Comparative Analysis and Expression Patterns of the PLP_deC Genes in Dendrobium officinale

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Abstract: Studies have shown that the type II pyridoxal phosphate-dependent decarboxylase (PLP_deC) genes produce secondary metabolites and flavor volatiles in plants, and TDC (tryptophan decarboxylase), a member of the PLP_deC family, plays an important role in the biosynthesis of terpenoid indole alkaloids (TIAs). In this study, we identified eight PLP_deC genes in Dendrobium officinale (D. officinale) and six in Phalaenopsis equestris (P. equestris), and their structures, physicochemical properties, response elements, evolutionary relationships, and expression patterns were preliminarily predicted and analyzed. The results showed that PLP_deC genes play important roles in D. officinale and respond to different exogenous hormone treatments; additionally, the results support the selection of appropriate candidates for further functional characterization of PLP_deC genes in D. officinale.

Keywords: Dendrobium officinale; PLP_deC; bioinformatics; expression pattern analysis; evolution

1. Introduction

Dendrobium officinale Kimura et Migo (also known as D. catenatum) is a perennial herb that is commonly used as a valuable Chinese herbal medicine and has a long evolutionary history among orchids. D. officinale is rich in alkaloids [1,2], and its genome, transcriptome, and metabolome indicate that D. officinale may also contain terpenoid indole alkaloids (TIAs). In this study, we identified eight PLP_deC genes in Dendrobium officinale (D. officinale) and six in Phalaenopsis equestris (P. equestris), and their structures, physicochemical properties, response elements, evolutionary relationships, and expression patterns were preliminarily predicted and analyzed. The results showed that PLP_deC genes play important roles in D. officinale and respond to different exogenous hormone treatments; additionally, the results support the selection of appropriate candidates for further functional characterization of PLP_deC genes in D. officinale.

Pyridoxal 5'-phosphate (PLP) is the active form of vitamin B6 and is used by a variety of enzymes in all organisms [8]. Previously, we classified all PLP-dependent enzymes into at least five structural groups based on their protein structures [8,9]. Among them, the type I group is the most common and contains aminotransferases, decarboxylases, and an enzyme that catalyzes α-, β-, or γ-eliminations. Type II encodes the enzymes involved in β-elimination reactions. Type III is primarily alanine-racemase
specific, while type IV enzymes typically include D-alanine aminotransferases. Type V enzymes are the most diverse, including glycogen and starch phosphorylases. One important group of PLP-dependent enzymes belongs to the PLP_deC family, which includes aromatic-L-amino acid decarboxylase (AAD), glutamic acid decarboxylase (GAD), and histidine decarboxylase (HDC) [10]. The biological functions of plant and animal AADs are closely related to their corresponding substrate selectivity and catalytic reactions; thus, some AADs, such as tyrosine decarboxylase (TYDC) and tryptophan decarboxylase (TDC), are further annotated based on their principal substrates [8,11]. These enzymes catalyze the decarboxylation of aromatic L-amino acids and are primarily involved in the synthesis of secondary metabolites in plants [12,13].

Numerous data indicate that PLP_deC exhibits tissue-specific and inducible transcript accumulation during plant development. In addition, several roles of PLP_deC in plant development have been identified. For example, TDC is a key enzyme that links primary and secondary metabolism with high substrate specificity [14,15]. In addition, the transcript levels of PLP_deC genes are affected by abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA), and abiotic stress [16,17].

In 2015, Chinese scientists announced that they had completed the genome sequence of the orchid Phalaenopsis equestris [18]. D. officinale and P. equestris (Schauer) Rchb.f. are epiphytes in the family Orchidaceae. The draft of the D. officinale genome sequence was reported recently [4,19]. To further understand the PLP_deC gene family in orchids, we identified 8 and 6 PLP_deC genes from the genomes of D. officinale and P. equestris, respectively, and analyzed their phylogenetic relationships, gene structures, cis-regulatory elements, tissue expression patterns, and expression profiles under MeJA, ABA, and SA treatments. Our results may not only improve the current understanding of the evolutionary expansion, sequence conservation, and functional differentiation of PLP_deC genes but also provide in-depth basic biological information for further studies of the evolution of these genes in Orchidaceae.

