INTRODUCTION

Coumarins are common plant-derived natural products that are characterized by its core structure, coumarin (1, Figure 1). These molecules exhibit various biological activities such as antibacterial (Schinkovitz et al., 2003; Stavri et al., 2003; Céspedes et al., 2006), antioxidant (Bajerova et al., 2014), anti-inflammatory (Witaicenis et al., 2013), rodenticidal (Lotfi et al., 1996), termiticidal (Schinkovitz et al., 2003; Stavri et al., 2003; Céspedes et al., 2014), and other activities (Stahmann et al., 1994; Murray, 1989; Runkel et al., 1996; Song et al., 2014). In addition, the role(s) of coumarins in plants have also been reported. Scopoletin in tobacco is accumulated during a hyper-sensitive response (Gachon et al., 2004) and is considered to be involved in virus resistance (Chong et al., 2002). In Arabidopsis thaliana, coumarins play a role as a chelator of iron ions in soil (Fourcroy et al., 2013; Schmid et al., 2013; Schmidt et al., 2014).

Based on their structural and biosynthetic properties, plant coumarins are categorized as follows: simple coumarins, furanocoumarins, and pyranocoumarins, and coumarins with modifications in the pyrone ring (Figure 1) (Keating and O’Kennedy, 1997). Simple coumarins harbor the hydroxy (-OH), alkoxy (-OR), and/or alkyl (-R) group(s) in their benzene ring: coumarin (1), umbelliferone (2: 7-hydroxycoumarin), esculetin (3: 6,7-dihydroxycoumarin), and scopoletin (4: 7-hydroxy-6-methoxycoumarin). Their hydroxy group is involved in conjugation to produce glycosides (Tal and Robeson, 1986; Taguchi et al., 2000, 2001; Shimizu et al., 2005; Kai et al., 2006; Bayoumi et al., 2008b; Wu et al., 2009). Furanocoumarins and pyranocoumarins have additional ring systems, a five- or six-member ring with an oxygen atom, which are fused to the benzene ring.

Plant researchers consider coumarins as a potential fluorescent and flavoring component (Poulton et al., 1980; Oba et al., 1981; Mock et al., 1999; Katerinopoulos, 2004; Bourgaud et al., 2006; Stanfill et al., 2007; Maggi et al., 2011; Krieger et al., 2013). Tracer experiments using cinnamate (10) or its derivatives have effectively shown that simple coumarin formation in plants proceeds via hydroxylation of the ortho-position (ortho-hydroxylation) of respective cinnamates, the adjacent position in the benzene ring to the side chain (Brown et al., 1960; Brown, 1962; Fritig et al., 1970; Bayoumi et al., 2008a), followed by formation of a lactone ring. Furanocoumarins and pyranocoumarins are derived from umbelliferone (2) by addition of prenyl group (Larbat et al., 2007; Karamat et al., 2013). 4-Hydroxycoumarin (7) in Apiaceae and Asteraceae plants is presumed to utilize another biosynthetic pathway that does not require ortho-hydroxylation (Liu et al., 2009). It has been previously suggested that coumestrol (8) in Leguminosae plants, which also comprises a coumarin core structure, is synthesized from isoflavonoids, circumventing the need for ortho-hydroxylation of cinnamates in its biosynthetic pathway (Veitch, 2013).

Due to its irreversibility, ortho-hydroxylation is considered a key step in the biosynthesis of simple coumarins. This review summarizes the research findings on ortho-hydroxylation enzymes (ortho-hydroxylases) of cinnamates that are involved in simple coumarin biosynthesis. The distribution of the ortho-hydroxylases in plants using a database search of EST homologs will be also discussed.

2-OXoglutarate-dependent dioxygenases involved in the biosynthesis of simple coumarins are the key enzymes of simple coumarin biosynthesis

In Arabidopsis, a 2-oxoglutarate-dependent dioxygenase (2OGD) encoded by the gene AtF6'H1 (locus: At3g13610) was found to exhibit ortho-hydroxylase activity to feruloyl coenzyme A (15: feruloyl-CoA) as a substrate, with a \( K_m \) value of 36 \( \mu \)M, yielding an ortho-hydroxylation product, 6-hydroxyferuloyl-CoA (19) (Kai et al., 2008). The AtF6'H1 enzyme exhibits no catalytic activity to \( p \)-coumaroyl-CoA (14), free ferulic acid (13), or feruloyl...
Ortho-hydroxylases involved in simple coumarins

