Functional Analysis of Arabidopsis CYP714A1 and CYP714A2 Reveals That They are Distinct Gibberellin Modification Enzymes

Takahito Nomura, Hiroshi Magome, Atsushi Hanada, Noriko Takeda-Kamiya, Lewis N. Mander, Yuji Kamiya, and Shinjiro Yamaguchi

Endogenous levels of bioactive gibberellins (GAs) are controlled by both biosynthetic and inactivation processes, and some cytochrome P450s are involved in this control mechanism. We have previously reported that CYP714B1 and CYP714B2 encode the enzyme GA 13-oxidase, which is required for GA1 biosynthesis, and that CYP714D1 encodes GA 16α,17-epoxidase, which inactivates the non-13-hydroxy GAs in rice. Arabidopsis has two CYP714 members, CYP714A1 and CYP714A2. To clarify the possible role of these genes in GA metabolism, enzymatic activities of their recombinant proteins were analyzed using a yeast expression system. We found that the recombinant CYP714A1 protein catalyzes the conversion of GA12 to 16-carboxylated GA12 (16-carboxy-16β,17-dihydro GA12), a previously unidentified GA metabolite. Bioassays of this GA product showed that CYP714A1 is an inactivation enzyme in Arabidopsis. This was confirmed by the extreme GA-deficient dwarf phenotype shown by CYP714A1-overexpressing plants. Intriguingly, the recombinant CYP714A2 protein catalyzed the conversion of ent-kaurenoic acid into steviol (ent-13-hydroxy kaurenolic acid). When GA12 was used as a substrate for CYP714A2, 12α-hydroxy GA12 (GA111) was produced as a major product and 13-hydroxy GA12 (GA13) as a minor product. Transgenic Arabidopsis plants overexpressing the CYP714A2 gene showed semi-dwarfism. GA analysis showed that the levels of non-13-hydroxy GAs, including GA4, were decreased, whereas those of 13-hydroxy GAs, including GA1 (which is less active than GA4), were increased in the transgenic plants. Our results suggest that the CYP714 family proteins contribute to the production of diverse GA compounds through various oxidations of C and D rings in both monocots and eudicots.

Keywords: Arabidopsis • Biosynthesis • cytochrome P450 • Gibberellin • Inactivation.

Abbreviations: 16-carboxy GA12, 16-carboxy-16β,17-dihydro GA12; 2ODD, 2-oxoglutarate-dependent dioxygenase; CaMV, Cauliflower mosaic virus; CPS, ent-copalyl diphasphate synthase; eui, elongated uppermost internode; GA13ox, GA 13-oxidase; GA2ox, GA 2-oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 3-oxidase; GAMT, GA methyltransferase; GC-MS, gas chromatography–mass spectrometry; GID1, GIBBERELLIN INSENSITIVE1; KAO, ent-kaurenoic acid oxidase; KO, ent-kaurene oxidase; MS, Murashige-Skoog; PAC, paclobutrazol; RNAi, RNA interference; RT-PCR, reverse transcription PCR; SEM, standard error of the mean; WT, wild type.

Introduction

Gibberellins (GAs) are a class of diterpenoid hormones that promote growth in various stages of plant lifecycle: seed germination, stem elongation, leaf expansion and flowering. GAs are biosynthesized from geranylgeranyl diphosphate (C20) via a multistep process (Fig. 1; reviewed by Yamaguchi 2008). More than 130 GA compounds have been identified, but only a few, such as GA3, GA4, GA7, and GA1, are known to be the bioactive forms. Arabidopsis mutants defective in GA biosynthesis show dwarfism, dark-green leaves and late flowering phenotypes (Fleet and Sun 2005). In Arabidopsis, GA4, a non-13-hydroxylated bioactive GA, is predominant in vegetative tissue, and is synthesized through the non-13-hydroxylation pathway (Fig. 1). GA1, a 13-hydroxylated bioactive GA, is synthesized from the early GA 13-hydroxylation pathway. The
Fig. 1 GA biosynthesis and inactivation pathways in Arabidopsis. Solid and dashed lines indicate GA biosynthesis and inactivation (2β-hydroxylation) pathways, respectively. GGDP, geranylgeranyl diphosphate; CPS, ent-kaurene synthase; KS, ent-kaurene synthase; KO, ent-kaurene oxidase; KAO, ent-kaurenoic acid oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 3-oxidase; GA2ox, GA 2-oxidase; GA13ox, GA 13-oxidase.

concentration of GA1 is very low in vegetative tissues, but is higher in developing siliques (Varbanova et al. 2007, Kanno et al. 2010). GA1 is less active than GA4 both in Arabidopsis (Talon et al. 1990, Yang et al. 1995, Cowling et al. 1998) and in rice (Ueguchi-Tanaka et al. 2007), and this difference is presumably attributable to the binding affinity of these hormones to the GA receptor, GIBBERELLIN INSENSITIVE1 (GID1) (Ueguchi-Tanaka et al. 2005, Nakajima et al. 2006).

Recent studies revealed that multiple GA inactivation pathways are important for controlling endogenous GA levels in flowering plants (Yamaguchi 2008). Conjugation of glucose, resulting in the formation of glucose ethers or glucose esters, has been found in a number of plant species. This might be an inactivation reaction, but no GA-glycosyl transferase gene has been identified yet (Schneider and Schliemann 1994). So far, three classes of GA inactivation enzyme genes have been identified: 2-oxoglutarate-dependent dioxygenase (2ODD), methyltransferase and cytochrome P450 monooxygenase. GA 2-oxidase (GA2ox), which encodes 2ODD, converts bioactive and intermediate forms of GAs to inactive forms by 2β-hydroxylation (Fig. 1; Schomburg et al. 2003, Thomas et al. 1999). Thus far, seven GA2ox (candidate) genes (GA2ox1-4 and -6-8; GA2ox5 is a pseudogene) have been reported in Arabidopsis (reviewed by Yamaguchi 2008). Given the presence of orthologous GA2ox genes in a number of plant species, such as rice (Sakamoto et al. 2001), pea (Lester et al. 1999, Martin et al. 1999) and poplar (Busov et al. 2003), this inactivation is most common in flowering plants. The GA methyltransferase genes (GAMT1 and GAMT2) have been identified in Arabidopsis (Varbanova et al. 2007). These genes are mainly expressed in developing and germinating seeds. The gamt1 and gamt2 double mutant exhibits elevated levels of bioactive GAs in siliques and shows resistance to a GA biosynthesis inhibitor, ancymidol, during germination. The rice cytochrome P450 monoxygenase CYP714D1 (EUI), represents a novel class of GA inactivation enzyme, which catalyzes 16β,17-epoxidation of non-13-hydroxy GAs (GA12, GA9 and GA4) (Zhu et al. 2006). The EUI gene was identified by map-based cloning of the recessive rice mutant, elongated uppermost internode (eu1). The mutant plants are morphologically normal until the drastic final internode elongation at the heading stage. Our GA analysis showed that exceptionally large amounts of the bioactive GAs GA4 and GA1 are accumulated in the uppermost internode of the eu1 mutant (Zhu et al. 2006). In contrast, overexpression of the P450 gene resulted in severe dwarfism in rice due to GA deficiency (Luo et al. 2006, Zhu et al. 2006).

