSO_4$ 0.157×10^{-6}, $COCl_2$ 0.253×10^{-6} and $Ca(NO_3)2$ 5.759×10^{-3} (w/v). The following nine pH treatment groups were set as pH 3.5, pH 4.0, pH 4.5, pH 5.0, pH 5.5, pH 6.0, pH 6.5, pH 7.0 and pH 7.5. The nutrient solution was adjusted to the appropriate pH with 0.1 M NaOH or 0.1 M HCl. They were then added to black hydroponic pots, each containing 3 L of nutrient solution. Tea cuttings were sequentially inserted into 0.5 cm thick black foam floats plate and then incubated in pots with 60 plants per pot, and three replications. Oxygen was applied daily in the morning/evening with an oxygen pump, every 12 h. The pH was calibrated once a day, while the nutrient solution was replaced once a week. On day 45 of culture, the rooting rate, mortality, number of new roots and root length were counted for each treatment (An et al. 2020).

Morphological observation of the tissue at the base of cuttings

The samples were fixed in FAA for 3 days, dehydrated in a graded ethyl alcohol series for 60 min each (10, 30, 50, 70, 85, and 95%) and then left in 100% ethyl alcohol overnight.

The samples were embedded in paraffin and then cut into thin sections of 7 μm by rotary microtome. The thin sections were double-stained with safranin and fast green. Observations were made with an optical microscope (Koufan et al. 2020).

Transcriptome sequencing

The ARs freshly protruding from the root primordia under the pH 4.5 treatment and the root primordia only under the pH 7.0 treatment were collected in a mixture of three biological replicates (30 d) for every five plants. Sampling locations are shown in Fig. S1 A. Samples were immediately stored in liquid nitrogen until transcriptome sequencing. At the same time, sufficient samples were taken from each treatment and stored in the – 80 °C refrigerator after quick freezing in liquid nitrogen for hormone content determination. RNA extraction and transcriptome sequencing of samples were completed by Shanghai Majorbio Co. Ltd.

RNA data processing and differential gene screening

The whole genome of "Shuchazao" cultivar registered on NCBI by Anhui Agricultural University was used as reference genome (version No.:GCF_004153795.1), Sequencing data were filtered using SOAPnuke (v1.5.2) (Li et al. 2008) and clear reads were mapped to the reference genome using HISAT2 (v2.0.4) (Kim et al. 2015). Bowtie2 (v2.2.5) (Langmead et al. 2012) was then used to compare clear reads to the internal coding gene set and RSEM (v1.2.12) (Li and Dewey. 2011) was used to determine the expression level of each gene. DES Eq. 2 (v1.4.5) (Love et al. 2014) was used to analyze differentially expressed genes. The screening threshold was: $|\log2FC| \geq 1$ and adjusted p-value < 0.05. GO enrichment analysis of genes/transcripts in the gene set was performed using the software Goatools (https://github.com/tanghaibao/GOatools) Generally, p-values were corrected by default. The GO function was considered significantly enriched when the corrected p-value (FDR) was less than 0.05 (Tang et al. 2015). KEGG pathway enrichment analysis was conducted on genes/transcripts in the gene set using R software. The calculation principle was the same as for GO function enrichment analysis. By default, the KEGG pathway was considered significantly enriched when the p-value (uncorrected) was less than 0.05.

qRT-PCR analysis

The accuracy of the RNA-seq results was verified by a qRT-PCR system with 2 × RealStar Fast SYBR qPCR Mix (Genestar, Beijing, China). Primers were designed across introns using Primer Premier 6.0 (Premier Biosof International, Quebec, Canada) and then tested for primer specificity at NCBI. The RNA returned by the company after transcriptome sequencing was reversed to cDNA (TAKARA, Dalian, China),diluted and used. The glyceraldehyde-3-phosphate dehydrogenase (*CsGAPDH*) gene of tea plant was used as an internal reference gene. The qRT-PCR was amplified as follows: 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Amplifications were run on an qTOWER3G fluorescence quantitative PCR instrument (Analytikjena, Germany) of Company. All verifications were performed in three biological replicates and three technical replicates. Standard deviations (SD) of the means were calculated using standard statistical methods. Gene expression was analyzed using the ΔΔCt data analysis method and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak. 2008).

Determination of auxin content in tea plant base

Standard curve construction

The auxin IAA standard was purchased from TMstandard Company, and contained 98.9% auxin. Accurately weighed 1.81 mg auxin standard and added to a constant volume of 25 ml of methanol, the IAA content in the mother liquor was 0.0724 mg/mL. The mother liquor was diluted to the following concentrations, 0.96, 1.92, 2.89, 5.79 and 14.48 μg/mL. Detection was performed on a Shimadzu LC-2040C ultra-high performance liquid chromatograph The method was as follows, C18 column (100 mm × 4.6 mm, 5 μm), PDA diode array detector, detection wavelength of 260 nm, flow rate at 0.5 mL/min, column temperature at 35 °C with automatic injection for injection volume of 10 μL. Mobile phase, methanol (A) and 0.1% acetic acid (B). Gradient elution of phase B was as follows, 75% 0 min, 75% 4 min, 70% 4.1 min, 70% 12 min, 60% 12.1 min, 60% 30 min, 30% 30.1 min, 30% 40 min, 60% 40.1 min, 60% 45 min, 75% 45.1 min and 75% 60 min (Fu et al. 2009). The standard curve was as follows, y = 37,642x—2208.9, $R^2 = 0.9999$, where y represents the peak area and x represents the concentration (Fig. S1B).