**FIGURE 1 | Coumarin biosynthetic pathway in plants.** Simple coumarins, coumarin (1), umbelliferone (2), esculetin (3), and scopoletin (4) have modifications in their benzene ring. They are biosynthesized from the phenylpropanoid pathway via ortho-hydroxylation of cinnamate (10), p-coumarate (11), caffeate (12), and ferulate (13), respectively. The ortho-positions are shown by red arrows. Oxygen atoms introduced by ortho-hydroxylation are also highlighted in red. The ortho-hydroxylases from Arabidopsis (AtF6′H1), Ruta graveolens (RgC2′H), and Ipomoea batatas (Ib1 and Ib2) were functionally analyzed. AtF6′H1 and Ib1 catalyze ortho-hydroxylation of feruloyl-CoA (15), whereas RgC2′H and Ib2 were capable of reacting to both feruloyl-CoA (15) and p-coumaroyl-CoA (14) as the substrates. After hydroxylation, trans/cis isomerization and lactonization occur, resulting in the production of their respective coumarins. Umbelliferone (2) is a key intermediate of prenylcoumarin biosynthesis, from which furanocoumarins and pyranocoumarins (examples: psoralen and xanthyletin, respectively) are derived. No report has described cloning and functional analysis of the hydroxylases that introduce an ortho-hydroxy group to cinnamate and caffeate to form coumarin (1) and esculetin (3), respectively (hashed arrows). Coumarins substituted in the pyrone ring are thought to be derived from different pathways.

Quinate. Deficient mutation of the AtF6′H1 gene in Arabidopsis causes a significant reduction in the accumulation of scopolin, a β-glucoside of scopoletin (4), indicating that AtF6′H1 catalyzes ortho-hydroxylation. Another 2OGD (AtF6′H2) encoded by a homologous gene (locus: At1g55290) exhibits an equivalent activity against CoA thioesters of cinnamates (K_m value for feruloyl-CoA: 14.5 µM); however, no significant change in scopolin levels was observed in the plant.
Further studies involving cloning and functional analysis of the 2OGD genes in plants have elucidated the mechanism of coumarin formation. Using Ruta graveolens, which accumulates franco-coumarins, a 2OGD (RgC2′H) was cloned as the key enzyme of coumarin biosynthesis (Vialart et al., 2011). RgC2′H shows hydroxylation activity not only to feruloyl-CoA (15, $K_m = 37 \mu M$), but also to $p$-coumaryl-CoA (14, $K_m = 50 \mu M$), forming scopoletin (4) and umbelliferone (2), respectively. Furano-coumarins are formed after addition of prenyl group to umbelliferone (2), which is detected in R. graveolens, whereas no scopoletin (4) was detected. This result indicates that RgC2′H exclusively catalyzes $p$-coumaryl-CoA (14), besides its activity against feruloyl-CoA (15) and $p$-coumaryl-CoA (14). Regulation of substrate supply to RgC2′H enzyme is likely to determine the structures of the products, namely, umbelliferone (2) or scopoletin (4).

The biosynthetic origin of the 1-oxygen atom of umbelliferone (2) in sweet potato root (Ipomoea batatas) is molecular oxygen; therefore, hydroxylase using a water molecule to introduce a hydroxy group was excluded as the candidate of ortho-hydroxylase enzyme(s) (Shimizu et al., 2008). 2OGDs from sweet potato were also cloned and functionally analyzed as the ortho-hydroxylases of CoA thioesters of the cin-namates (Matsumoto et al., 2011). The 2OGDs were then categorized into two groups based on their substrate specificities. Enzymes belonging to the first one, designated as Ib1s, showed ortho-hydroxylation activity to feruloyl-CoA (15, $K_m = \text{approximately} \ 10 \mu M$), whereas those of Ib2s catalyzed both $p$-coumaryl-CoA (14, $K_m = 7.3–14 \mu M$) and feruloyl-CoA (15, $K_m = 6.1–15.2 \mu M$) as the substrates to yield umbelliferone (2) and scopoletin (4), respectively. Root tissues of sweet potato accumulate moderate levels of scopoquin. After fungal and elicitor treatments, the production of umbelliferone (2) is still unknown, whereas approaches to biosynthesis of coumarin (1) have been performed using sweet clover (Gestetner and Conn, 1974) and lavender (Brown et al., 1960; Stoker and Bellis, 1962). Esculetin (3) formation is also remained to be elucidated. Ib1s from sweet potato showed a trace activity to caffeoyl-CoA (Matsumoto et al., 2011). Therefore, catalysis of these reactions by members of the 2OGD family is reasonable using cinnamate (10) or caffeate (12) esters, or their free acid, respectively. Enzymatic information of ortho-hydroxylase homologs would tell mechanism of these coumarins. There is still a possibility that other enzyme families such as flavin monoxygenases or another oxidase family would also contribute to this reaction (Schlaich, 2007). Furthermore, in cassava or chicory, modification steps involving the conversion of umbelliferone (2) to esculetin (3) or daphnetin (20; 7,8-dihydroxycoumarin) have been detected by tracer analysis, indicating a biosynthetic grid of simple coumarin formation (Sato and Hasegawa, 1972; Bayoumi et al., 2008a).

Although the details of the biosynthesis of simple coumarins are still unclear, the three examples of ortho-hydroxylases serve as key information for future researches on elucidating the mechanism of coumarin biosynthesis in plants. Substrate specificities of the ortho-hydroxylases from plants that accumulate coumarins will be also clue to know the metabolic grid of coumarin biosynthesis.