Recently we reported that other rice CYP714 members, CYP714B1 and CYP714B2, encode GA 13-oxidase (GA13ox), a key enzyme of the early GA 13-hydroxylation pathway (Fig. 1; Magome et al. 2013). The cyp714b1 cyp714b2 double mutants showed decreased levels of 13-hydroxy GAs, including GA1, but increased levels of non-13-hydroxy GAs, including GA4. Like eu1 plants, the double mutant plants had elongated uppermost internodes at the heading stage. Our findings on the function of rice CYP714 members suggest that CYP714 proteins may also be involved in GA metabolism in other plant species. Arabidopsis has two CYP714 members, CYP714A1 and CYP714A2, and it has been reported earlier that CYP714A1- or CYP714A2-overexpressing Arabidopsis plants show a severe dwarf phenotype (Zhang et al. 2011). Moreover, CYP714A1 and CYP714A2 double knockout plants show increased above-ground biomass and early flowering with an increased level of bioactive GA4. Based on these results, a possible functional overlapping of the CYP714A1 and CYP714A2 proteins in GA inactivation was proposed (Zhang et al. 2011). However, their enzymatic functions have not yet been identified. In this study, we investigated the possible role of these proteins in GA metabolism using a heterologous yeast expression system. We found that CYP714A1 encodes a GA deactivation enzyme, which catalyzes 16-carboxylation of GA12. Intriguingly, we found that CYP714A2 acts as a 13-oxidase or 12α-oxidase of GAs or GA precursors, depending on the substrate. GA analysis of Arabidopsis plants that overexpress CYP714A2 showed that CYP714A2 could contribute to GA 13-hydroxylation in planta, indicating a functional kinship with rice CYP714B. Possible roles of CYP714A1 and CYP714A2 in GA metabolism are discussed.
Results

Functional analysis of the CYP714A1 and CYP714A2 proteins in a yeast expression system

We have previously shown that CYP714D1/EUI encodes the enzyme GA 16α,17-epoxidase, which inactivates the non-13-hydroxy GAs in rice (Zhu et al. 2006). More recently, we have reported that CYP714B1 and CYP714B2 genes encode GA13ox in rice (Magome et al. 2013). In Arabidopsis the CYP714A1 and CYP714A2 proteins share 73.1% identity in their amino acid sequences, and both are distantly related to rice CYP714Bs and CYP714D1 proteins (38.5–47.6%) (Supplementary Table S1). To clarify the possible function of these cytochrome P450s in GA metabolism, enzymatic activities of recombinant CYP714A1 and CYP714A2 proteins were analyzed using a yeast expression system. We first analyzed the enzymatic activity by incubating a microsomal fraction of CYP714A1 or CYP714A2-expressing yeast with various GAs as putative substrates, as we did for CYP714D1 (Zhu et al. 2006), but only small amounts of putative products were obtained in all cases. Such a reduced or loss of activity of plant P450s in the yeast microsomal fraction has been reported previously (Helliwell et al. 1999, Nomura et al. 2005). We therefore added various GAs (GA 12, GA9, GA4, GA53, GA20 and GA1) to a liquid culture of CYP714A1- or CYP714A2-expressing yeast cells, and the products were analyzed by full-scan gas chromatography–mass spectrometry (GC-MS).

Recombinant CYP714A1 protein catalyzes 16-carboxylation of GA12

Among the GA compounds mentioned above, GA12 (1) (bold numbers in parentheses refer to compounds indicated in Supplementary Fig. S1) was clearly metabolized by the CYP714A1-expressing yeast cells. A large fraction of the added GA12 was metabolized to a predominant product (designated metabolite A), whereas no conversion occurred when the control yeast carrying the empty vector was used (Fig. 2). Metabolite A was subsequently identified to be a 16-carboxylated GA12 (16-carboxy-16β,17-dihydro GA12, designated in this paper as 16-carboxy GA12, 4) by the retention time on GC and the mass spectrum listed in Table 1.

![Image](https://example.com/image.png)

**Fig. 2** Recombinant CYP714A1 protein has GA12 16-carboxylation activity. Shown are total ion chromatograms (mass-to-charge ratio 50 to 600) of GA12 (upper, methyl ester derivative, indicated by an open triangle) and metabolite A (lower, methyl ester derivative, indicated by a closed triangle) after incubation of GA12 with control (empty vector) or CYP714A1-producing yeast culture. Metabolite A was identified as 16-carboxy GA12 (16-carboxy-16β,17-dihydro GA12, 4) by the retention time on GC and the mass spectrum listed in Table 1.

<table>
<thead>
<tr>
<th>Metabolite and reference sample</th>
<th>Retention time on GC (KRI)</th>
<th>Characteristic ions, m/z (relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA12, metabolite A(^a)</td>
<td>2641</td>
<td>406 [M(^+)] (3), 374 (100), 359 (16), 342 (15), 314 (46), 287 (60), 259 (56), 227 (84), 199 (93)</td>
</tr>
<tr>
<td>Authentic 16-carboxy-16β,17-dihydro GA12(^e)</td>
<td>2640</td>
<td>406 [M(^+)] (3), 374 (100), 359 (16), 342 (15), 314 (47), 287 (61), 259 (55), 227 (85), 199 (93)</td>
</tr>
<tr>
<td>Authentic 16-carboxy-16α,17-dihydro GA12(^e)</td>
<td>2677</td>
<td>406 [M(^+)] (4), 374 (100), 359 (19), 342 (9), 314 (40), 287 (69), 259 (60), 227 (74), 199 (91)</td>
</tr>
<tr>
<td>GA12, metabolite B(^d)</td>
<td>2798</td>
<td>523 [M(^+)-15(^-)] (1), 435 (100), 375 (27), 315 (4), 285 (5), 239 (6), 225 (7), 147 (28), 73 (75)</td>
</tr>
<tr>
<td>Authentic 16,17-dihydroxy-16α,17-dihydro GA12(^d)</td>
<td>2796</td>
<td>523 [M(^+)-15(^-)] (2), 435 (100), 375 (29), 315 (4), 285 (6), 239 (9), 225 (9), 147 (28), 73 (80)</td>
</tr>
<tr>
<td>GA12, metabolite C(^d)</td>
<td>2658</td>
<td>450 [M(^+)] (1), 435 (24), 418 (42), 403 (9), 328 (37), 300 (100), 287 (18), 259 (28), 241 (95), 73 (100)</td>
</tr>
<tr>
<td>GA12, metabolite D(^d)</td>
<td>2736</td>
<td>464 [M(^+)] (28), 449 (64), 432 (22), 389 (85), 357 (14), 329 (31), 301 (26), 223 (61), 73 (100)</td>
</tr>
</tbody>
</table>