2. Results

2.1. Sequence Analysis of PLP_deC Family Members

HMMER software was used to identify candidate genes in the genomes of D. officinale and P. equestris. All candidate genes were submitted to Pfam and SMART for verifying the presence of the PLP_deC domain. The sequences without the conserved domain and the redundant sequences were removed. Finally, a total of 14 PLP_deC-family sequences were obtained in D. officinale and P. equestris. The basic information of each PLP_deC-family sequence was listed in Table 1. We found that in D. officinale, the molecular weights ranged from 34.48 kDa (DoGAD4) to 79.29 kDa (DoAAD2), with an average molecular weight of 57.64 kDa, and the theoretical isoelectric points ranged from 5.27 (DoHDC1) to 7.52 (DoAAD1), with an average value of 6.12. In P. equestris, the molecular weights ranged from 53.44 kDa (PeHDC1) to 57.26 kDa (PeGAD3), with an average molecular weight of 55.53 kDa, while the theoretical isoelectric points ranged from 5.53 (PeGAD1) to 8.55 (PeGAD3), with an average of 6.52. These results showed that most genes have an acidic pl. We analyzed the subcellular localization of 8 and 6 PLP_deC-family protein sequences in D. officinale and P. equestris by Target P 1.1. and the results showed the presence of a signal peptide in DoAAD1 and DoAAD3, suggesting their cellular localization in extracellular with a probability of 0.735 and 0.561. The proteins of DoHDC1, PeAAD1, PeAAD2 and PeHDC1 are possibly located in other cellular compartments, e.g. chloroplast transit peptide and mitochondrial, whereas the location of the remaining eight proteins is unknown (Table 2).
Table 1. Sequence analysis of pyridoxal phosphate-dependent decarboxylase (PLP\_deC) family members.

| Gene Name | Gene ID     | Location | pI   | MW (kDa) | Pyridoxal\_deC Domain |
|-----------|-------------|----------|------|----------|-----------------------|
| PeGAD1    | PAXXG012610 | scaffold3| 5.53 | 55.66    | ✓                     |
| PeAAD1    | PAXXG064070 | scaffold28| 6.43 | 54.31    | ✓                     |
| PeAAD2    | PAXXG110580 | scaffold67| 6.68 | 55.69    | ✓                     |
| PeGAD2    | PAXXG162190 | scaffold130| 5.96 | 56.84    | ✓                     |
| PeGAD3    | PAXXG162200 | scaffold130| 8.55 | 57.26    | ✓                     |
| PeHDC1    | PAXXG233410 | scaffold291| 5.96 | 53.44    | ✓                     |
| DoAAD2    | Dendrobium\_GLEAN\_10136748 | scaffold166| 6.32 | 79.29    | ✓                     |
| DoGAD3    | Dendrobium\_GLEAN\_10126118 | scaffold506| 5.88 | 68.10    | ✓                     |
| DoAAD3    | Dendrobium\_GLEAN\_10085660 | scaffold2906| 5.83 | 62.7     | ✓                     |
| DoGAD4    | Dendrobium\_GLEAN\_10051783 | scaffold549| 6.28 | 34.48    | ✓                     |
| DoGAD2    | Dendrobium\_GLEAN\_10051784 | scaffold549| 6.2  | 51.98    | ✓                     |
| DoAAD1    | Dendrobium\_GLEAN\_10046548 | scaffold7413| 7.52 | 42.84    | ✓                     |
| DoGAD1    | Dendrobium\_GLEAN\_10045094 | scaffold7529| 5.67 | 73.82    | ✓                     |
| DoGAD4    | Dendrobium\_GLEAN\_10046449 | scaffold7738| 5.27 | 47.84    | ✓                     |

Note: ✓ shows conserved domain of Pyridoxal\_deC.