**QUEST FOR THE CANDIDATE SEQUENCES OF ORTHO-HYDROXYLASES IN PLANTS**

The substitution patterns involving the phenyl group of cin-namates have been extensively characterized. Furthermore, the CoA moiety is a prerequisite for their activity. The alignment of the amino acid sequences of previously reported ortho-hydroxylases is presented in Figure 2, which shows a moderately high sequence identity (approximately 59–64% amino acid iden-ty), with conserved amino acid residues. Investigation of sub-strate specificities of 2OGDs using chimeric proteins revealed the significance of C-terminal sequence elements of gibberellin 20-oxidases of Cucurbita maxima (Lange et al., 1997) and flavonane$3\beta$-hydroxylase of Petunia sp. (Wellmann et al., 2004). They reported that the C-terminal sequences comprising 33–54 amino acid residues are involved in substrate recognition.

Taking advantage of these results, a TBLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997) was performed to explore candidate EST sequences of ortho-hydroxylases involved in the biosynthesis of simple coumarins, using the C-terminal sequences of AtF6′H1 (54 amino acid residues, Supplementary Material 1).

The results (maximum target sequences: 1000; Supplementary Material 2) showed that the hit sequences belonged to the 2OGD family, with maximum scores within the range of 42–111 and minimum E-values within the range of $1 \times 10^{-27}$–$1 \times 10^{-2}$. The highest scoring hits were observed in the Brassicaceae plants. Although it was necessary to analyze the accumulation of simple coumarins, these clones would show ortho-hydroxylase activity, thus indicating its involvement in simple coumarin formation. Plant species belonged to Spindales, Malvales, Malpighiales, Fabales, Rosales, FAGales, Vitales, Solanales, Lamiales, Gentianales, and Asteriales also showed significantly high scores and low E-values, whereas other plant species with 2OGD sequences were of relatively lower levels of similarity. In plants that accumulate simple coumarins, 2OGDs with higher levels of similarity are likely to exhibit ortho-hydroxylase activity. In Fabales, Lotus japonicus, Gly cine max, Vigna unguiculata, and Medicago truncatula harbored ESTs with highly similar sequences. Coumarin is accumulated in Melilotus alba, a Fabales
Shimizu Ortho-hydroxylases involved in simple coumarins

FIGURE 2 | Comparison of amino acid sequences of ortho-hydroxylases from the plants. Amino acid sequences are aligned using ClustalW2 (McWilliam et al., 2013, http://www.ebi.ac.uk/Tools/msa/clustalw2/). A FASTA file of the protein sequences is available as Supplementary Material 3.
plant (Brown et al., 1960; Stoker and Bellis, 1962; Gestetner and Conn, 1974). These EST sequences in Fabales plants could serve as clues in the search for ortho-hydroxylases in cinnamate (10) from M. alba. In addition, sequences from Euphorbia spp. or Manihot esculenta, which accumulate esculetin (Masamoto et al., 2003; Bayoumi et al., 2008a; Nazemiyeh et al., 2009; Shi et al., 2009), showed high similarities. The biosynthetic pathway of simple coumarins containing esculetin in these plants would be elucidated through the functional analysis of these sequences. Species from the rest of the orders were less similar to the partial sequence of AtF6′H1.

Kawai et al. (2014) conducted an extensive phylogenetic analysis of 2OGD sequences, where the ortho-hydroxylases involved in simple coumarin biosynthesis belonged to DOXC30-clade. These enzymes were not detected in Oryza sativa or other vascular plants that arose from more basal lineages (Stevens, 2014). There is no report about coumarin accumulation in O. sativa. The tendency decrease in the level of similarity in the EST sequences supports the results of the present study; therefore, it is unlikely that the hit sequences showing less similarity than that of O. sativa (max score: 45; minimum E-value: 2 e−4) exhibited ortho-hydroxylation of cinnamates to form simple coumarins. However, the boundary line dividing the ortho-hydroxylase sequence involved in simple coumarin biosynthesis and the other 2OGDs remains unclear. Liriodendron tulipifera, a Magnoliales plant that arose from a more basal lineage than monocots, accumulates scopoletin (4) (Kang et al., 2014). Cinnamomum cassia, which is Laureales plant, also contains coumarin (1) (Choi et al., 2001). However, no significant similarity in the C-terminal sequence of AtF6′H1 was observed by TBLASTN search for ESTs in Magnoliales and Laureales plants. An unknown biosynthetic pathway of simple coumarins without 2OGD enzymes perhaps exists in plants.

Candidates of ortho-hydroxylases are mainly distributed in dicots, indicating that the biosynthesis of simple coumarins is a newer pathway of plant secondary metabolism, compared to flavonoids, which extensively occur in the plant kingdom (Harborne and Baxter, 1999; Williams and Grayer, 2004). Furthermore, biosynthetic pathways comprising apparently different enzyme sets evolutionally converged to form the coumarin core structure. Further analysis of plant ortho-hydroxylases at the molecular level would provide more details on the evolution of plant coumarins.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00549/abstract

Supplementary Material 1 | The C-terminal sequences of the ortho-hydroxylases involved in biosynthesis of simple coumarins.

Supplementary Material 2 | Results of TBLASTN search in EST sequences.

Supplementary Material 3 | FASTA file of the protein sequences.

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