\(^a\)Kovats retention index.
\(^b\)m/z, mass-to-charge ratio (M\(^+\), molecular ion).
\(^c\)Methyl ester derivative.
\(^d\)Methyl ester-trimethylsilyl ether derivative.

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was determined to be 16,17-dihydroxy-16\(\alpha\),17-dihydro GA\(_{12}\) (S), which was likely converted from 16\(\alpha\),17-epoxy GA\(_{12}\) in the presence of acetic acid during purification, as previously reported (Zhu et al. 2006). Metabolite C appeared to be 17-hydroxy-16\(\alpha\),17-dihydro GA\(_{12}\) (6) (Gaskin and Macmillan 1991), whereas metabolite D was unknown. GC-MS analysis of other GA compounds added as substrates showed no conversion, with the exception of GA\(_{9}\) (2), which was converted to a putative oxidized product (16\(\alpha\),17-dihydroxy-16,17-dihydro GA\(_{9}\), 3) and two unknown products (data not shown).

When we used [17,17-\(^2\)H\(_2\)] GA\(_{12}\) as a substrate for CYP714A1, the product, 16-carboxy GA\(_{12}\) (4) carried a deuterium after derivatization to methyl esters, although there is no deuterium (hydrogen) at C17 (data not shown). Previous studies have shown that aldehydes RCH\(_2\)CHO are formed in addition to epoxides upon oxidation of the olefins RCH = CH\(_2\) by rat liver microsomes in the presence of NADPH and O\(_2\) (Mansuy et al. 1984). These aldehydes were not derived from the rearrangement of the epoxide, but are formed by the migration of a hydrogen within an intermediate with the active oxygen–iron complex. [16-\(^2\)H] 16-carboxy GA\(_{12}\) was produced from [17,17-\(^2\)H\(_2\)] GA\(_{12}\) by a similar mechanism, which might then have been further oxidized to form [16-\(^3\)H] 16-carboxy GA\(_{12}\). We have used this labeled product as an internal standard to quantify endogenous ones in plants (see below).

**Recombinant CYP714A2 protein has ent-kaurenoic acid 13-hydroxylation and GA\(_{12}\) 12\(\alpha\)-hydroxylation activity**

Next we examined the enzymatic activity of CYP714A2. Among the various GAs described above, only GA\(_{12}\) (1) was converted by the CYP714A2-expressing yeast culture; a large fraction of the added substrate was metabolized to a predominant product (metabolite E) (Fig. 3A). This metabolite was identified as 12\(\alpha\)-hydroxy GA\(_{12}\) (GA\(_{111}\), 13) by comparison of the retention time on GC and the mass spectrum with that of an authentic sample (Table 2, Supplementary Fig. S1). We also found two minor products (F and G) (Fig. 3A, Table 2). Intriguingly, product F was identified as 13-hydroxy GA\(_{12}\) (GA\(_{53}\), 14), while product G was identified as 12\(\alpha\),13-dihydroxy GA\(_{12}\) (GA\(_{129}\), 16) by comparison with respective authentic samples on GC-MS. The ratio of these GA\(_{12}\) products was 100 (12\(\alpha\)-hydroxy, 13); 6 (13-hydroxy, 14); 6 (12\(\alpha\),13-dihydroxy, 16), calculated based on the relative intensity on the total ion chromatogram. The conversion of GA\(_{12}\) (1) to GA\(_{53}\) (14) by 13-hydroxylation is believed to be a key step of the early GA 13-hydroxylation pathway (Fig. 1) (Magome et al. 2013). But as shown above, 13-hydroxylation activity of the recombinant CYP714A2 protein was low when GA\(_{12}\) (1) was used as a substrate. This result implies the presence of other substrates more preferential than GA\(_{12}\) (1) for the 13-hydroxylation activity. Therefore, we tested three upstream precursors (ent-kaurenoic acid, 7; ent-7\(\alpha\)-hydroxy kaurenoic acid, 8; and GA\(_{12}\)-7-aldehyde, 9) of GA\(_{12}\) (1) for this assay. When ent-kaurenoic acid was added as a substrate, steviol (ent-13-hydroxy kaurenoic acid, 10) was detected as the sole product (metabolite H) (Fig. 3B, Table 2). In addition, another metabolite, which is probably ent-7\(\alpha\),13-dihydroxy kaurenoic acid (11) (Gaskin and Macmillan 1991), was detected as the sole product of ent-7\(\alpha\)-hydroxy kaurenoic acid metabolism (B) (Supplementary Table S2). When GA\(_{12}\)-7-aldehyde (9) was added as a substrate, a large amount of 12\(\alpha\)-hydroxylated (15) and a small amount of 13-hydroxylated (12) derivatives (Gaskin and Macmillan 1991) were detected as probable metabolites (Supplementary Table S2, Supplementary Fig. S1), as was the case with the
We also tested the bioactivity of 16-carboxy GA12 (ent-bifunctional enzyme that preferentially catalyzes the C-13 hydroxylation of the ent-kaurane carbon skeleton (ent-kauranol, 7; ent-7α-hydroxy kaurenoic acid, 8) and the C-12 hydroxylation of the ent-gibberellane carbon skeleton (GA12-7-aldehyde, 9; GA12, 1)).