Determination of auxin content

A frozen sample was taken and placed in a mortar and pestle for rapid grinding. Quickly and accurately weighed 0.2000 g, placed in a test tube, added 3 ml of methanol and sealed. The sample was extracted by ultrasound at 15 °C for 100 min in a constant temperature water bath and filtered through a 0.45 μM filter membrane to obtain the sample to be tested. The operation was the same as in **1.7.1**. The resulting peak areas were taken into the standard curve to obtain the auxin concentrations.

Analysis of homeopathic elements in promoter region

The 2500 bp sequence upstream of the ATG of the AR development-related genes in the tea plant genome was intercepted(http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). After analyzing the homeopathic elements,it was visualised and mapped using TBtools software.

Statistical analysis

Parametric analyses were performed using ANOVA and means were compared using Tukey's test($p < 0.05$), which was conducted using SPSS 21.0 software. All data were expressed as the mean of at least three replicates and one SD. All figures were plotted in the GraphPad Prim 9.0.

Result analysis

Effects of environmental pH on the differentiation of AR in tea cuttings

The number and length of ARs at the base of tea plants cuttings showed a trend of increasing and then decreasing with rising pH. The highest number of ARs was found at pH 4.5 and pH 5.0 treatments. In particular, the longest average root length, the highest rooting rate, the highest number of new roots and the lowest mortality rate were observed at pH 4.5, with significant difference compared to other treatment groups (Fig. 1A, B). These indicators were second at pH 5.0 only to pH 4.5. The results showed that an environment of pH 4.5–5.0 was favorable to the differentiation and development of tea plants roots. Further, pH 4.5 was the most suitable acidic environment for the differentiation of tea plants roots under the experimental conditions.

By comparing the morphological changes of cells at the base of the cuttings during AR generation and development under pH 4.5 and pH 7.0, it was found that root primordia was generated at day 22 in both pH conditions. Furthermore, the root primordia were formed by the proliferation and differentiation of parenchyma cells from the central sheath to the phloem. However, root primordia began to form root tips and break through root primordium epidermis at day 30 under pH 4.5, compared to 38 d in pH 7.0. This suggested that pH 4.5 environment promoted the differentiation of root primordia to form AR (Fig. 2).

Analysis of gene expression levels in AR differentiation

Differences in gene expression between pH 4.5 and pH 7.0 at the base of the cuttings were compared by transcriptome sequencing. At this time, the root primordia were just protruding from the AR under the pH 4.5 environment, while at the root primordia stage under pH 7.0. The results showed that there were 5741 DEGs, including 1844 up-regulation genes and 3897 down-regulation genes (Fig. S2). The results of GO analysis and KEGG analysis of DEGs are shown in Fig. S3 and Fig. S4, respectively.

After reviewing the reported AR differentiation-related genes from other plants, the genes with the highest similarity and functional domains in the genomes of tea plants were also compared and considered as homologous sequences of tea plants. In the transcriptome data, the following nine genes were significantly differentially expressed, with six positively [*CsKNAT4* (LOC114273157), *CsLBD18* (LOC114307927), *CsLBD33* (LOC114275146), *CsNAC2* (LOC114287191), *CsNAC100* (LOC114260242) and *CsWRKY30* (LOC114314481)] and one negatively regulated gene [*CsWRKY75* (LOC114293712)] for AR differentiation being up-regulated. The expression of a negatively regulated gene [*CsIAA14* (LOC114321959)] and an auxin transport vector [*CsAUX/LAX3* (LOC114282558)] were down-regulated. In order to clarify the expression levels of these genes, validation was performed by qRT-PCR and the results were consistent with the transcriptomic data (Fig. 3). Gene names and primer sequences are shown in Table. S1.

Analysis of homeopathic elements in the promoter region

The homeopathic elements in the 2500 bp sequence upstream of the start codon of the above nine genes were analyzed. The results showed that all genes contained brassinosteroid response elements. *CsKNAT4, CsLBD18, CsLBD33, CsNAC2, CsWRKY30* and *CsIAA14* contained auxin response elements (Fig. 4). It suggested that the differential expression of the above genes might be related to the levels of IAA, BR as well as signal transduction.

The pH significantly affects the synthesis and signal transduction related genes expression of BR and IAA

The interaction between phytohormones BR and IAA regulated the generation and development of AR during root differentiation. Analysis of the KEGG pathway showed significant differences in the expression levels of several genes involved in BR and IAA synthesis as well as signaling at pH 4.5 compared to pH 7.0. In particular, the expression levels of key negatively regulated gene (*CsIAA14*) in the IAA signaling pathway and key positively regulated gene (*CsBZR1*) in the BR signaling pathway were significantly down-regulated. It demonstrated that the signaling pathways of IAA and BR were promoted and inhibited at pH 4.5, respectively, which was consistent with previous report of antagonistic regulation