Table 2. Subcellular localization of PLP\_deC proteins.

| Gene Name | Chloroplast Transit Peptide | Mitochondrial Targeting Peptide | Signal Peptide | Other | Location | Reliability Class |
|-----------|-----------------------------|-------------------------------|----------------|-------|----------|------------------|
| DoAAD1    | 0.040                       | 0.026                        | 0.735          | 0.346 | S        | 4                |
| DoAAD2    | 0.093                       | 0.239                        | 0.029          | 0.806 | *        | 3                |
| DoAAD3    | 0.039                       | 0.109                        | 0.561          | 0.525 | S        | 5                |
| DoGAD1    | 0.218                       | 0.279                        | 0.203          | 0.148 | *        | 5                |
| DoGAD2    | 0.135                       | 0.068                        | 0.180          | 0.727 | *        | 3                |
| DoGAD3    | 0.207                       | 0.190                        | 0.106          | 0.161 | *        | 4                |
| DoGAD4    | 0.015                       | 0.261                        | 0.229          | 0.061 | *        | 5                |
| DoHDC1    | 0.196                       | 0.194                        | 0.134          | 0.846 | _        | 2                |
| PeAAD1    | 0.038                       | 0.201                        | 0.091          | 0.893 | _        | 2                |
| PeAAD2    | 0.128                       | 0.098                        | 0.088          | 0.900 | _        | 2                |
| PeGAD1    | 0.324                       | 0.135                        | 0.110          | 0.383 | _        | 2                |
| PeGAD2    | 0.406                       | 0.188                        | 0.134          | 0.243 | *        | 4                |
| PeGAD3    | 0.283                       | 0.595                        | 0.056          | 0.072 | *        | 4                |
| PeHDC1    | 0.064                       | 0.116                        | 0.219          | 0.845 | _        | 2                |

Note: Confidence levels range from 1 to 5, and 1 shows the highest predicted reliability with difference > 0.8; 2: 0.8 > difference > 0.6; 3: 0.6 > difference > 0.4; 4: 0.4 > difference > 0.2; 5: 0.2 > difference. Location: C, M, and S, represent chloroplast, mitochondrial and extracellular, respectively. _ other location, * unknown location.

2.2. Analysis of the Gene Structures, Conserved Motifs, and Phylogenetic Relationships of PLP\_deC Genes

To clarify the evolutionary relationships among the PLP\_deC genes, we compared the PLP\_deC proteins from Arabidopsis thaliana (A. thaliana), Oryza sativa (O. sativa), D. officinale and P. equestris. We used the maximum likelihood (ML) method to construct a phylogenetic tree using IQ-TREE software. As shown in Figure 1, the 42 PLP\_deC genes could be divided into three subfamilies: GAD, HDC, and aromatic-L-AAD. The PLP\_deC genes in D. officinale and P. equestris were named according to their relative homology with A. thaliana and O. sativa genes. Among them, the GAD subfamily was the largest, with 18 PLP\_deC genes, and the HDC subfamily was the smallest, with 6 members.
PeGAD3 0.283 0.595 0.056 0.072 * 4
PeHDC1 0.064 0.116 0.219 0.845 _ 2

Note: Confidence levels range from 1 to 5, and 1 shows the highest predicted reliability with difference > 0.8; 2: 0.8 > difference > 0.6; 3: 0.6 > difference > 0.4; 4: 0.4 > difference > 0.2; 5: 0.2 > difference. Location: C, M, and S, represent chloroplast, mitochondrial and extracellular, respectively. _ other location, * unknown location.

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Figure 1. Phylogenetic analysis of PLP_deC genes from Dendrobium officinale, Phalaenopsis equestris, Oryza sativa, and Arabidopsis thaliana. The maximum likelihood (ML) tree was created using IQ-TREE with 8 D. officinale (Do), 6 P. equestris (Pe), 15 O. sativa (Os) and 12 A. thaliana (At) PLP_deC protein sequences. The red, green, and blue colors indicate GADs, HDCs, and AADs, respectively. Bootstrap supports are indicated at each branch.