Table 2 GC-MS profiles of GA12 and ent-kaurenoic acid metabolites by recombinant CYP714A2 protein

<table>
<thead>
<tr>
<th>Metabolite and reference sample</th>
<th>Retention time on GC (KRI)</th>
<th>Characteristic ions, m/z (relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA12 metabolite E</td>
<td>2560</td>
<td>448 <a href="2">M+</a>, 416 (24), 388 (16), 298 (34), 239 (55), 209 (65), 207 (76), 181 (45), 180 (47), 73 (100)</td>
</tr>
<tr>
<td>Authentic 12z-hydroxy GA12</td>
<td>2560</td>
<td>448 <a href="2">M+</a>, 416 (29), 388 (19), 298 (45), 239 (65), 209 (69), 207 (78), 181 (50), 180 (49), 73 (100)</td>
</tr>
<tr>
<td>Authentic 12β-hydroxy GA12</td>
<td>2580</td>
<td>448 <a href="3">M+</a>, 416 (30), 388 (20), 298 (42), 239 (51), 209 (68), 207 (78), 181 (46), 180 (46), 73 (100)</td>
</tr>
<tr>
<td>GA12 metabolite F</td>
<td>2517</td>
<td>448 <a href="26">M+</a>, 416 (6), 389 (10), 251 (17), 235 (16), 208 (81), 207 (100), 193 (24), 181 (60), 73 (64)</td>
</tr>
<tr>
<td>Authentic GA5</td>
<td>2518</td>
<td>448 <a href="21">M+</a>, 416 (6), 389 (8), 251 (17), 235 (16), 208 (78), 207 (100), 193 (24), 181 (62), 73 (72)</td>
</tr>
<tr>
<td>GA12 metabolite G</td>
<td>2643</td>
<td>536 <a href="23">M+</a>, 504 (7), 477 (9), 433 (25), 420 (14), 251 (17), 193 (62), 181 (55), 147 (30), 73 (100)</td>
</tr>
<tr>
<td>Authentic 12z,13-dihydroxy GA12</td>
<td>2643</td>
<td>536 <a href="22">M+</a>, 504 (7), 477 (10), 433 (24), 420 (14), 251 (16), 193 (65), 181 (56), 147 (31), 73 (100)</td>
</tr>
</tbody>
</table>

ent-Kaurenoic acid metabolite H 4 2473 404 [M+] (9), 389 (3), 348 (3), 214 (8), 193 (100), 180 (7), 73 (58)

Authentic steviol (ent-13-hydroxy kaurenoic acid) 2473 404 [M+] (9), 389 (3), 348 (3), 214 (8), 193 (100), 180 (6), 73 (48)

* Kovats retention index.
* m/z, mass-to-charge ratio (M+, molecular ion).
* Methyl ester-trimethylsilyl ether derivative.

GA12 substrate. These results suggest that CYP714A2 is a bifunctional enzyme that preferentially catalyzes the C-13 hydroxylation of the ent-kaurane carbon skeleton (ent-kaurenoic acid, 7; ent-7α-hydroxy kaurenoic acid, 8) and the C-12 hydroxylation of the ent-gibberellane carbon skeleton (GA12-7-aldehyde, 9; GA12, 1).

Biological activity of CYP714A1 and CYP714A2 products

To clarify the role of CYP714As in Arabidopsis, we examined the biological activity of the products of these enzymes using the Arabidopsis ga1-3 mutant (Table 3). The ga1-3 mutant has a null mutation in the ent-copalyl diphosphate synthase (CPS) gene in an early step of the GA biosynthesis pathway, resulting in the inability of the seeds to germinate without exogenous treatment with GA (Sun and Kamiya 1994). Due to their commercial unavailability, we used 16-carboxy GA12 (4) and 12α-hydroxy GA12 (13) prepared enzymatically in this assay (see Materials and Methods). We found that the bioactive GA4 was the most effective (100% germination with 1 μM GA4) in inducing ga1-3 seed germination, and GA1, a 13-hydroxylated bioactive GA, was effective to a lesser extent (32% germination with 1 μM GA1). Treatment with 1 μM GA12 (1), a common substrate for CYP714A1 and CYP714A2, promoted 74.3% germination. In contrast, treatment with 16-carboxy GA12 (4), which is a product of CYP714A1, and 12α-hydroxy GA12 (13) and GA3 (14), which are products of CYP714A2, hardly restored the non-germination phenotype of the ga1-3 mutant. Similarly, steviol (10) exhibited weaker germination-inducing activity than did ent-kaurenoic acid (7) (Table 3). We also tested the bioactivity of 16-carboxy GA12 (4) and 12α-hydroxy GA12 (13) on the effect of rosette growth of the ga1-3 mutant, and found that these GA compounds hardly restore growth, unlike GA12 (Supplementary Table S3). These results suggest that CYP714A1 and CYP714A2 have the potential to play a role in reducing GA activity in Arabidopsis.

Overexpression of CYP714A1 causes extreme dwarfism, whereas that of CYP714A2 causes semi-dwarfism in Arabidopsis

The results from biological activity studies described above indicated that CYP714A1 and CYP714A2 potentially encode distinct GA inactivation enzymes. To further understand the role of these enzymes, we generated transgenic Arabidopsis plants that overexpressed CYP714A1 or CYP714A2 under the control of the Cauliflower mosaic virus (CaMV) 35S promoter. We found that the majority of CYP714A1-atOE lines exhibited extreme dwarfism that resembled that of ga1-3, while CYP714A2-atOE plants showed semi-dwarfism (Fig. 4A, B). Their dwarfism was restored by exogenously applied GA4 (Fig. 4C). Both CYP714A1-atOE and CYP714A2-atOE showed a late-flowering phenotype, as seen in known GA biosynthesis mutants, including ga1-3, under constant light (Fig. 4B). The CYP714A2-atOE plants retained their fertility under the normal growth condition, whilst the CYP714A1-atOE plants needed exogenous application of GA4 to restore their fertility (data not shown). We analyzed transcripts of CYP714A1-atOE and CYP714A2-atOE, and confirmed high accumulation [over 1000-fold compared with wild type (WT) of each transcript of the transgene] (Supplementary Fig. S2). Expression of the GA biosynthesis genes GA20ox1 (GA 20-oxidase1) and GA3ox1 (GA 3-oxidase1), both of which are negatively regulated by GA.
activity (Xu et al. 1995, Cowling et al. 1998), was highly induced in both overexpressor plants (Supplementary Fig. S2).