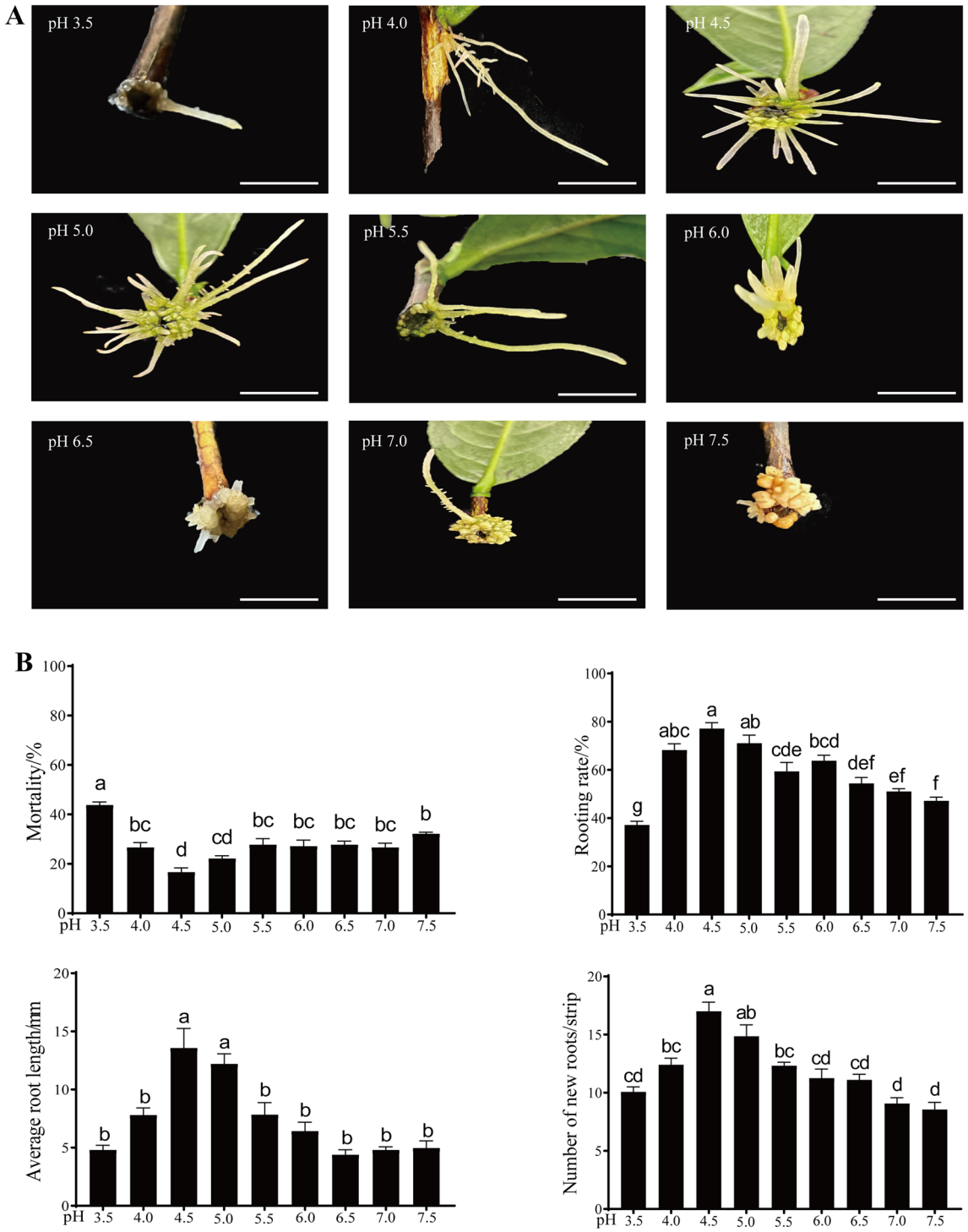

Fig. 1 Effects of different pH treatments for 45 d on the AR of cuttings. **A** The base of the cuttings for 45 d. Bar = 5 mm. **B** Effects of different pH treatments on the phenotypic indicators of AR in tea cuttings. The x-axis represents the pH value of the treatment and the y-axis represents the value of the indicator. The error bar represents ± SEs, n ≥ 3. Different letters showed significant differences ($P < 0.05$), while the same letters showed no significant differences ($P > 0.05$)

of root differentiation by IAA and BR (Fig. S5).The high performance liquid chromatography (HPLC) was used to determine the auxin content at the base of the cuttings under both treatments, showing that the auxin content was 65.4% higher at pH 4.5 than at pH 7.0 (Fig. S6). 11 DEGs in the IAA, BR synthesis and signal transduction

