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ORIGINAL ARTICLE

Hypoxia enhances lignification and affects the anatomical structure in hydroponic cultivation of carrot taproot

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Abstract

Key message **Hypoxia enhances lignification of carrot root.**

Abstract Hypoxia stress was thought to be one of the major abiotic stresses that inhibiting the growth and development of higher plants. The genes encoding the plant alcohol dehydrogenase (ADH-P) were induced when suffering hypoxia. To investigate the impact of hypoxia on the carrot root growth, carrot plants were cultivated in the hydroponics with or without aeration. Morphological characteristics, anatomical structure, lignin content, and the expression profiles of *DcADH-P* genes and lignin biosynthesis-related genes were measured. Six *DcADH-P* genes were identified from the carrot genome. The expression profiles of only three (*DcADH-P1, DcADH-P2*, and *DcADH-P3*) genes could be detected and the other three (*DcADH-P4, DcADH-P5*, and *DcADH-P6*) could not be detected when carrot cultivated in the solution without aeration. In addition, carrot roots had more lignin content, aerenchyma and less fresh weight when cultivated in the solution without aeration. These results suggested that hypoxia could enhance the lignification and affect anatomical structure of the carrot root. However, the expression levels of the genes related to lignin biosynthesis were down-regulated under the hypoxia. The enhancement of lignification may be the consequence of the structure changes in the carrot root. Our work was potentially helpful for studying the effect of hypoxia on carrot growth and may provide useful information for carrot hydroponics.

Keywords *ADH-P* gene · Anatomical structure · Carrot · Gene expression · Hypoxia · Lignin

Introduction

Hydroponics is a highly efficient way to cultivate vegetables without the use of soil (Kimura and Rodriguez-Amaya 2003; Yu and Matsui 1993). The production of hydroponic vegetables was about 35,000 ha in 2011 (Hickman 2011). Moreover, hydroponics was often employed in the research of plant science, especially in the research of plant root growth and development (Hickman 2011; Kimura and Rodriguez-Amaya 2003). In the environmental toxicant biomonitoring, the root elongation of higher plants has been the most commonly used index. Root elongation was also an important endpoint of plant development (Park et al. 2016; Ratsch 1983). However, roots are easily destroyed during the process of experiment when cultivated in the soils. In hydroponics, this problem will be relieved. Hydroponics is a rapid and low-cost screening tool for root characteristics at the early growth stages of many plants. One obvious advantage of hydroponics is that the subsequent and non-destructive investigation of plant root growth can be carried out (Tuberosa et al. 2002).

Hypoxia is a serious impedimental factor in plant growth and results in yield losses (Bai et al. 2011; Christianson et al. 2010; Geigenberger 2003). Deficiency of oxygen can dramatically affect the efficiency of cellular adenosine triphosphate (ATP) production. In *Arabidopsis*, the expression of the gene encoding alcohol dehydrogenase (ADH) was induced when suffering from hypoxia. The loss-of-function mutants of *ADH* genes in rice, maize, and *Arabidopsis* showed poor tolerance when suffering from hypoxia. *ADH* genes are necessary for plants to survival under hypoxia

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(Christianson et al. 2010; Peng et al. 2001). When plants are grown under anoxic conditions, anaerobic fermentation will increase transiently and then carbohydrate catabolism will be restricted. Cellular metabolism and plant development processes would be affected seriously, too (Fukao and Bailey-Serres 2004). To survive, plants have to make some alternation to adapt to the oxygen deficiency. The appearances of aerenchyma and adventitious roots were important alterations, which allow submerged tissues to capture oxygen (Drew et al. 2000; Geigenberger 2003; Suralta and Yamauchi 2008). Space formation within aerenchymatous organs was caused by middle lamella cell separation or cell death (Schussler and Longstreth 1996). In addition, the cell wall of wheat roots was found to thicken under hypoxia in the previous study (Albrecht and Mustroph 2003).

As the second most abundant organic component, lignin plays important roles in response to abiotic stress in plants (Boerjan et al. 2003; Moura et al. 2010). The degree of lignification, development of transfusion tissues, and epidermal accessory structure of roots are involved in the stress tolerance of plants (Cervilla et al. 2009; Nobel 2005). Lignin is the main component of plant secondary cell wall that imparts rigidity and hydrophobicity of the secondary cell wall (Boerjan et al. 2003; Bonawitz and Chapple 2010; Zhao and Dixon 2011). Previous studies demonstrated that the lignification of plants would be triggered by abiotic stresses, such as drought, low temperatures, mineral deficiency and ultraviolet-B (UV-B) (Alvarez et al. 2008; Moura et al. 2010; Rozema et al. 1997; Thomashow 1999). In the lignin biosynthesis pathway, phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD), hydroxycinnamoyltransferase (HCT), *p*-coumaroylshikimate/quinate 3′-hydroxylase (C3′H), caffeoyl CoA *O*-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), caffeic acid *O*-methyltransferase (COMT), peroxidase (PER) and laccase (LAC) are important participants (Vanholme et al. 2010; Weng and Chapple 2010). In addition, DcPAL, DcCCoAOMT, DcCAD, and DcPER1 were reported to play positive roles in carrot root development (Wang et al. 2016). However, whether hypoxia can trigger lignification in carrot roots under hypoxia is unclear.

Carrot (*Daucus carota* L.) is an important root vegetable and has been one of the top ten vegetable crops due to its abundant nutritional value. Taproot, the prized part of carrot, is the edible part and main object of research (Cavagnaro et al. 2011; Xu et al. 2014b; Wang et al. 2015). The hydroponics of carrot will be a useful way to study the growth and development of carrot roots. To our knowledge, relevant studies about the carrot hydroponics remain rare.

In this study, to investigate the response of carrot roots to hypoxia, carrot plants were cultivated in the environment with or without aeration by hydroponic cultivation. The expression profiles of lignin-related genes and the *DcADH-P* genes were measured through quantitative real-time PCR (qRT-PCR). The lignin contents of the roots at 30 and 60 days after sowing (DAS) were measured. The microscopy method was also performed to investigate the lignin distribution and cell development. The results obtained from this study will be helpful for studying the impact of hypoxia on plant growth and provide useful information for carrot hydroponics.