CYP714A1-overexpressor plants show severe GA deficiency and reduced sensitivity to GA4

We further examined the CYP714A1-overexpressor plants that showed an extreme dwarf phenotype. GA analysis of CYP714A1-atOE plants (lines a4 and c2) showed that bioactive GA4 was under the detection level limit (Fig. 5A, Supplementary Table S4). In addition, the levels of GA intermediates such as GA12 (4), GA15, GA24 and GA53 (14) were significantly reduced in the overexpressor plants. The GA12 metabolite 16-carboxy GA12 (4) accumulated in the CYP714A1-atOE plants, but accumulated comparably in WT plants (Fig. 5A). In our yeast expression assay described above, GA12 (4) and GA9 (2) were found to be substrates for the recombinant CYP714A1 protein, but GA4 was not. This was unlike rice CYP714D1/EUI, which used non-13-hydroxy GAs including GA4, but not 13-hydroxy GA including GA1, as substrates (Zhu et al. 2006). To determine whether the bioactive GA4 could be a substrate for CYP714A1 in planta, we examined the effect of three bioactive GAs, GA4, GA16 and 16,17-dihydro GA4 (racemic mixture), on the growth of CYP714A1-atOE plants (lines a4 and c2). We expected that 16,17-dihydro GA4 would not be inactivated by CYP714A1 and CYP714D1 because of its structural protection at C-16 and C-17. The rosette growth of CYP714A1-atOE plants was comparable to that of ga1-3 mutant plants in the absence of exogenous GA (Fig. 5B). The effect of the tested compounds on the growth of WT plants can be summarized as follows: GA4 > 16,17-dihydro GA4 > GA1. Dose–response curves of CYP714A1-atOE and ga1-3 were nearly overlapping when the plants were treated with GA1 or 16,17-dihydro GA4 (Fig. 5B). However, the dose–response curves were clearly different between CYP714A1-atOE and ga1-3 when GA4 was used; CYP714A1-atOE plants were less responsive to GA4 than ga1-3 (Fig. 5B). One possible explanation for these results is that GA4 but not GA1 and 16,17-dihydro GA4 might be a substrate for CYP714A1 in planta.

**Table 3** Biological activities of GAs on the germination of Arabidopsis ga1-3 mutant

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>0 ± 0.0</td>
<td>0 ± 0.0</td>
</tr>
<tr>
<td>GA12</td>
<td>10 nM</td>
<td>1.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>100 nM</td>
<td>15.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>74.3 ± 2.2</td>
</tr>
<tr>
<td>16-carboxy GA12</td>
<td>10 nM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>100 nM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>12α-hydroxy GA12</td>
<td>10 nM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>100 nM</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>13-hydroxy GA12</td>
<td>10 nM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>100 nM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>GA4</td>
<td>10 nM</td>
<td>16.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>100 nM</td>
<td>72.3 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>GA1</td>
<td>10 nM</td>
<td>0.0 ± 0.0</td>
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<tr>
<td></td>
<td>100 nM</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>32.0 ± 2.5</td>
</tr>
<tr>
<td>ent-Kaurenoic acid</td>
<td>100 nM</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>2.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>16.3 ± 4.1</td>
</tr>
<tr>
<td>Steviol (ent-13-hydroxy kaurenoic acid)</td>
<td>100 nM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1 µM</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
<td>3.3 ± 3.3</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of triplicate tests
Mock, treatment without GA.

Fig. 4 Arabidopsis plants that overexpress the CYP714A1 or CYP714A2 gene. (A) Phenotype of CYP714A1-atOE and CYP714A2-atOE plants (24 days old). Bar = 1 cm. (B) Phenotype of ga1-3, CYP714A1-atOE and CYP714A2-atOE plants (54 days old). Bar = 5 cm. (C) Restoration of growth of CYP714A1-atOE and CYP714A2-atOE plants by exogenous application of GA4. Sixteen-day-old seedlings of ga1-3, CYP714A1-atOE and CYP714A2-atOE plants are shown. They were first grown on 1/2 MS agar media for 6 d and then transferred to and grown on 1/2 MS agar media without or with GA4 (2 or 0.2 µM).
Fig. 5 Analyses of CYP714A1-overexpressing plants. (A) Endogenous GA levels in the aerial part of CYP714A1-atOE plants. GAs are shown according to the order in the biosynthesis pathway (Fig. 1). Results for GA<sub>4</sub> are also indicated in the inset with a different y axis scale to clarify the differences among the plants. GA analyses were repeated twice with similar results (Supplementary Table S4). Representative data (1st) are shown. ND, not detected; †, could not be quantified due to trace level. (B) GA<sub>4</sub> but not GA<sub>1</sub> and 16,17-dihydro GA<sub>4</sub> is a likely substrate for CYP714A1. Six-day-old seedlings grown on 1/2 MS agar media were transferred to 1/2 MS agar media containing various concentrations of GAs. Rosette radii of 16-day-old seedlings were measured (n = 13–16). Error bars represent SEM. *Significant difference between ga1-3 and CYP714-atOEs (Student's t-test with Bonferroni correction, P < 0.001).
CYP714A2-overexpressor plants accumulate steviol and 13-hydroxy GAs

GA analysis of CYP714A2-atOE plants (lines b2 and d1) showed that the levels of non-13-hydroxy GAs [GA12 (1) GA15, GA24 and GA3] were decreased in these semi-dwarf plants (Fig. 6, Supplementary Table S5). In contrast, 13-hydroxy GAs (GA6, GA18, GA20 and GA3) except GA3 (14), were highly accumulated in these plants. Our functional analysis indicates that CYP714A2 is a bifunctional enzyme that preferentially catalyzes C-13 hydroxylation of the ent-kaurane carbon skeleton and C-12 hydroxylation of the ent-gibberellane carbon skeleton (Fig. 3). We found that the level of ent-kaurenoic acid (7) was significantly decreased in CYP714A2-atOE plants; instead, steviol (10) accumulated to an extent more than 15-fold greater than in WT plants (Fig. 6). The level of 12α-hydroxy GA12 (13), another metabolite of CYP714A2 from GA12 (1), was rather low in CYP714A2-atOE plants. This is possibly due to the depletion of GA12 (1) precursors by 13-hydroxylation activity of CYP714A2 in the earlier steps of the GA biosynthesis pathway. Given that the bioactivity of GA3 is much lower than that of GA4 in plants (Talon et al. 1990, Yang et al. 1995, Cowling et al. 1998; also shown in Fig. 5B), our results indicate that the semi-dwarism of CYP714A2-atOE plants is caused by depletion of GA4.