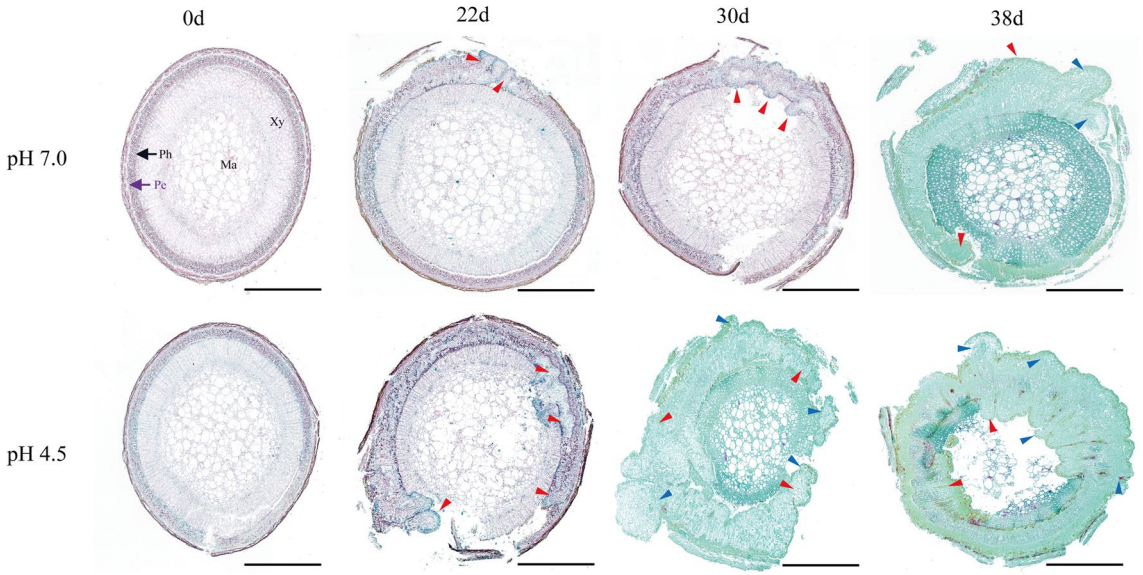

Fig. 2 Histological observation of ARs formation process of tea cuttings at pH 4.5 and pH 7.0. Paraffin section observation of founder cells at 0 d, 22 d, 30 d and 38 d during AR development. ARs appeared at pH 4.5 for 30 days, and pH 7.0 for 38 days. Bar = 1 mm. Arrows: purple, pericycle (Pe); dark,phloem (Ph); red, root primordia; blue, ARs formed by differentiation from root primordia. Xylem (Xy); marrow (Ma)

pathways were randomly selected for qRT-PCR. The gene name and primer sequence were shown in Table. S1.The results showed that the expression levels of the 10 genes were consistent with the transcriptome, except for gene LOC114300015 (*CsYUCCA6-like*) (Fig. S7). This result also indicated that pH 4.5 could promote the synthesis of IAA. The above results suggested that the increase in auxin content and signal transduction at pH 4.5 might be responsible for promoting AR differentiation. In contrast, BR played an opposite role to IAA in regulating AR differentiation.

BR and IAA affect gene expression and regulate AR

Tea plant cuttings were treated with 20 nM BL and 10 μM IAA at pH 4.5 and pH 7.0, respectively. After 45 d, the rooting rate, mortality rate, number of new roots and average root length were calculated. The results showed that the IAA treatment significantly reduced the mortality rate and increased the rooting rate of cuttings under both pH conditions. The BL treatment, on the other hand, had no significant effect, indicating that the differences in mortality rate and rooting rate between pH 4.5 and pH 7.0 were caused by IAA. Number and length are important indicators to evaluate the condition of the root system. In this study, the IAA treatment significantly increased the number and length of roots at pH 7.0 and also slightly increased the two indicators at pH 4.5. In contrast, BL treatment could inhibit the number of roots at pH 4.5 to the level at pH 7.0, but promoted root length at pH 7.0, with less effect than IAA. In conclusion, IAA and BR acted antagonistically in regulating AR production in tea plants. During the AR elongation stage, both BR and IAA served to promote root growth (Fig. 5).

In order to investigate whether IAA and BR regulate root primordia differentiation by inducing the expression of AR differentiation-related genes, the expression levels of these genes(Sect. "Analysis of gene expression levels in AR differentiation") at AR production stage were examined by qRT-PCR (Fig. 6). The results showed that there were significant differences in gene expression levels as influenced by BL and IAA. *CsKNAT4, CsNAC2, CsNAC100, CsWRKY30* and *CsLBD18* genes were significantly up-regulated by IAA induction at pH 7.0 but were not affected by BL. The expression of these genes was significantly promoted by IAA at pH 4.5, but was down-regulated to the expression level at pH 7.0 under BL treatment. This indicated that they were mainly involved in regulating root mass generation at pH 4.5 and were induced by IAA, and BL could inhibit this process. There was no significant difference in the expression level of *CsLBD33* gene between BL and IAA treatments. The expression level of *CsWRKY75* gene was down-regulated by IAA, which might be related to its negative regulation