To further analyze the gene structures and conserved motifs of the PLP_deC family members in D. officinale and P. equestris, a total of 10 motifs were identified from the amino acid sequences of the PLP_deC family members using the Multiple EM for Motif Elicitation (MEME) software (Figure 2). We checked these motifs to verify they are known domains by pfam and the motif logos were generated using online MEME program (Figure S1). Group I, which includes GAD sequences, harbors all the motifs; group II, which includes AAD sequences, harbors motifs 5, 6, 9, and 10; and group III, which includes HDC sequences, harbors motifs 4, 5, and 10. These results show that most PLP_deC genes in the same subfamily have highly similar motifs, which supports their close evolutionary relationships and the reliability of the constructed phylogenetic tree. Remarkably, motif 10 and motif 5 are present in all subfamilies, but they are not part of the PLP_deC domain. Thus, we speculate that they may perform other specific functions. In addition, we used the online Gene Structure Display Server to analyze the gene structures. The results showed that the number of exons in PLP_deC genes ranged from 2 to 14. For example, there are 3–8 exons in subfamily GAD, 2–14 exons in subfamily AAD, and 5 or 7 exons in subfamily HDC. These results indicate that the number of exons in the PLP_deC gene family has increased or decreased during evolution, providing a basis for functional differences among the homologous PLP_deC genes (Figure S2).
were lower than 0.3, the Ka/Ks values of the three homologous pairs (PeAAD1–DoAAD1, DoAAD2–DoAAD4, PeAAD1–DoAAD3, DoAAD2–PeAAD2, PeHDC1–DoHDC1, and PeGAD2–PeGAD3) were between 0.3 and 1, and the Ka/Ks values of gene pairs (PeAAD1–DoAAD3, DoAAD2–DoAAD4, and PeGAD1–DoGAD1) were between 0.3 and 1, and the Ka/Ks value of one of the homologous pairs (PeAAD1–DoAAD3, DoAAD2–PeAAD2, PeHDC1–DoHDC1) were greater than 1 (Table 3). These data indicated that most homologous PLP_deC gene pairs are subjected to purifying selection. To further understand the influence of selection on the homologous gene pairs, we performed a sliding window analysis. The grey regions shown in Figure 3 represent the conserved domains. The Ka/Ks values for the conserved domains were typically less than 1, indicating that these pairs have purifying selection. These findings suggest that purifying selection may have played a key role in the evolution of this gene family.

Table 3. The Ka, Ks, and Ka/Ks values of gene pairs.

| Gene Pair | Ka    | Ks    | Ka/Ks |
|-----------|-------|-------|-------|
| PeGAD1    | 0.6314| 0.8256| 0.7645|
| DoGAD2    | 0.194 | 0.37  | 0.5243|
| PeAAD1    | 0.3143| 0.5474| 0.5742|
| DoAAD2    | 0.1117| 0.76  | 0.147 |
| PeHDC1    | 0.1642| 0.5726| 0.2868|
| PeGAD2    | 0.2015| 0.1384| 1.4559|
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| Gene Pair          | Ka    | Ks    | Ka/Ks |
|--------------------|-------|-------|-------|
| PeGAD1 DoGAD1      | 0.6314| 0.8256| 0.7645|
| DoGAD2 DoGAD4      | 0.194 | 0.37  | 0.5243|
| PeAAD1 DoAAD3      | 0.3143| 0.5474| 0.5742|
| DoAAD2 PeAAD2      | 0.1117| 0.76  | 0.147 |
| PeHDC1 DoHDC1      | 0.1642| 0.5726| 0.2868|
| PeGAD2 PeGAD3      | 0.2015| 0.1384| 1.4559|

2.4. D. officinale PLP_deC Gene Expression in Different Tissues

To understand the gene expression pattern of the PLP_deC genes in *D. officinale*, we performed an overall in silico analysis of gene expression profiles in eight tissues (root, root tip, stem, leaf, sepal, column, lip, and flower bud). These PLP_deC genes exhibited distinct organ-specific expression and could be divided into three groups. As shown in Figure 4, in group A, two genes (*DoAAD1* and *DoAAD2*) were highly expressed in the flower bud and lip columns, indicating that these PLP_deC genes may be involved in the development of these tissues. In addition, *DoAAD2* had a high expression level in the sepal, indicating that *DoAAD2* may be involved in sepal development. The four PLP_deC genes in group B were generally expressed in low amounts in all eight tissues. Remarkably, a homologous gene pair (*DoGAD2*–*DoGAD4*) exhibited similar patterns of expression and had relatively low transcript abundance in different tissues. In group C, *DoAAD3* had low expression in all tissues. *DoGAD3* had higher expression levels in the column and sepal. Overall, the PLP_deC genes with high expression levels may be involved in tissue growth and differentiation in *D. officinale.*
2.5. Identification of Cis-Acting Elements of PLP_deC Genes