Materials and methods

Experiment in hydroponics

'Kurodagosun' was chosen as the experimental material and cultivated in a climate-controlled chamber by hydroponics at the state key laboratory of crop genetics and germplasm enhancement in Nanjing Agricultural University (32°04′N, 118°85′E). 'Kurodagosun' is a widely cultivated variety and was used in the genome sequencing (Xu et al. 2014a; Wang et al. 2018). Carrot seeds were germinated in the petri dishes. After a few days, seeds with germs were transferred into 96-well plates whose ends were cut off. The edges of the plates were not used to cultivate seedlings. Plates with seeds were placed at a tank (25 × 16 × 6 cm) with two shelves. The solution (Hoagland) was renewed every 5 days. Seedlings of the carrot with two true leaves were divided into two parts and placed in two tanks. One tank was continuously aerated through rubber tubes by an air compressor and another one without air transport was used as control. The oxygen concentrations of the two conditions were about 3 and 8 mg/L, respectively. The temperature of the chamber was held at 25 °C for 16 h during daytime with 320 μmol/m/s light intensity and 18 °C for 8 h in the dark.

Plants were sampled at 30 and 60 DAS and stored at − 80 °C for molecular research. Plants at 30 and 60 DAS were also harvested to measure lignin content and perform microscopy analysis. Total of 15 carrot seedlings were used for measuring root length, root fresh weight, and root diameter in different developmental stages and different oxygen environments, respectively.

Identification, sequence structure, and phylogenetic analysis of carrot alcohol dehydrogenases

To identify carrot *DcADH-P* genes at the genome level, the data of carrot genome was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/genome/?term=Daucus) and CarrotDB (Iorizzo et al. 2016; Xu et al. 2014a). The candidate *ADH-P* genes were obtained by using HMMER 3.0 with the Hidden Markov models (HMMs) of ADH_N

(PF08240) domain (Eddy 2011; Finn et al. 2013, 2015). The alcohol_DH_plants (cd08301) domain was the signature structure of plant *ADH* genes. Then the proteins coding by candidate genes were analyzed with InterPro (http://www.ebi.ac.uk/interpro/search/sequence-search). The analysis of intron was performed *via* GSDS 2.0 (Hu et al. 2014). In addition, multiple alignments of the 6 *DcADH-P* proteins were performed by DNAMAN version 5.2.2 (Lynnon Biosoft, Quebec, Canada). The phylogenetic tree was constructed via MEGA 6 with Neighbor-joining method under the Jones–Thornton–Taylor (JTT) model. The bootstrap replication was set as 1000 (Tamura et al. 2013).

Measurement of lignin content

Lignin content was extracted and measured according to previous description with the following modifications (Cai et al. 2006; Cervilla et al. 2009; Wang et al. 2016). Briefly, approximately 2 g carrot roots were initially ground with liquid nitrogen. The powders were then mixed with 6 mL of the 99.5% ethanol. The mixture was centrifuged at 10,000×*g* for 20 min. The precipitates were collected and exposed at the room temperature overnight. About 10 mg dried precipitates were then mixed with 1 mL of the 2 M HCl, and 0.1 mL of the thioglycolic acid. The mixture was then transferred into a 2-mL plastic tube. The tubes were tightly sealed and incubated at 100 °C for 8 h. After boiling, tubes were cooled on ice. Cooled mixture was then centrifuged at 12,000×*g* for 20 min at 4 °C to get a pellet. The obtained pellet was then washed with 1 mL of the deionized water and resuspended in 1 mL of 1 M NaOH. The mixed solution was stored in a gently agitated shaker at 25 °C for 18 h. Following the 18-h agitation, the mixture was centrifuged at 12,000×*g* for 20 min. The upper liquid was transferred into a new tube and mixed with 1 mL of concentrated HCl. The mixture was stored at 4 °C for 6 h to precipitate lignin thioglycolate. Finally, the mixture was centrifuged at 12,000×*g* for 20 min and the sediment was then dissolved in 1 mL of the 1 M NaOH. The absorbance of the 1 M NaOH at 280 nm was measured as blank control. The calibration curve was made using a commercial alkali lignin (Sigma-Aldrich).

Histochemical staining and UV fluorescence microscopy

Plants grown under conditions with or without aeration were sampled at 30 and 60 DAS for histochemical staining to investigate the lignin distribution. Phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde was used to store the carrot root samples. To perform safranin-*O*/fast green staining, xylene was used to deparaffinize the root samples. Subsequently, ethanol was used to dehydrate the deparaffinized root samples. The dehydrated sections were then stained in 1% safranin-*O* for 2 h and counterstained with 0.5% fast green for 15 s. After staining, ethanol was used to remove excess stain and neutral balsam was used to mount the sections. Finally, the lignified parts were stained into red, whereas the cellulosic tissues were stained into green. When explored under the UV excitation, lignified cell walls could exhibit autofluorescence (Donaldson and Knox 2012; Jia et al. 2015). Fluorescence microscopy was used to search the lignin containing components in the root.

Total RNA isolation

A RNA extraction kit (Tiangen, Beijing, China) was used to extract the total RNA of the carrot roots according to the manufacturer's instructions. cDNA was synthesized using a PrimerScript RT reagent kit (TaKaRa, Dalian, China). The cDNA was diluted 15-fold for qRT-PCR analysis.

Gene expression analysis

In this study, 12 genes involved in the lignin biosynthesis and 6 *DcADH-P* genes were chosen for qRT-PCR analysis. The 12 genes involved in the lignin biosynthesis of carrot were reported in a previous study. Primers of genes related to lignin synthesis for qRT-PCR was designed according to previous description (Wang et al. 2016). The primers of the 6 *DcADH-P* genes were designed through Primer 5.0 (Lalitha 2000) (Table 1). The qRT-PCR analysis was performed via MyIQ Real-Time PCR Detection System (Bio-Rad, CA,

Table 1 Primers of *DcADH-P* genes for qRT-PCR

Genes	Forward primer (5′–3′)	Reverse primer (5′–3′)
DcADH-P1	TCTTCACTTCCCTCTGCC	CCAAATCCGTTACTCCCT
DcADH-P2	AACCTCGTTCGGACCTTC	TTCGCCTTTCAGCATCAA
DcADH-P3	CAGCGAGTACACTGTCATG	CACTGTGGAGCCTTTCTTA
DcADH-P4	GAAACCGTTGGTGATAGA	TAGCCTCCCAGAAGTAAA
DcADH-P5	CTTGGCGATAGAGGAAAT	TATGCGAGGAAACAGAGG
DcADH-P6	GAGGAGGTGGAAGTGAGT	TCCGAGGAAATAGATGAA

USA). The cycling conditions were maintained as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s, at last a melting curve (65–95 °C, at increments of 0.5 °C) was generated. The relative gene expression profile was calculated with the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). The internal control was *DcACTIN* in this study (Tian et al. 2015). Each PCR reaction had three biological replicates and technical replicates.