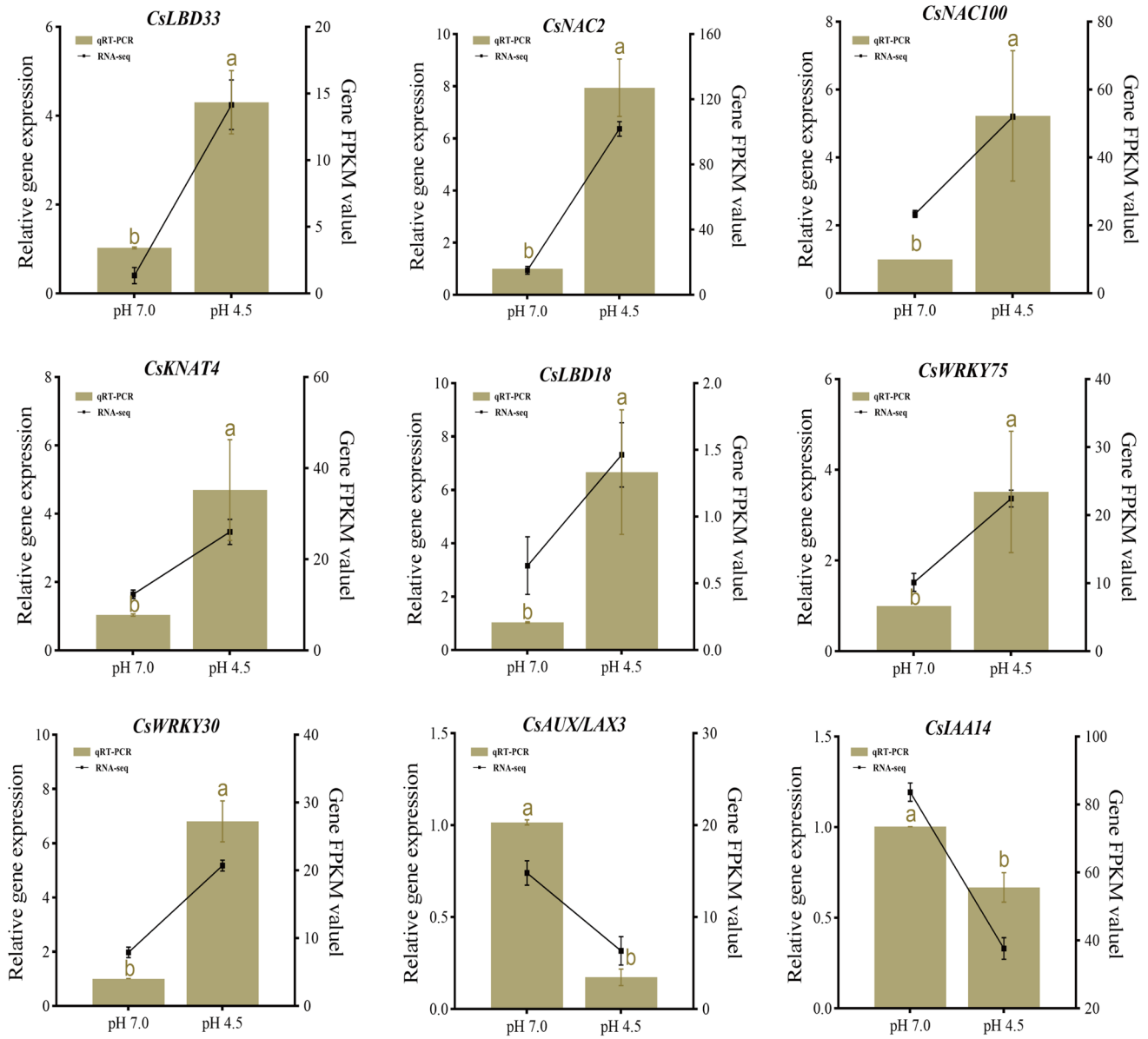

Fig. 3 qRT-PCR was used to validate differentially expressed genes in the transcriptome of AR differentiation-related. The relative expression was calculated as $2.^{-\Delta\Delta Ct}$. The left y-axis is the result of qRT-PCR and the right y-axis is the result of RNA-seq. The error bar represents ± SEs, n ≥ 3.Different letters showed significant differences ($P < 0.05$), while the same letters showed no significant differences ($P > 0.05$)

of root hair generation. The expression of *CsAUX/LAX3* was up-regulated under BL and IAA treatment but down-regulated under pH 4.5, which was associated with the ability of exogenous IAA and BL to promote the aggregation of IAA in plants. The expression of *CsIAA14* gene was significantly up-regulated by BL and down-regulated by IAA, showing opposite effects of inhibiting or promoting auxin signal transduction. In conclusion, up-regulation of auxin content and signal transduction induced expression of *CsKNAT4, CsNAC2, CsNAC100, CsWRKY30* and *CsLBD18* genes might be responsible for promoting AR production. BR might inhibit auxin signaling by promoting the expression of *CsIAA14* gene, thereby reducing AR.

Discussion

Rooting rate, length and the number of AR were the key indicators for evaluating the maturity of cutting propagation (An et al. 2020). These three indicators under pH 4.5 were

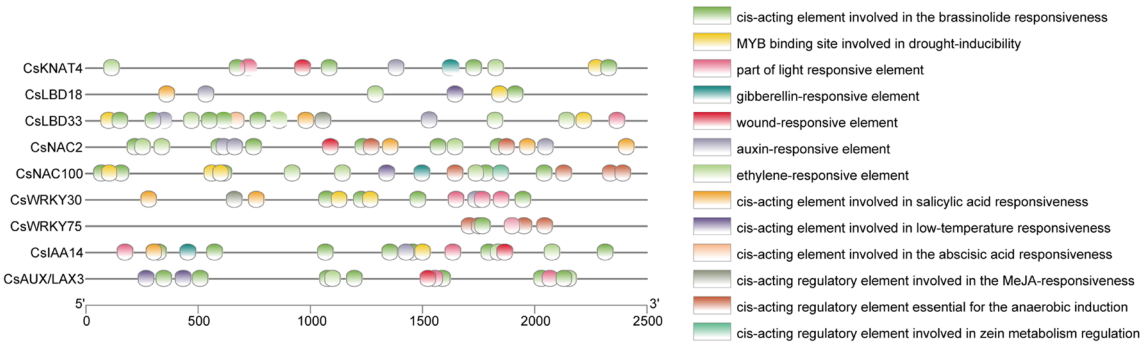

Fig. 4 Analysis of homeopathic elements in the promoter regions of differentially expressed genes related to AR differentiation