Plant growth and development are regulated by different cis-elements in genes. Therefore, we used the PlantCARE database to identify and analyze cis-elements in the PLP_deC genes and identified three categories of cis-elements: plant growth and development, phytohormone response, and biotic and abiotic stress response. The cis-acting elements in the growth and development category included the CAT-box for meristem expression, O2-site for zein metabolic regulation, MRE and Box 4 for light response, and others. We identified 35 Box-4 motifs, which compose the largest portion of the growth and development category. All the PLP_deC genes contained Box-4 elements (Figure 5a), indicating that the expression of all these genes is closely related to light. In the phytohormone response category, TCA elements for SA response and P-boxes and TATC-boxes for gibberellic acid response were identified. Notably, TGACG motifs for MeJA response accounted for 47% of the phytohormone response category (Figure 5c), while ABRE elements for ABA response accounted for 33%. The last category was biotic and abiotic-stress-response elements, including AREs and GC motifs for anerobic response, TC-rich repeats for defence and stress response, and MBSs and LTRs for low temperature response. Our data suggested that the PLP_deC genes may respond to abiotic stresses in D. officinale.
were strongly upregulated (by more than 110-fold and 50-fold, respectively). The expression of two
PLP_deC genes (DoAAD2 and DoHDC1) reached their highest expression levels after 72 hours, and
DoAAD1 and SA treatments.

In the MeJA treatment, three PLP_deC genes (DoAAD3, DoGAD2, and DoGAD3) were strongly upregulated (by more than 250-fold) (Figure 6). In the ABA treatment, we found that DoAAD1, DoAAD2, DoAAD3, DoGAD1, DoGAD3, and DoGAD4 reached their highest expression levels after 72 hours, and DoGAD1 and DoGAD3 were strongly upregulated (by more than 110-fold and 50-fold, respectively). The expression of two PLP_deC genes (DoGAD2 and DoHDC1) peaked at 96 h, and DoGAD2 was strongly upregulated (more than 250-fold) (Figure 6). In the MeJA treatment, three PLP_deC genes (DoAAD3, DoGAD3, and DoHDC1) showed strong upregulation at 2 h (Figure 7), while five PLP_deC genes (DoAAD1, DoAAD2, DoGAD1, DoGAD2, and DoGAD4) were strongly upregulated after 4 h of treatment. In the SA treatment, the expression levels of DoAAD1, DoAAD3, DoGAD1, DoGAD2, and DoHDC1 were strongly upregulated at 2 h (Figure 8). However, DoAAD2 and DoGAD3 were strongly upregulated after treated with SA for 72 h of treatment (over 645- and 508-fold, respectively).

2.6. Analysis of the Expression Patterns of PLP_deC Genes in D. officinale

In plants, many stress responses are modulated or mediated by various signaling pathways that are inseparable from gene expression and regulation [21]. To investigate the responses of the PLP_deC genes to different hormone treatments, we used qRT-PCR to analyze their expression under MeJA, ABA, and SA treatments.

In the ABA treatment, we found that DoAAD1, DoAAD2, DoAAD3, DoGAD1, DoGAD3, and DoGAD4 reached their highest expression levels after 72 hours, and DoGAD1 and DoGAD3 were strongly upregulated (by more than 110-fold and 50-fold, respectively). The expression of two PLP_deC genes (DoGAD2 and DoHDC1) peaked at 96 h, and DoGAD2 was strongly upregulated (more than 250-fold) (Figure 6). In the MeJA treatment, three PLP_deC genes (DoAAD3, DoGAD3, and DoHDC1) showed strong upregulation at 2 h (Figure 7), while five PLP_deC genes (DoAAD1, DoAAD2, DoGAD1, DoGAD2, and DoGAD4) were strongly upregulated after 4 h of treatment. In the SA treatment, the expression levels of DoAAD1, DoAAD3, DoGAD1, DoGAD2, and DoHDC1 were strongly upregulated at 2 h (Figure 8). However, DoAAD2 and DoGAD3 were strongly upregulated after treated with SA for 72 h of treatment (over 645- and 508-fold, respectively).
**Figure 6.** The expression levels of PLP\_deC genes in *D. officinale* under abscisic acid (ABA) treatment. The x-axis represents the treatment time, and the y-axis represents the gene expression level. Error bars indicate the mean and standard deviation (SD). The asterisks indicate significant difference relative to the time 0. ** significant difference (p < 0.01), * significant difference (p < 0.05).