Data analysis

Data were analyzed by using the office 2007 software. In addition, Student's *t* test was used to analyze the data in this study at the 0.05 significance through SPSS 17.0 software.

Results

Growth analysis of carrot plants

To study the response of carrot roots to hypoxia in hydroponics, carrot plants were cultivated in the conditions with or without aeration. Plants were sampled at 30 and 60 DAS, respectively. The growth status of carrot plants under the two treatments is displayed in Fig. 1. Carrots cultivated in nutrition solution had a large number of fibrous roots at the middle and tip parts of taproots. The fibrous roots seemed to originate from the cortex. These fibrous roots were long and intertwined with each other. The length, fresh weight and diameter of the carrot roots were measured (Fig. 2). Carrot plants treated with aeration grew much better than that treated without aeration. Morphological characters of carrot roots under different oxygen concentrations were obviously different. At 30 DAS, the fresh weight of the seedling roots cultivated in the higher O_2 concentration (with aeration) was significantly higher than that in lower O_2 concentration (without aeration). At 60 DAS, the fresh weight of the carrot root treated with or without aeration were 32.0 and 29.7 g, respectively. Moreover, the root diameter was about 29.4 and 27.0 mm, respectively.

Identification, evolution analysis of carrot alcohol dehydrogenases

To identify the *ADH-P* genes from the carrot genome, HMMER 3.0 was used with the ADH_N (PF08240) domain. Initially, 69 candidate genes were detected from the carrot genome. Then, InterPro database was used to analyze the protein structure of the candidate genes. Six DcADH-P proteins were found to have NAD(P)-binding domain and a signature structure of alcohol_DH_plants (cd08301) (Supplementary Table 1). As a result, these six genes were determined and were designated as *DcADH-P1, DcADH-P2, DcADH-P3, DcADH-P4, DcADH-P5*, and *DcADH-P6*. Multiple alignments indicated that the six DcADH-P proteins have 72% sequence similarity (Supplementary Fig. 1).

In the study of gene evolution, the absence of introns was thought to be an important evidence. Here, the introns of the six *DcADH-P* genes were analyzed (Fig. 3b). *DcADH-P1* and *DcADH-P3* had 8 introns, and *DcADH-P2, DcADH-P4, DcADH-P5*, and *DcADH-P6* had 9 introns. Furthermore, a phylogenetic tree was constructed based on the full-length amino acid sequence of six carrot *DcADH-P* genes with 16 homologues from other plant species (Fig. 3a). *DcADH-P1, DcADH-P2*, and *DcADH-P3* aligned within the same

Fig. 1 Growth status of carrots under different oxygen environments. Carrots were cultivated under two different oxygen environments in hydroponics: no air entering (NAE) and with air entering (WAE). The samples were collected at 30 and 60 days after sowing, respectively. Black lines in the lower right corner of each plant represent 2 cm in that pixel

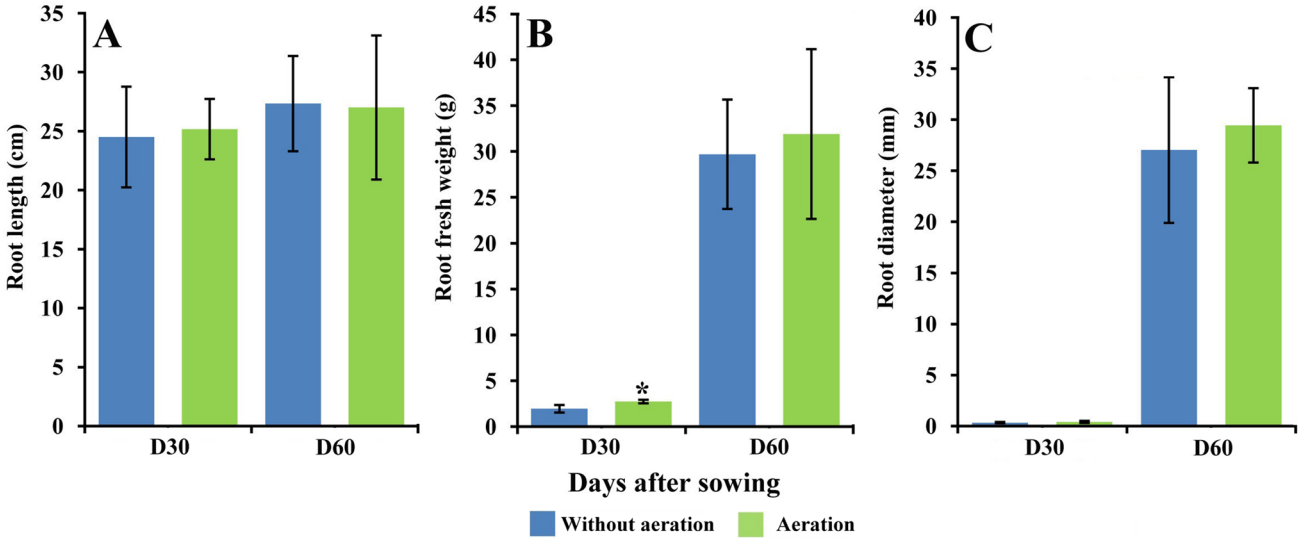

Fig. 2 Morphological characteristics of carrot roots. **a** Root length (cm) of carrot roots; **b** root fresh weight (g) of carrot roots; **c** root diameter (mm) of carrot roots. Student's *t* test was used to identify the differences under different oxygen concentrations ($P < 0.05$; *control versus treatment). Error bars represent standard deviation (SD). *DAS* days after sowing. (Colour figure online)

sub-lineage group. *DcADH-P4, DcADH-P5*, and *DcADH-P6* aligned into other sub-lineages.