Expression patterns of CYP714A1 and CYP714A2

We next examined the expression profiles of the CYP714A1 and CYP714A2 genes in WT Arabidopsis. Quantitative RT-PCR analysis showed that both genes are mainly expressed in siliques, and substantially expressed in roots (Supplementary Fig. S3A). These expression profiles are consistent with the developmental data set of AtGenExpress, which shows that both these genes are predominantly expressed in developing seeds and siliques (Supplementary Fig. S3B, C) (Schmid et al. 2005).

Phenotype and endogenous GA levels of cyp714a1 and cyp714a2 mutants

To understand the physiological roles of CYP714A1 and CYP714A2, we examined their T-DNA insertion mutants. CYP714A1 and CYP714A2 are located tandemly on chromosome 5 (Fig. 7A). We obtained one cyp714a1 allele, cyp714a1-1 (SAIL_721E01) and two cyp714a2 alleles, cyp714a2-1/ela2 (SALK_137272) and cyp714a2-2 (WISC_367A10), from the Arabidopsis Biological Resource Center (Columbus, OH, USA) (Fig. 7B). Transcripts of the disrupted genes were clearly decreased compared with WT in each mutant (Supplementary Fig. S4). Because T-DNAs of cyp714a1-1 and cyp714a2-2 are located in their respective first introns, it is conceivable that each transcript detected contains intact mRNA. Even so, the

![Fig. 6 Endogenous GA levels in the aerial part of CYP714A2-overexpressing plants. GAs are shown according to the order in the biosynthesis pathway (Fig. 1). Results for GA6, ent-kaurenoic acid (7), steviol (ent-13-hydroxy kaurenoic acid, 10) are also indicated in the inset with a different y axis scale to clarify the differences among the plants. GA analyses were repeated twice with similar results (Supplementary Table S5). Representative data (1st) are shown. ND, not detected; †, could not be quantified due to trace level.](http://pcp.oxfordjournals.org/)

level of each intact mRNA in these mutants was estimated at best as less than one-tenth of the WT level in cyp714a1-1, and at best as less than one-thousandth of the WT level in cyp714a2-2 (Supplementary Fig. S4). We measured the endogenous GA levels in siliques and found that 16-carboxy GA 12 (4) was significantly decreased in cyp714a1-1 (Supplementary Table S6). However, the levels of other GAs, including bioactive GA, and GA, of cyp714a1-1, were comparable to those of WT. GA analysis of the cyp714a2-1 and cyp714a2-2/ela2 mutants showed that 12a-hydroxy GA12 (13) was decreased in the late stage of siliques of both mutants (Supplementary Table S7). However, other than this change, there is no clear difference in the gross GA profiles in both lines of mutants compared with WT (Supplementary Table S7). Consistent with the GA profiles, these cyp714a1 and cyp714a2 mutants are morphologically similar to WT under the normal growth condition (data not shown). Flowering times of these mutants were almost the same as those of WT plants under long- and short-day conditions (Supplementary Fig. S5). As described above, both these genes are highly expressed in developing seeds and siliques (Supplementary Fig. S3). We examined the seed phenotype and found that the cyp714a1-1 mutant showed weak resistance to the GA biosynthesis inhibitor paclobutrazol (PAC) during seed germination (Fig. 7C). Only 2.6 and 1.0% of WT seeds germinated in the presence of 50 and 100 μM PAC, respectively, while 13.2 and 9.6% of cyp714a1-1 seeds germinated under similar conditions.

**Discussion**

Previously we found that the CYP714D1/EUI gene encodes GA 16z,17-epoxidase, and Magome et al. (2013) reported that the CYP714B1 and CYP714B2 genes encode GA13ox in rice. Here, our functional analysis demonstrated that *Arabidopsis* CYP714A1 and CYP714A2 also encode distinct GA modification enzymes that oxidize various sites of C and D rings of GA compounds. Our study indicates that the metabolism of GA carried out by CYP714 family proteins is common to monocots and eudicots.

Functional analysis of the recombinant CYP714A1 protein in yeast cells showed that CYP714A1 preferentially catalyzes the conversion of GA 12 (1) to 16-carboxy GA12 (4) (Fig. 2). Bioactivity of this GA12 metabolite (Table 3) and the GA-deficient phenotype of the CYP714A1-overexpressing *Arabidopsis* plants (Fig. 4) indicate that the 16-carboxylation of GA12 (1) is an inactivation reaction. The severe GA deficiency of the CYP714A1 overexpressor plants can thus be explained by the depletion of precursor GAs, including GA12 (1) (Fig. 5A), similar to what occurs in CYP714D1-overexpressing rice plants (Zhu et al. 2006). Because P450s are monooxygenases (Schuler and Werck-Reichhart 2003), 16-carboxy GA12 (4) is probably produced via a multi-step reaction from GA12 (1), and no potential intermediates have been detected in our yeast expression assay. To the best of our knowledge, the occurrence of 16-carboxy GA12 (4) has not been reported in any organisms until now. We detected relatively high levels of this GA metabolite in the aerial part of the seedlings and in siliques of WT plants (Fig. 5A and Supplementary Table S6), indicating that 16-carboxy GA12 (4)
is a major GA$_{12}$ metabolite in Arabidopsis. We also found increased levels of 16-carboxy GA$_{12}$ (4) in CYP714A1-atOE plants (Fig. 5A and Supplementary Table S4) and decreased levels of this metabolite in the cyp714a1-1 mutant (Supplementary Table S6). These results support the idea that CYP714A1 catalyzes the conversion of GA$_{12}$ (1) to 16-carboxy GA$_{12}$ (4) in planta. Besides 16-carboxy GA$_{12}$ (4), three minor metabolites (B, C and D) were detected in our yeast expression assay (Table 1). Among them, metabolite B (16,17-dihydroxy-16,17-dihydro GA$_{12}$, 5), which is most likely a non-enzymatically hydrated form of 16,17-epoxy GA$_{12}$, is a GA$_{12}$ metabolite produced by the CYP714D1 protein (Zhu et al. 2006). This suggests that CYP714A1 also possesses GA 16,17-epoxidase activity, and that the modes of catalysis of CYP714A1 and CYP714D1 therefore are in part similar to each other.