much higher than other treatment groups, and the mortality rate was also the lowest. The cell morphology at the base of cuttings differed between the stages of root primordium differentiation to form AR at pH 4.5 and pH 7.0, similar to the results of exogenous auxin treatment to promote AR in plants. (Quan et al. 2017). Therefore, it could be hypothesized that in the early stages, IAA was transported from the upper end to the lower end of the cutting morphology, promoting the proliferation and differentiation of the thin-walled cells from the central sheath to the phloem to form the root primordia. However, the synthesis of IAA or signal transduction was affected at the cutting base due to pH, further leading to differences in AR production in the root primordia. At pH 4.5, the content of auxin increased, inducing loosening and degradation of the cell wall at the base of the cuttings. At the same time, cell proliferation and differentiation favored the production of AR. After GO enrichment analysis of DEGs screened for transcriptome data, the highest enrichment was found for the plant secondary cell wall generation, plant cell wall generation, xylan biosynthesis and xylan metabolic process (Fig. S4 A, B). The results indicated that the cell wall was extensively disrupted and stretched, creating the conditions for AR to grow from the root primordia, which was consistent with the results of the histological sections.

The differentiation of plant roots were largely hormonally mediated (Zhang et al. 2018; Zhao et al. 2009). A growing number of studies showed that the regulation of endogenous hormone signaling was closely linked to the formation of AR. The balance of high levels of IAA and low levels of BR was crucial for the maintenance of apical resting center cells and meristem. IAA could increases the expression levels of several genes encoding BR catabolic enzymes, including *BAS1, SOB7, BAT1* and *BEN1*. Conversely, BR could inhibit its signal transduction by regulating the expression of the *IAA*, an inhibitor of the IAA signaling pathway. This might form a feedback loop that helped maintain the balance between the two hormone (Bao et al. 2004; Juthamas and Wang. 2015). In the transcriptome, BR synthesis and signal transduction were inhibited and IAA synthesis and signal transduction were promoted. This balance of high levels of IAA and low levels of BR promoted the formation and development of AR. Studies of AR formation in purple grass, sugarcane and mulberry cuttings showed significant differences in the expression of several genes involved in BR and IAA signaling pathways, similar to the results of the present study (Devi et al. 2021; Li et al. 2020; Cao et al. 2018).

CsKNAT4 gene could regulate the root angulation and was expressed during the formation of root apical meristem as well as in the associated regions of cambium. The homologous gene *AtKNAT6* in *Arabidopsis* was highly expressed when the root primordia were differentiated into AR (Qi and Zheng. 2013). The transcription factors *CsLBD18* and *CsLBD33* were related to callus formation and played roles in the initiation and boundary establishment of plant lateral organs. Their up-regulated expression activated cell wall loosening factors that degraded cell wall and promoted root differentiation (Lee et al. 2017). Homologous of *CsNAC2* and *CsNAC100* genes were demonstrated to have positive regulatory effects on AR formation in soybean and rice, respectively. Their up-regulated expression could increase rooting number and shorten rooting time (Redillas et al. 2012a, b). *CsWRKY30* gene was involved in wound repair and stress response in cuttings, and its up-regulation in expression promoted plant root regeneration (Ding et al. 2015). Up-regulation of *CsWRKY75* gene expression inhibited the generation of root hairs (Rishmawi et al. 2014). Down-regulation of *CsIAA14* gene enhanced IAA signal transduction (Fukaki et al. 2002). Down-regulation of *CsAUX/LAX3* gene, the gene encoding the IAA input vector, indicated that that IAA transport was diminished. It was hypothesized that IAA may not be synthesized and transported to the base of the cuttings, but synthesized and accumulated in the cells at the base of the cuttings. Exogenous BL and IAA could significantly affect root differentiation at pH 4.5 and pH 7.0. The expression levels of