Expression profiles of *DcADH-P* genes in carrot roots

According to previous reports, the expression profile of *ADH-P* gene was induced under the hypoxia in *Arabidopsis*. Here, the expression profiles of the six carrot *DcADH-P* genes under different oxygen environments were measured by qRT-PCR (Fig. 4). As a result, expression profiles of three carrot *DcADH-P* genes (*DcADH-P1, DcADH-P2*, and *DcADH-P3*) could be detected and the other three (*DcADH-P4, DcADH-P5*, and *DcADH-P6*) could not be detected. The expression profiles of *DcADH-P1, DcADH-P2*, and *DcADH-P3* were significantly induced in carrots cultivated in the low oxygen concentration compared with that cultivated in the high oxygen concentration. In addition, the expression profiles of the three genes (*DcADH-P1, DcADH-P2*, and *DcADH-P3*) at 60 DAS were significantly higher than that at 30 DAS under low oxygen concentration environment.

Anatomical structure of carrot roots

To observe the anatomical structure, carrot roots treated with or without aeration were sectioned and stained by safranin-*O*/fast green (Fig. 5). Roots cultivated in the environment with abundant oxygen had less aerenchyma (space) compared with plants grown with limited oxygen. The vascular pericycle was destroyed and pitted with aerenchyma under limited oxygen environment. By contrast, the vascular pericycle was complete under abundant oxygen environment. Furthermore, the secondary xylem was also destroyed and pitted with aerenchyma in carrot roots without aeration at 60 DAS. The root xylem was more tight and complete under the abundant oxygen environment. Vessels had better development under the abundant oxygen environment.

The micrographs under the white light and fluorescence were used to determine the effect of hypoxia on carrot root lignification (Fig. 6). In the carrot roots, lignin was mainly accumulated in the cell walls of tracheary elements of the xylem. As shown in Fig. 6b, lignification of carrot roots under the low-oxygen environment was enhanced. However, the number of vessels in the carrot roots cultivated in the higher oxygen environment was more.

Lignin content of carrot roots

To investigate the lignification of carrot roots, the lignin content of carrot roots at 30 and 60 DAS were measured (Fig. 7). These carrot roots under the low (without aeration) and abundant (with aeration) oxygen conditions were collected at 30 and 60 DAS, respectively. During the development, the lignin contents were significantly decreased in the roots of carrot. The content changed from 163.96 to 16.55 mg/g during the development under the low-oxygen environment. Similar results were obtained under the abundant oxygen environment (from 118.17 to 11.86 mg/g). Moreover, the lignin contents in the roots without aeration were significantly higher than that with aeration.