The substrate preference of CYP714A1 protein is currently unclear. The recombinant CYP714A1 was able to catalyze oxidation of GA$_{9}$ (2; data not shown) as well as GA$_{12}$ (1), indicating that CYP714A1 is capable of catalyzing oxidation of multiple GA substrates. Our previous in vitro results obtained with the recombinant CYP714D1 protein indicated that 13-hydroxylation negatively affects the substrate preference of this enzyme: CYP714D1 catalyzed oxidation of non-13-hydroxy GAs (GA$_{12}$, GA$_{9}$ and GA$_{6}$) but not 13-hydroxy GAs (GA$_{20}$, GA$_{19}$ and GA$_{17}$) (Zhu et al. 2006). The recombinant CYP714A1 protein, unlike CYP714D1 protein, did not catalyze oxidation of 13-hydroxy GAs, but also did not catalyze oxidation of a non-13-hydroxy GA, GA$_{16}$, in yeast cells (data not shown). However, CYP714A1-atOE plants were less sensitive to the applied GA$_{4}$ than was the ga1-3 mutant, a difference that was not observed for GA$_{16}$ or 16,17-dihydro GA$_{9}$ (Fig. 5B). This observation could be explained by inactivation of GA$_{4}$ by CYP714A1 in planta. One possible explanation for this discrepancy is that the uptake of GA$_{4}$ by yeast cells from liquid media might not have been efficient for some unknown reason. To eliminate this possibility, in vitro functional analysis of CYP714A1 using a microsome fraction would be needed.

In contrast to the severe dwarf phenotype of the CYP714A1 overexpressor plants (Fig. 4A, B), the loss of function mutant cyp714a1-1 shows a subtle GA-related phenotype in the presence of a GA biosynthesis inhibitor (Fig. 7C). In addition, our GA analysis of siliques of this mutant showed that there was no significant change in bioactive GA levels (GA$_{4}$ and GA$_{1}$) compared with those in WT, while the levels of 16-carboxy GA$_{12}$ (4) were significantly decreased (27% of the WT level) (Supplementary Table S6). It has been reported that another cyp714a1-1/ela1 mutant (SALK_016089) also had no visible phenotype (Zhang et al. 2011). One possible reason for the small impact of the disruption of this gene on bioactive GA levels is the presence of other GA inactivation enzymes in the siliques. Five CYP714A2-GA$_{20}$ genes (GA$_{20}$ox1, -2, -3, -4 and -6), encoding 20DD enzymes, are expressed in Arabidopsis siliques (Rieu et al. 2008). Reduced fertility, possibly due to the unequal elongation of pistils and stamens, was observed in the ga20x quintuple mutant but not in each single mutant (Rieu et al. 2008). Furthermore, the GA 5-methyltransferase genes (GAMT1 and GAMT2) are also highly expressed in the siliques (Varbanova et al. 2007). To clarify the physiological role of the CYP714A1 gene in siliques, preparation of multiple mutants with defects in other GA inactivation enzyme genes might be required.

Our functional analysis of the recombinant CYP714A2 protein showed that this P450 enzyme possesses 12α-hydroxylating activity and/or 13-hydroxylation activities using early GA intermediates in the GA biosynthesis pathway (ent-kaurenoic acid, 7; ent-7α-hydroxy kaurenoic acid, 8; GA$_{12}$-7-aldehyde, 9; and GA$_{12}$, 1) as substrates (Fig. 1). In particular, ent-kaurenoic acid (7) and ent-7α-hydroxy kaurenoic acid (8) were predominantly metabolized into steviol (ent-13-hydroxy kaurenoic acid, 10) and probably to ent-7α,13-dihydroxy kaurenoic acid (11), respectively (Fig. 3B, Table 2, Supplementary Table S2). These results suggest that CYP714A2 may contribute to producing 13-hydroxy GAs in Arabidopsis, even though GA$_{12}$ is not its substrate (as has been predicted), but, rather, more upstream precursors (Fig. 1). The following results and facts support our speculation. First, steviol (10) exists in WT Arabidopsis plants (Fig. 6, Supplementary Table S5). Second, steviol (10) and almost all 13-hydroxy GAs accumulated in Arabidopsis plants that overexpressed CYP714A2 (Fig. 6, Supplementary Table S5). Third, CYP714A2 is predominantly expressed in developing seeds and siliques (Supplementary Fig. S3), in which 13-hydroxy GAs, including GA$_{1}$, are highly accumulated in Arabidopsis (Varbanova et al. 2007, Kanno et al. 2010). Finally, other CYP714 members, such as rice CYP714B1 and CYP714B2, encode GA13ox, although they catalyze the 13-hydroxylation of GA$_{12}$ (1) but not that of ent-kaurenoic acid (7) (Magome et al. 2013). Arabidopsis plants that overexpress CYP714B1 or CYP714B2 show semi-dwarfism with increased levels of 13-hydroxy GAs, including GA$_{1}$ (Magome et al. 2013). However, our GA analysis of siliques of cyp714a2-1 and cyp714a2-2 showed that there is no significant change in GA 13-hydroxyl-
CYP714A2 to GA metabolism is shown in Fig. 8. GA biosynthesis (Wang et al. 2012) role in the production of diterpenoid phytoalexins, but not in subfamily, showing that CYP714A1 (a KO paralog) likely plays a been reported for the rice CYP714A/CYP714D family members (indicated by letters) based on sequence identity 40%; they are further grouped into sub-families (indicated by letters) based on sequence identity >50% (Schuler and Werck-Reichhart 2003). In this study, we demonstrated that although CYP714A1 and rice CYP714D1 (38.8% identity; Supplementary Table S1) are classified in different subfamilies, they are functionally similar as GA inactivation enzymes (C16, C17 oxidation). Both CYP714A2 and rice CYP714Bs (41.2–42.0% identity) possess GA 13-hydroxylation activity. Furthermore, we have also demonstrated that CYP714A1 and CYP714A2 (73.1% identity) possess distinct enzyme activities, even though they are classified in the same subfamily. The diversity of CYP714 family members makes it difficult to speculate on the function of the CYP714 proteins based only on their primary structures. Recently, a similar functional diversity has been reported for the rice CYP701A1/ent-kaurene oxidase (KO) subfamily, showing that CYP701A8 (a KO paralog) likely plays a role in the production of diterpenoid phytoalexins, but not in GA biosynthesis (Wang et al. 2012).