**Figure 7.** The expression level of PLP\_deC genes in *D. officinale* under methyl jasmonate (MeJA) treatment stress. The x-axis represents the treatment time, and the y-axis represents the gene expression level. Error bars indicate the mean and standard deviation (SD). The asterisks indicate significant difference relative to the time 0. ** significant difference (p < 0.01), * significant difference (p < 0.05).

**Figure 8.** The expression levels of PLP\_deC genes in *D. officinale* under salicylic acid (SA) treatment. The x-axis represents the treatment time, and the y-axis represents the gene expression level. Error bars indicate the mean and standard deviation (SD). The asterisks indicate significant difference relative to the time 0. ** significant difference (p < 0.01), * significant difference (p < 0.05).
3. Discussion

The type II PLP_deC enzymes are an important group of carboxylases among the PLP-dependent enzymes. Many data indicate that PLP_deCs show developmental, tissue-specific, and inducible transcript accumulation during plant development [19,22]. In this paper, we identified 8 and 6 PLP_deC genes from the D. officinale and P. equestris genomes, respectively. According to the phylogenetic analysis, all the PLP_deC genes from A. thaliana, O. sativa, D. officinale, and P. equestris were clustered into GAD, AAD, and HDC subclasses based on their high sequence similarity, which is consistent with the ML tree of PLP_deC genes from the genomes of 18 species and previously published articles [17]. However, some of these genes might have evolved with different functions. For example, in tomato, SIHDC19 and SIHDC6 do not act on histidine but prefer tyrosine as their substrate [17]. Furthermore, many HDCs are biased toward serine rather than histidine based on biochemical analysis [18]. Therefore, although their sequences have high similarity, PLP_deC genes have individual substrate specificities; we should perform an in-depth biochemical characterization to understand their precise functions [23].

Gene duplication is a common phenomenon in species and contributes to the generation of biodiversity during evolution [24]. To date, the chromosome assemblies of the D. officinale and P. equestris genomes have not yet been finished [25], and thus, the homologous genes of D. officinale and P. equestris cannot yet be clearly shown on the chromosomes. Therefore, we are unable to determine the type of replication events that have occurred between these species. To further understand the evolutionary patterns of the PLP_deC genes, we calculated the Ka and Ks values of homologous gene pairs. We predicted that two gene pairs (PeGAD1–DoGAD1 and PeAAD2–AAD2) are evolved from the genome-wide duplication events shared by D. officinale and P. equestris, because their values of Ks are 0.7 to 1.1 [26]. The Ka/Ks values in this experiment were less than 1 for all the homologous gene pairs except for PeGAD2–PeGAD3, implying that these gene pairs have undergone purifying selection during evolution. In addition, we noticed four homologous gene pairs (PeGAD1–DoGAD1, PeAAD2–AAD2, PeAAD1–DoAAD3, and PeHDC1–DoHDC1) that had the comparatively high Ka/Ks values (>0.5), showing that these gene pairs have undergone rapid evolutionary diversification after duplication events in the course of evolution [24].

Analysis of D. officinale PLP_deC gene expression in different tissues can help us better understand the tissue specificity of the PLP_deC genes. Therefore, expression profiles for all the PLP_deC genes were established using published RNA-sequence data. Among them, DoAAD1, DoAAD2, and DoGAD3 were highly expressed in different tissues, indicating that these PLP_deC genes play important roles during D. officinale growth and development. For example, GADs are involved in many cellular processes, including pollen-tube development in Arabidopsis and Picea wilsonii [25,27]. In this study, some cis-acting elements associated with particular tissues were identified in the PLP_deC gene promoter regions, such as the O2-site required for seed expression and the CAT-box required for meristem organization. The corresponding PLP_deC genes (such as DoAAD1 and DoAAD2) might play important role in the formation of reproductive organs.