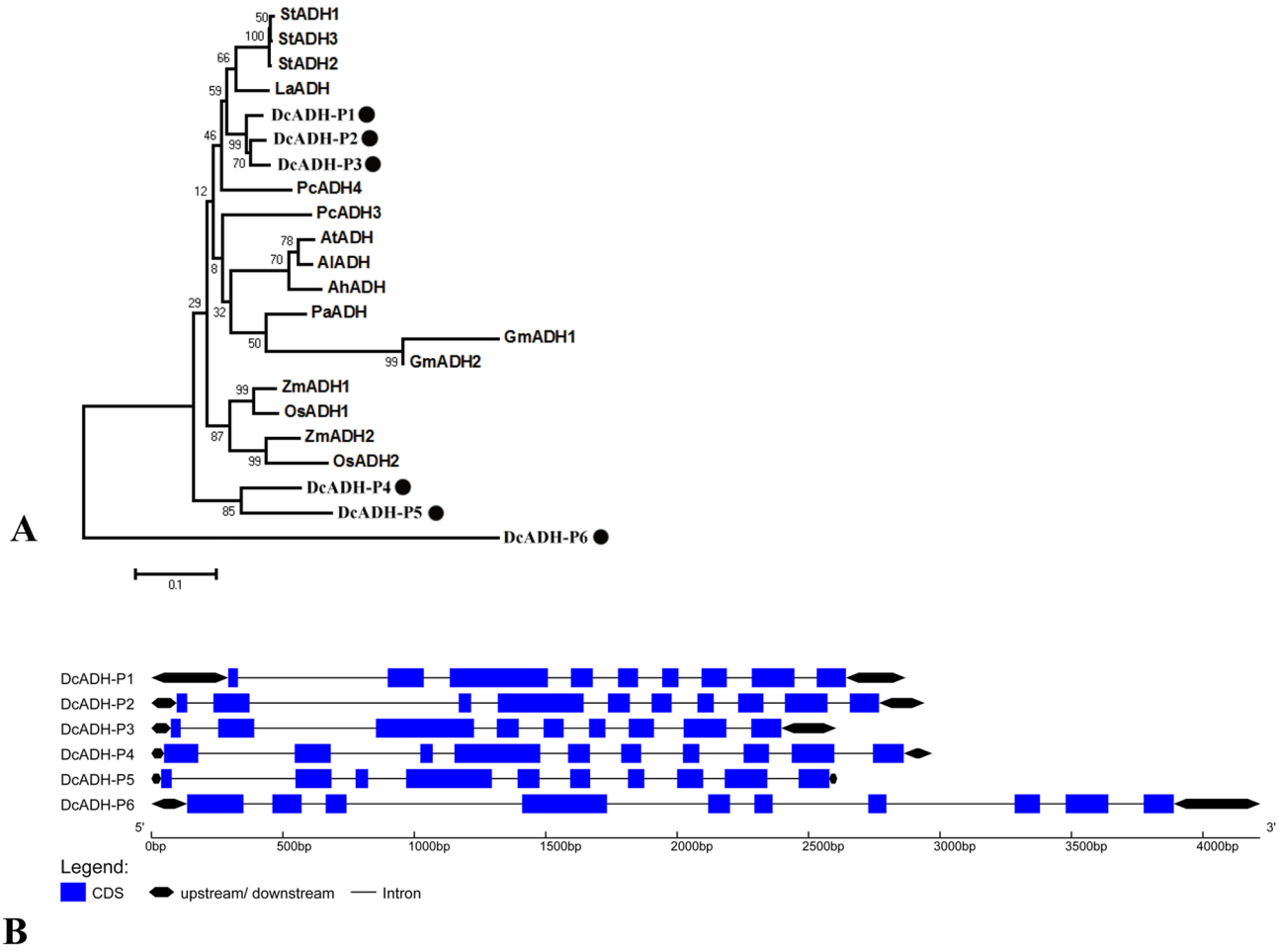

Fig. 3 The evolution analysis of carrot *DcADH-P* genes. **a** Phylogenetic tree of carrot DcADH-P proteins. The phylogenetic tree was constructed via MEGA 6.0 on the basis of full-length amino acid sequences of DcADH-P proteins and 16 ADH-P proteins from other species with Neighbor-joining method. Bootstrap analysis was conducted with 1000 replicates. The DcADH-P proteins were marked with solid circles. **b** The component of introns in carrot *DcADH-P* genes. The intron analysis was performed via GSDS 2.0. Blue blocks represent the CDS sequence and black blocks represent the upstream or downstream sequences. (Colour figure online)

Expression profiles of lignin biosynthesis-related genes in carrot roots

To study the molecular mechanisms of lignin accumulation, genes related to lignin biosynthesis were selected for expression profiles analysis (Fig. 8). Among the 12 selected genes, most of them were differentially expressed under different oxygen environments. The expression levels also changed significantly during the development of carrot roots. During the carrot root development, the expression profiles of most genes decreased significantly in spite of the oxygen environment. However, the expression profile of *DcCCR* showed an opposed trend which increased during the development of carrot roots. The transcription of most genes showed higher levels in the high oxygen environment. At 30 DAS, the expression profiles of most genes were similar except *DcPER1*. At 60 DAS, *Dc4CL, DcHCT, DcCCoAOMT, DcF5H, DcCOMT, DcCAD*, and *DcLAC1* increased significantly when carrot roots were surrounded with abundant oxygen. By contrast, the transcription of *DcC3'H* showed an opposite trend.

Discussion

Impact of hypoxia on expression profiles of *DcADH-P* genes in carrot roots

Plant *ADH-P* genes contribute much in the survival of plants under hypoxia. Hypoxia could induce the expression of *ADH-P* genes in *Arabidopsis*, maize, and rice (Baxter-Burrell et al. 2003; Ismond et al. 2003; Kürsteiner et al. 2003). The plant *ADH* gene family is a small gene family comprised two or three members (Chang and Meyerowitz 1986).

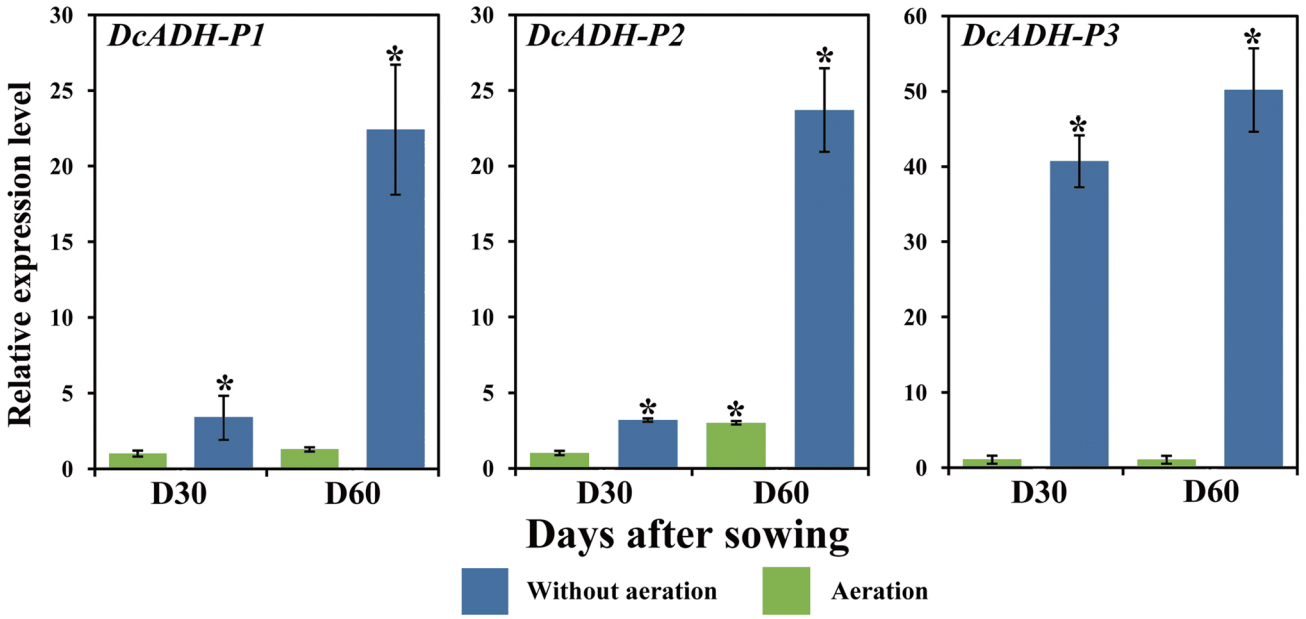

Fig. 4 Expression profile of *DcADH-P* genes at different growth stages and under different oxygen situations. The value on the left Y-axis indicates the relative gene expression profiles. The X-axis represents the two stages of carrot root development, corresponding to 30 and 60 days after sowing. The expression profiles of the genes were measured by qRT-PCR. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method. Student's *t* test was used to do the statistic analysis ($P<0.05$; *control versus treatment). Error bars represent standard deviation (SD) of three replicates. *DAS* days after sowing. (Colour figure online)

Here, we found six *DcADH-P* genes in the carrot genome. The six *DcADH-P* genes did not align within the same sub-lineage group in the phylogenetic tree. The sequences of *DcADH-P1, DcADH-P2*, and *DcADH-P3* were located near the *ADH-P genes* reported in other species. In contrast, the sequences of *DcADH-P4, DcADH-P5*, and *DcADH-P6* were located far from the other three *DcADH-P* genes. These results suggested that there could be some duplication events among them during the evolution (Gabaldón and Koonin 2013).