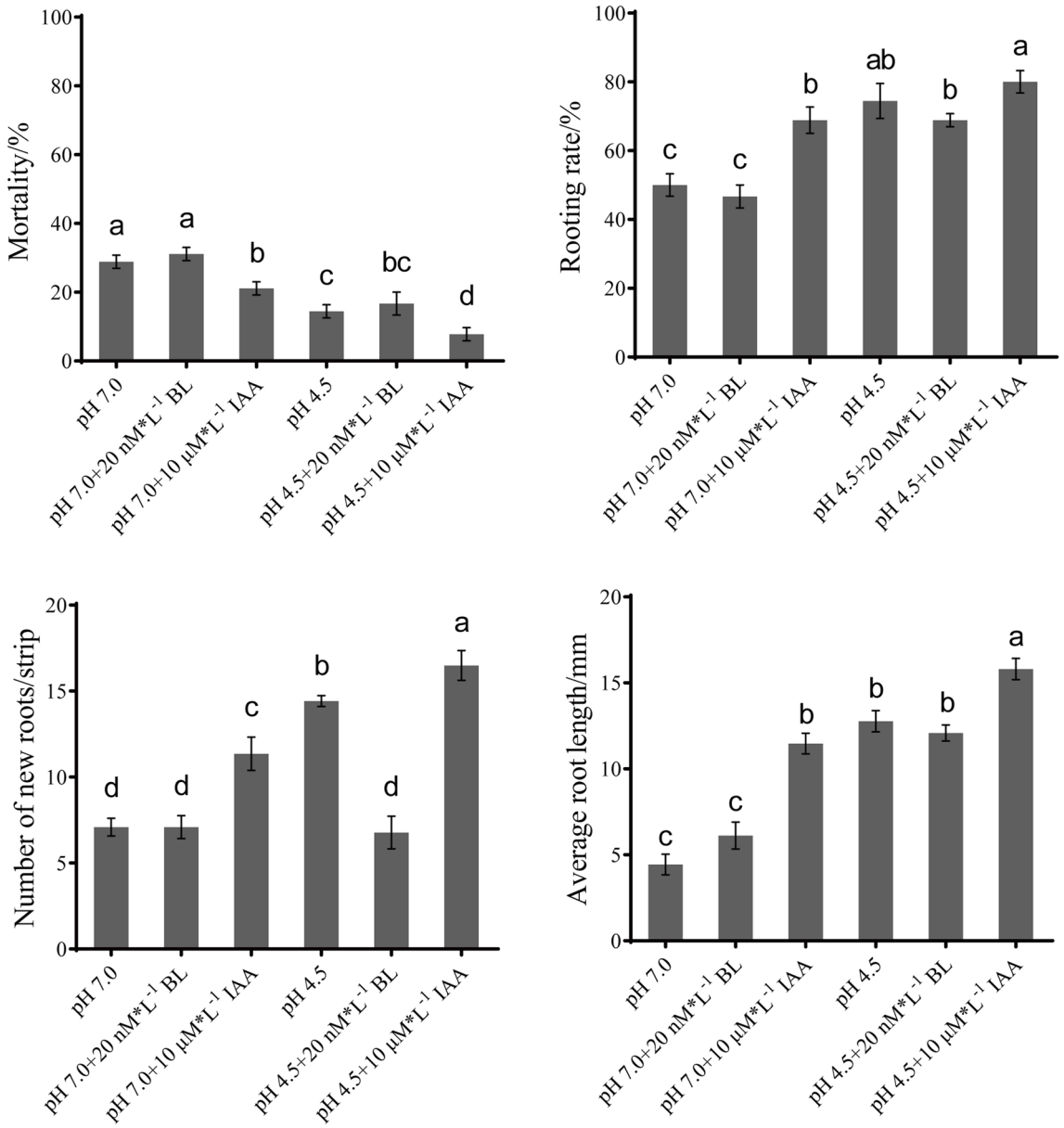

Fig. 5 Phenotypic indicators of tea plant cuttings after 45 d. The x-axis represents the pH value and hormone treatment and the y-axis represents the value of the indicator. The error bar represents ± SEs, n ≥ 3. Different letters showed significant differences ($P < 0.05$), while the same letters showed no significant differences ($P > 0.05$)

CsKNAT4, CsNAC2, CsNAC100, CsWRKY30 and *CsLBD18* genes were always up-regulated when AR was promoted and down-regulated when AR was inhibited, positively correlating with AR production. Notably, the expression of *CsIAA14* gene was always significantly up-regulated when BL was induced, which hindered auxin signaling. In contrast, IAA could alleviate this inhibition to a certain extent, which in turn promoted AR differentiation. This might be because IAA promoted the expression level of BR catabolism-related enzymes coding gene, reducing BR content and mitigating the induction of *CsIAA14* gene. The significant increase in IAA content at the base of the cuttings at pH 4.5 compared to pH 7.0 also indicated a significant down-regulation of *CsIAA14* gene expression by IAA content. This study provided valuable insights into the response of AR differentiation-related genes to phytohormones.

This study showed that pH 4.5 had the most significant promoting effect on the differentiation of tea plant roots and was the most suitable acidic environment for the differentiation of tea plant roots. The suitable acidic pH conditions promoted auxin synthesis and signal transduction. Auxin initiated the expression of genes related to AR differentiation