Many studies have suggested that the expression levels of PLP_deC genes are also influenced by abiotic and biotic stresses [28–30]. Furthermore, plant hormones such as ABA, SA, and ethylene also modulate the expression of these genes [17,29,30]. In this study, we identified a number of cis-acting elements in the promoter regions of PLP_deC genes in both D. officinale and P. equestris, such as MBS, MRB, Box 4, and ABRE. We found that these PLP_deC genes contain at least one abiotic stress cis-element, which showed that they may contribute to abiotic and abiotic stress responses. To further investigate the responses of the PLP_deC genes to different hormones, we analyzed their expression with the treatments of MeJA, ABA, and SA by qRT-PCR. We observed that the PLP_deC genes had significantly differential expression patterns under different treatments. Some of the PLP_deC genes showed strong upregulation under the treatments, indicating that these genes play key roles in the abiotic stress responses of D. officinale. For example, DoAAD2 was strongly upregulated (645-fold) after 72 h of SA treatment. Overall, we found that the PLP_deC genes of D. officinale responded to abiotic
stress, such as MeJA, ABA, and SA stresses. These results provide strong evidence that the PLP_deC genes in plants are involved in abiotic stress responses.

4. Materials and Methods

4.1. Materials and Treatments

Seedlings of *D. officinale* were planted on Murashige and Skoog (MS) medium and placed in a tissue culture chamber at a constant temperature of 25 °C (16 h light/8 h dark) for 1 month. The tissue culture seedlings were then transferred to MS medium containing 1.0 mg/L 6-BA (biosharp, Shanghai, China), 0.1 mg/L NAA (Aladdin, Shanghai, China), and 30 g/L sucrose (Aladdin, Shanghai, China). The induced protocorms (PLBs) were supplemented with 1/2 MS liquid medium containing 0.1 mg/L α-naphthylacetic acid (NAA), 0.1 g/L whey protein hydrolysate, and 30 g/L sucrose (pH 5.8), and then cultured in darkness at 25 °C for 2 months. The PLBs were cut into 0.5 cm × 0.5 cm pellets, and 7 g of the pellet was inoculated into an Erlenmeyer flask containing 40 mL of MS medium. MeJA (100 µM methyl jasmonate; Aladdin, Shanghai, China), SA (100 µM salicylic acid; Aladdin, Shanghai, China), and ABA (100 µM abscisic acid; Aladdin, Shanghai, China) were filtered through 0.22 µm filter membrane and added to the MS medium, based on previously published articles [31]. After induction with SA and MeJA, samples were harvested at 0, 2, 4, 8, 24, 48, and 72 h. The original bulbs under ABA induction were harvested at 0, 24, 48, 72, 96, and 168 h. All samples were immediately stored at −80 °C after harvesting for RNA extraction. All results were based on three biological repeats and each biological repeat had three technical replicates. The extraction of total RNA from PLBs was carried out with Plant Total RNA Isolation Kit (Sangon Biotech, Shanghai, China) using 300 mg tissue homogenized in liquid nitrogen according to the manufacturer’s protocol, which was subsequently reverse transcribed into the first DNA strand using a One Step RT-qPCR Kit (BBI Life Science, Shanghai, China).

4.2. Screening and Identification of the PLP_deC Genes

We obtained the HMM (Hidden Markov Model) configuration file for the PLP_deC domain (Pfam00282) from Pfam (http://pfam.xfam.org/). All PLP_deC genes were then identified in the *D. officinale* and *P. equestris* genomes using HMMER software (E-value = 0.001) [32]. All candidate genes were submitted to Pfam and SMART for verifying the presence of the PLP_deC domain. The sequences that did not contain the conserved domain and the redundant sequence of the repeat were removed, and finally, the PLP_deC genes were obtained. In Table S1 are listed the sequences of the PLP_deC genes of *O. sativa* and *A. thaliana* described in previous studies (add citations) and used in the present work [17].