To measure the expression profiles of *DcADH-P* genes under the different oxygen environments, we performed qRT-PCR analysis on the six *DcADH-P* genes. The expression profiles of only three *DcADH-P* genes (*DcADH-P1, DcADH-P2*, and *DcADH-P3*) could be detected and the other three genes (*DcADH-P4, DcADH-P5*, and *DcADH-P5*) could not be detected. Those results suggested that the three *DcADH-P* genes (*DcADH-P4, DcADH-P5*, and *DcADH-P6*) may not response to the hypoxia. The expression profiles of *DcADH-P1, DcADH-P2*, and *DcADH-P3* in the carrot cultivated without aeration were significantly higher than that cultivated with aeration. Furthermore, the expression profiles of the three genes (*DcADH-P1, DcADH-P2*, and *DcADH-P3*) were significantly induced at 60 DAS compared with that at 30 DAS under low-oxygen environment. These results suggested that carrots cultivated in the solution without aeration suffering from hypoxia.

Impact of hypoxia on anatomical structure of carrot roots

Hypoxic environment is injurious to the growth and development of plants. Therefore, plants have to make some alterations to adapt to the low-oxygen environments. Lysigenous aerenchyma and adventitious roots were generated to adapt oxygen deficiency. Maize, soybeans, as well as some wetland species were reported to produce lysigenous aerenchyma when suffering flooding (Bacanamwo and Purcell 1999; Drew et al. 1989; Kawai et al. 1998; Morgan 1994). Lysigenous aerenchyma not only serves as a pathway for oxygen transfer, but also reduces the expenditure of oxygen by reducing the quantity of O_2-consuming cells. However, the formation of lysigenous aerenchyma was the result of cell separation or cell death and dissolution (Drew et al. 2000; Schussler and Longstreth 1996). Here, safranin-*O*/fast green staining was used to observe the anatomical structure of carrot roots. Safranin-*O*/fast green staining was a useful method to investigate plant anatomical structure and tissues containing lignified cell walls which has been used in previous studies (Jia et al. 2014; Wang et al. 2016). In the present study, carrot roots cultivated in the solution without aeration had more aerenchyma (space), and the aerenchyma size was bigger. Similar results appeared in maize roots suffering hypoxia under laboratory conditions (Ober and Sharp 1996).

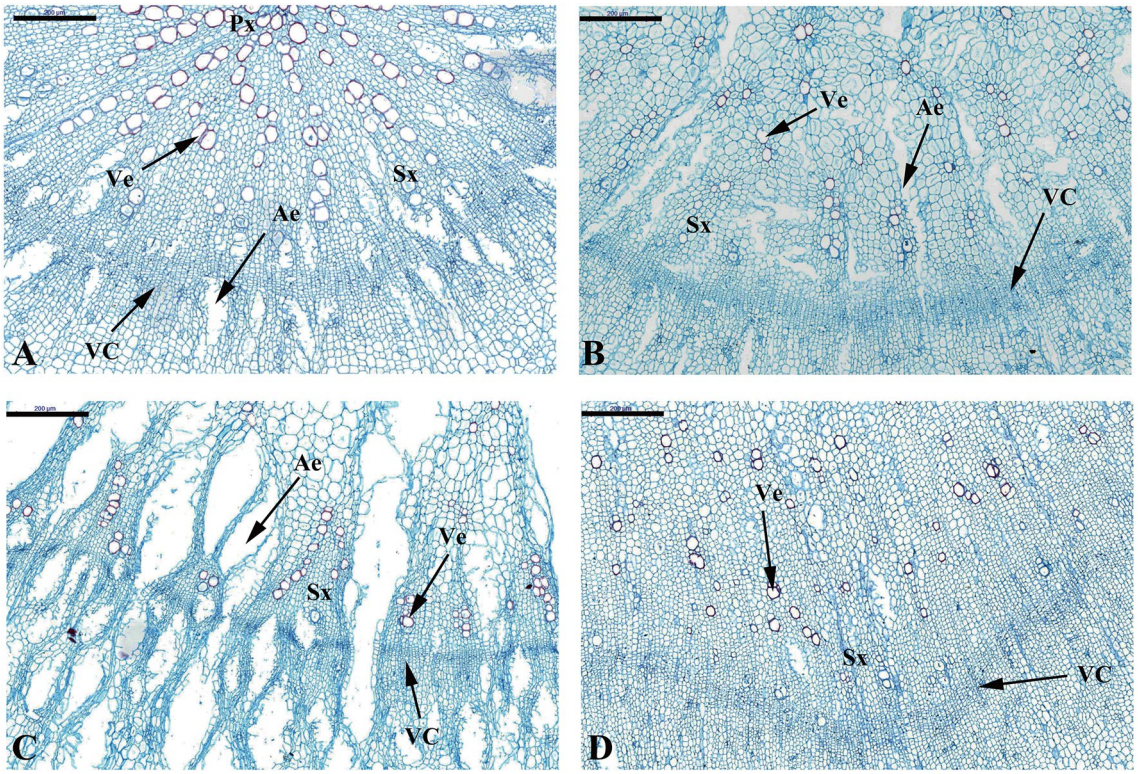

Fig. 5 Microstructure of carrot roots under different oxygen environments and at different growth stages. Carrot roots treated with or without aeration were sectioned and stained by safranin-*O*/fast green. The part around the vascular cambium of a root cross section is shown. **a** The 30 DAS carrot root cultivated without aeration. **b** The 30 DAS carrot root cultivated with aeration. **c** The 60 DAS carrot root cultivated without aeration. **d** The 60 DAS carrot root cultivated with aeration. Staffs in the figure represent the magnification. *Ep* epidermis, *Xy* xylem, *SP* secondary phloem, *Ve* vessel, *VC* vascular cambium, *Ae* aerenchyma