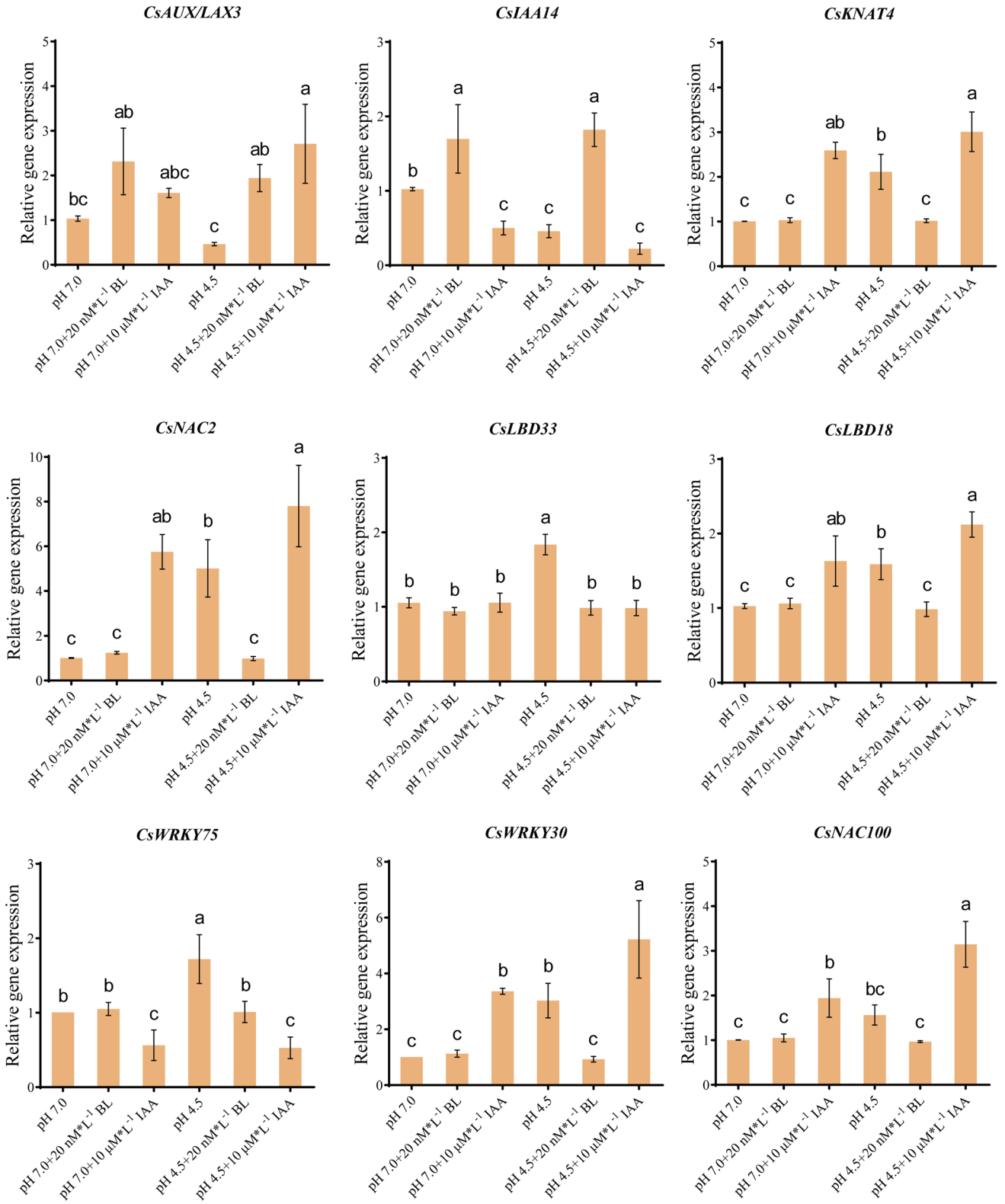

Fig. 6 Gene expression at the stage of AR differentiated in the base of tea cuttings. The relative expression was calculated as $2.^{-\Delta\Delta Ct}$. The left y-axis is the result of qRT-PCR. The error bar represents ± SEs, n ≥ 3. Different letters showed significant differences ($P < 0.05$), while the same letters showed no significant differences ($P > 0.05$)

and promoted it. BR was involved in the regulation of AR formation and elongation through the regulation of auxin signaling. In addition, the DEGs identified in this study were potential candidates for future functional analysis. It could provide a theoretical basis for further revealing the molecular mechanism that tea plants can only differentiation normally in acid environment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11103-023-01383-z.

Acknowledgements We thank the equipment support provided by Institute of Agro-bioengineering and the College of Life Sciences of Guizhou University.

Authors' contributions The corresponding authors DGZ and YZ provided design ideas and experimental guidance for the paper, and revised the paper. KL, the author of this article, designed and operated the experiment according to the ideas of the corresponding author, and wrote the manuscript. All authors read and approved the manuscript.

Funding This work was supported by National Natural Science Foundation of China, "Functional analysis of Brassinosteroid transcription factor CsBZRs in Camellia sinensis under cold stress" (Grant numbers: 32160077). Talent program of the Guizhou Academy of Agricultural Sciences, "Research on functional genomics and high-value utilization technology of important economic plants such as Eucommia ulmoides"[(2022) No.02]. Guizhou Provincial Talent Office "Guizhou Provincial Characteristic Plant Germplasm Resources Utilization and Innovation Talent Base" (RCJD2018-14). Guizhou Provincial High-level Innovative Talent Training Project [QKH Talent (2016) No. 4003].

Data Availability The dataset used and/or analyzed in this study can be obtained from the author according to reasonable requirements.

Declarations

Competing interests The authors declare that they have no competing interests. The authors have no relevant fnancial or non-fnancial interests to disclose.

Ethical approval and consent to participate We does not contain any studies with human or animal subjects.

Consent for publication My manuscript doesn't contain any individual person's data in any form (including any individual details, images or videos).

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