4.3. Sequence Attribute Analysis and Phylogenetic Tree Construction of PLP_deC Genes

To analyze the sequence attributes and characteristics of the amino acids of the PLP_deC family members, the isoelectric points (pIs) of the obtained PLP_deC amino acid sequences were determined using online analysis with the ProtParam tool (https://web.expasy.org/protparam/) [33] and subcellular localization of 8 and 6 PLP_deC-family protein sequences in *D. officinale* and *P. equestris* by Target P 1.1 (http://www.cbs.dtu.dk/services/Target P). Properties such as molecular weight (MW) were predicted. The obtained PLP_deC protein sequences were aligned using Clustal X [34], implemented in MEGA 5.0 [35], and a maximum likelihood (ML) phylogenetic tree was generated using IQ-TREE software [36] with 1000 bootstrap replicates. The PLP_deC genes were classified according to the phylogenetic relationships. If two different species of genes are located in the phylogenetic tree at the same node and the sequence similarity is more than 80%, we consider two of these are homologous genes [37]. The conserved motifs on the orchid sequences of PLP_deC were defined by MEME (http://meme-suite.org/) using the following parameters: maximum number of motifs = 10, number of repetitions—any, and only motifs with an E-value < 0.01 were retained for further analysis. The motif
logos of the PLP_deC domains were generated using online MEME program (Figure S2) [38], and GSDS was used to determine the exon–intron structure (http://gsds.cbi.pku.edu.cn/) [39].

4.4. Calculation of Ks and Ka Values of the PLP_deC Genes

The protein sequences of the gene pairs were first aligned using Clustal X 2.0, and then the multiple sequence alignments of proteins and the corresponding cDNA sequences were converted to codon alignments using PAL2NAL (http://www.bork.embl.de/pal2nal/) [40]. Finally, the resulting codon alignment was used to calculate Ks and Ka using DnaSP 5.0 (http://www.ub.edu/dnasp/) [41].

4.5. Analysis of Cis-Acting Elements of the PLP_deC Genes

A 2000 bp sequence upstream of the translation start site (ATG) of each PLP_deC gene was obtained, and an analysis of the upstream regulatory promoter elements was performed using the online tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [42].

4.6. Expression of the D. officinale PLP_deC Genes in Different Tissues

To obtain expression data for D. officinale, we searched the NCBI SRA database (PRJNA348403) for RNA-sequence data from different tissues [27] (Additional file: Table S2). The raw data were stripped of adapters and low-quality reads (and bases) and then rRNA and virus reads were filtered out by using Trimmomatic software with the default parameters. The clean reads were aligned to the D. officinale genome using Hisat2 [43] with the options -dta and -no-unal. The aligned outputs were converted from SAM to BAM format by using SAMtools [44]. Then, the Stringtie software was used to estimate the transcript abundances with FPKM method. The heat map of the PLP_deC genes expression profiles was obtained by TBtools software (https://github.com/CJ-Chen/TBtools/releases).

4.7. Quantitative Fluorescence Analysis of the PLP_deC Genes

We used the CFX96 Touch™ Real-Time PCR Detection System (Singapore) to perform a quantitative fluorescence analysis of the PLP_deC genes in the cDNA samples from D. officinale protocorms with three replicates. β-actin [45–47] was used as an internal reference, the relative expression levels of genes were calculated using the $2^{-\Delta\Delta CT}$ method [48], and the primers were designed using Primer Premier 5.0 software (Table S3). Each reaction contained the following: 8 µL of SYBR Premix Ex Taq II (2x), 2 µL of template cDNA, 1 µL of forward and reverse primers, and addition of ddH2O to a final volume of 20 µL. The reaction conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 52 °C for 15 s, and 72 °C for 30 s.

To determine whether the differential expression was significant, the difference in the relative expression of each target gene in the different treatment groups was analyzed by Student’s t-test in SPSS 25.0 software [49].

5. Conclusions

Overall, we conducted a comprehensive analysis of PLP_deC genes in both D. officinale and P. equestris. Comparative analysis has shown that eight PLP_deC genes from D. officinale could be divided into three subfamilies: GAD, HDC, and AAD. Most genes have an acidic pI. Purifying selection may have played a key role in the evolution of this PLP_deC genes in D. officinale based on the Ka/Ks value. Among them, both DoAAD1 and DoAAD2 were highly expressed in the column, flower bud, and lip. Under three hormone treatments, MeJA, ABA, and SA, the PLP_deC genes responded to abiotic stresses. These results provide preliminary biological information for further studies of the evolution of PLP_deC genes in Orchidaceae.
Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/1/54/s1.

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