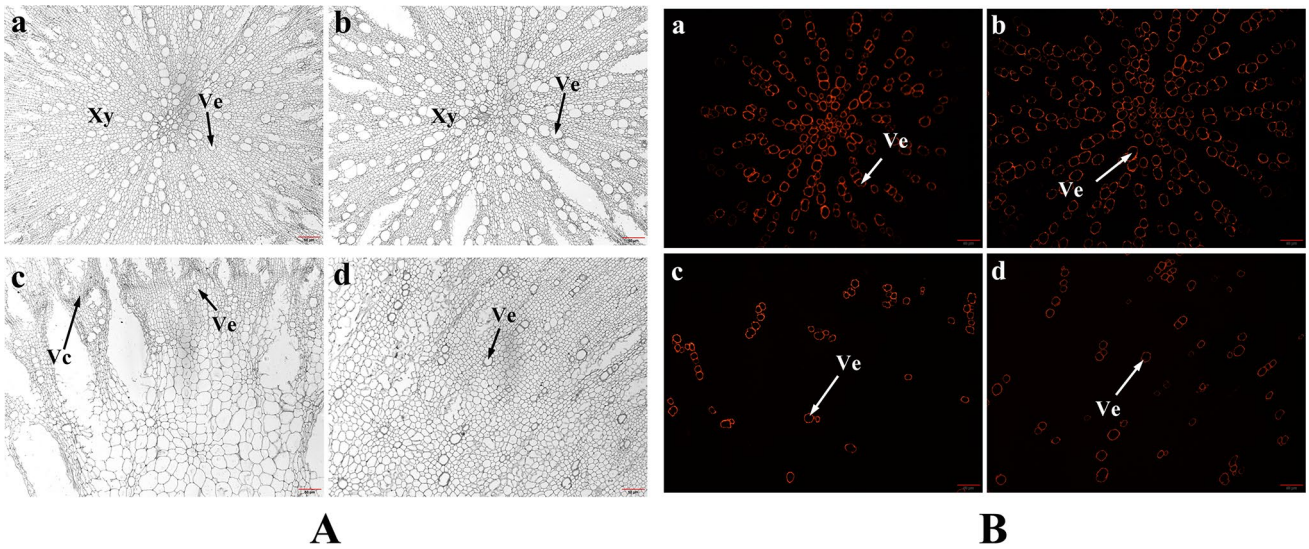

Fig. 6 Transverse sections of carrot roots. **a** Transverse sections of carrot roots under white light. a The 30 DAS carrot root cultivated without aeration. b The 30 DAS carrot root cultivated with aeration. c The 60 DAS carrot root cultivated without aeration. d The 60 DAS carrot root cultivated with aeration. **b** Fluorescence micrographs of transverse sections of carrot roots. a The 30 DAS carrot root cultivated without aeration. b The 30 DAS carrot root cultivated with aeration. c The 60 DAS carrot root cultivated without aeration. d The 60 DAS carrot root cultivated with aeration. Scale bars are 50 μm in length. *Ve* vessel, *Xy* xylem, *VC* vascular cambium

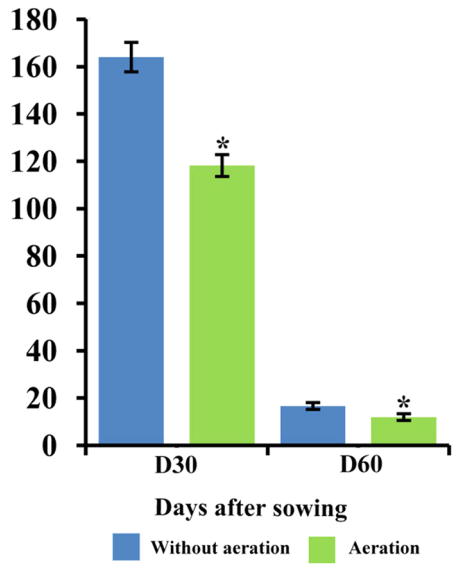

Fig. 7 Lignin content of carrot roots at different growth stages and cultivated in different oxygen environments. Student's *t* test was used to identify the differences under different oxygen concentrations ($P < 0.05$; *control versus treatment). Error bars represent standard deviation (SD) of three replicates. DAS days after sowing. (Colour figure online)

Impact of hypoxia on lignin content of carrot roots

Lignin biosynthesis was believed to occur during the process of normal tissue development and can be triggered under many various biotic and abiotic stresses (Bonawitz and Chapple 2010; Moura et al. 2010). However, the study on stressing situations changing lignin content is still little. Lignin is the second most abundant compound in plant organs (Boerjan et al. 2003). In vascular plants, lignin was found to be the main component of the secondary cell wall (Müse et al. 1997). In carrot roots, the lignin was mainly deposited in the cell walls of tracheary elements in the xylem (Wang et al. 2016). During maize stem development, the lignin content continuously increased (Jung and Casler 2006). Similar results were reported in the stem development of other plants (Shen et al. 2009). Here, lignin contents decreased during the root development which is consistent with the results from a previous study (Wang et al. 2016). In addition, the result of analysis on lignin content and autofluorescence under UV excitation revealed that the lignification of carrot roots cultivated in the solution without aeration was enhanced. Compared with the carrots cultivated in the soil, the lignin contents of hydroponic carrot roots were significantly increased. These results suggested that hypoxia may enhance the lignification of carrot roots.

Impact of hypoxia on expression profiles of lignin biosynthesis-related genes in carrot roots

Based on previous reports, the response of plants to hypoxia not only appeared in the biochemical and physiological reconfiguration, but also in the change of gene expression level (Christianson et al. 2010; Narsai et al. 2011). In this study, 12 genes involved in the biosynthesis of lignin were selected and analyzed. Among these genes, *DcPAL, DcCCoAOMT, DcCAD*, and *DcPER1* were reported to be positively related to lignin accumulation during the developmental of carrot root. The expression profiles of these four genes in this study were consistent with the profiles reported in previous study, during carrot root development (Wang et al. 2016). *PAL* encodes the enzyme of the first step in monolignol synthesis and was also reported to be a key gene in the phenylpropanoid pathway (Dixon et al. 2002; Kao et al. 2002). In the present work, the transcription level of *DcPAL* was found to decrease during the root development and increase when carrot roots were treated with aeration. In *Arabidopsis*, the expression of *PAL* and *4CL* were reported to be coordinately regulated (Lee et al. 1995). Herein, *DcPAL* and *Dc4CL* showed similar expression trend. 4CL is an important enzyme in lignin synthesis and is generally thought to encode the third step of phenylpropanoid pathway (Costa et al. 2005). In *Populus tremuloides*, the down-regulation of *4CL* led to the reduction of lignin contents (Hu et al. 1999). Similar result appeared during the development of carrot roots. However, the transcription level of *Dc4CL* increased when in the environment with aeration (lignin content decreased). Similar results appeared in the expression trends of *DcC4H, DcHCT, DcCCoAOMT, DcCCR, DcF5H, DcCOMT, DcCAD*, and *DcLAC1* in carrot roots in the environment with or without aeration.

Lignin is the main component of the secondary cell walls in vascular plants. The secondary cell walls biosynthesized during the development of vascular plant build a strong xylem thus providing mechanical support for the plant (Boudet 2000). However, the development of plants would be negatively affected by hypoxia.(Geigenberger 2003; Schussler and Longstreth 1996). In cotton, genes involved in cell wall synthesis were down-regulated when suffering from hypoxia (Christianson et al. 2009). The development of carrot roots would be restricted and the initial structure would also be destructed during hypoxia. These results suggested that the enhanced lignification in carrot roots may due to the decrease of contains and destroyed structure in the carrot roots when suffering from hypoxia.

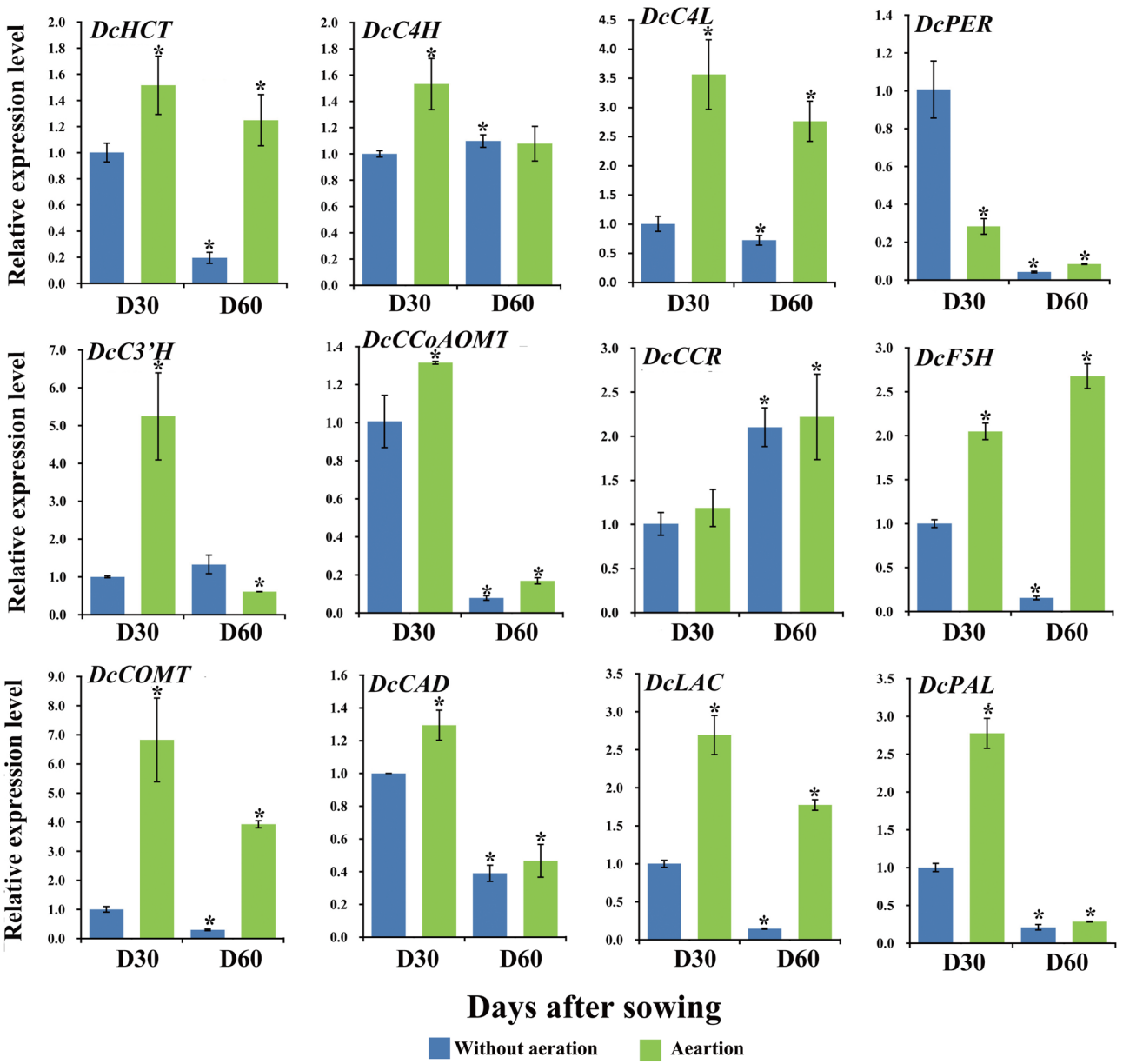

Fig. 8 Expression profile of genes related to lignin biosynthesis at different growth stages and under different oxygen situations. The expression profiles of the genes were measured by qRT-PCR. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method. Student's *t* test was used to do the statistic analysis ($P < 0.05$; *control versus treatment). Error bars represent standard deviation (SD) of three replicates. *DAS* days after sowing. (Colour figure online)

Conclusion

Here, six *DcADH-P* genes were identified from carrot genome. Among them, three *DcADH-P* genes were significantly induced when carrot cultivated without aeration in hydroponics, while the other three could not be detected. This result suggested that carrot cultivated without aeration suffered from hypoxia. In addition, the carrot roots cultivated without aeration had more aerenchyma (space). Analysis of the lignification of carrot roots showed that the roots of carrot cultivated without aeration had more lignin content. The same result appeared in the autofluorescence under UV excitation analysis. Our results indicated that hypoxia could enhance the lignification of carrot root. The current study will be useful for investigating the hypoxia on carrot growth and development and may also provide information for improving carrot hydroponics.

Author contribution statement Conceived and designed the experiments: Xiong AS and Que F. Performed the

experiments: Que F, Wang GL, Fen K, Xu ZS, and Wang F. Analyzed the data: Que F and Wang GL. Contributed reagents/materials/analysis tools: Xiong AS. Wrote the paper: Que F. Revised the paper: Xiong AS and Wang GL. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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