The current model for the contribution of CYP714A1 and CYP714A2 to GA metabolism is shown in Fig. 8. CYP714A1 would play a role in the inactivation of non-13-hydroxy GAs such as GA12 (1), GA9 (2) and possibly GA16 by C16-carboxylation or a similar oxidation. CYP714A2 would also play a role in the C13-hydroxylation using ent-kaurenoic acid (7) and ent-7α-hydroxy kaurenoic acid (8) as substrates. This leads to the production of weakly bioactive GA1 by the early GA 13-hydroxylation pathway. Although it is unclear whether ent-kaurenoic acid oxidases (KAOs) metabolize 13-hydroxy substrates, accumulation of GA1 in the CYP714A2 overexpressor plants (Fig. 6, Supplementary Table S5) supports our idea. Besides 13-oxidase, CYP714A2 would also act as a 12α-oxidase when GA12,7-aldehyde (9) and GA12,13 (1) are its substrates. Therefore, both CYP714A1 and CYP714A2 would compete for the non-13-hydroxy substrates (Fig. 8). These competitive activities would determine the ratio of the strongly active GA (GA1) and the weakly active one (GA14), as is the case with the CYP714A1 and CYP714B8 in rice (Magome et al. 2013).

The physiological roles of CYP714A1 and CYP714A2 are unclear. Intriguingly, Zhang et al. (2011) reported that CYP714A1 and CYP714A2 double knockdown plants made by the combinatorial use of T-DNA insertion and RNA interference (RNAi) showed increased above-ground biomass and early flowering with increased levels of bioactive GA1. These phenotypes might be explained as a consequence of double blocking of the CYP714A1 and CYP714A2 pathways (Fig. 8). However, GA1 levels of the double knockdown plants were comparable to those of WT (Zhang et al. 2011), indicating that GA 13-
hydroxylation is not blocked in these plants. In addition, the transcript levels of CYP714A1 and CYP714A2 are very low in above-ground tissues, except for developing seeds and siliques in WT (Supplementary Fig. S3). These results seem inconsistent with the outstanding GA overdose phenotype. Further studies would be needed to reveal the cause of the double knockdown phenotype.

The steviol (10) synthase activity of CYP714A2 shows promise for applications other than plant growth regulation. Steviol (10) is an aglycone of stevioside, a natural sweetener produced by stevia (S. rebaudiana) (reviewed by Brandle and Telmer 2007). We found that steviol (10) can be accumulated by overexpressing the CYP714A2 gene in yeast and Arabidopsis (Figs 3B, 6), demonstrating the potential for efficient stevioside production in heterologous organisms using biotechnological approaches.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as the WT. The T-DNA tagged mutants cyp714a1-1 (SAIL_721E01), cyp714a2-1/ela2 (SALK_137272) and cyp714a2-2 (WISC_367A10) were provided by the Arabidopsis Biological Resource Center (Columbus, OH, USA). ga1-3 mutant (backcrossed six times to Col-0 plants) has been described previously (Mitchum et al. 2006). Plants were grown on soil (mold:vermiculite = 1:1, v/v) or 1/2 Murashige–Skoog (MS) agar media (Mitchum et al. 2006). Plants were grown on soil (mold:vermiculite = 1:1, v/v) or 1/2 Murashige–Skoog (MS) agar media (Murashige and Skoog 1962) under constant light conditions (approximately 60 μmol m⁻² s⁻¹) at 22°C unless otherwise specified.

Heterologous expression in yeast and identification of CYP714As metabolites

Coding regions of CYP714A1 and CYP714A2 were amplified from cDNA clones using sets of primers (Supplementary Table S8). The PCR products were ligated into pCR2.1 vector plasmid (Life Technologies Corporation, Carlsbad, CA USA). After confirmation of the nucleotide sequence, the plasmid DNAs were digested with BamHI and KpnI, and the DNA fragment containing the cloned DNAs was ligated into pYeDP60 vector. The resulting plasmid was transformed into yeast strain WAT11 engineered to co-produce the Arabidopsis NADPH P450 reductase-1 (Pompon et al. 1996). Functional analyses of CYP714A1 and CYP714A2 were performed by in vivo bioconversion (Pompon et al. 1996). The transformed colonies were inoculated into 10 ml of SGI medium (Pompon et al. 1996) and were grown at 30°C for 1 d in a shaking incubator (200 rpm). One milliliter of SGI-cultured yeast medium was inoculated into 10 ml of SLI medium (Pompon et al. 1996) and grown at 28°C for 1 d until the cell density reached 4 × 10⁷ cells ml⁻¹. The medium was diluted with fresh SLI medium to obtain 4 × 10⁶ cells ml⁻¹. Five milliliters of the diluted medium was incubated with 1 μg of a GA substrate at 28°C for 16 h until the cell density reached 5 × 10⁷ cells ml⁻¹. The GA products were extracted with ethyl acetate. The ethyl acetate phase was partitioned against 0.5 M K₂HPO₄ buffer (pH 9). The buffer phase was adjusted to pH 3 with 6 N HCl and extracted with ethyl acetate. The acidic ethyl acetate phase was evaporated to dryness. The residual solid was dissolved in 5% methanol (including 1% acetic acid) and loaded onto a Bond Elut C18 column (ODS, Agilent Technologies, Santa Clara, CA USA) that was eluted with 80% methanol after washing with 1% acetic acid. The metabolites of ent-kaurenoic acid (7), ent-7α-hydroxy kaurenoic acid (8) and GA12-7-aldehyde (9) were extracted with n-hexane, followed by n-hexane-ethyl acetate (1:1) and ethyl acetate. The extracts obtained with these three kinds of solvents were combined and evaporated to dryness. The residual solid was dissolved in 90% methanol and passed through a Bond Elut C18 column. The column eluates were evaporated to dryness and derivatized with diazomethane and/or N-methyl-N-(trimethylsilyl)trifluoroacetamide. GC-MS analysis of the samples was performed as described previously (Zhu et al. 2006).

Chemical synthesis of 16-carboxy GA12 (16-carboxy-16β,17-dihydro GA12) trimethyl ester

Following procedures reported by Gaskin et al. (1992) and Seto et al. (1995), an authentic sample was prepared from GA12 dimethyl ester by treatment with diboran to give a mixture of epimeric 16,17-dihydro-17-ols, which were oxidized with Dess-Martin reagent to the corresponding aldehydes. Base-catalyzed equilibration then afforded the 16α-epimer, which was oxidized with Jones reagent to 16-carboxy-16β,17-dihydro GA12 (4). Then, diazomethane treatment produced the trimethyl ester. Details will be published elsewhere.

Enzymatic preparation of 16-carboxy GA12 and 12α-hydroxy GA12

16-Carboxy GA12 (4) and 12α-hydroxy GA12 (13) were prepared by incubation of GA12 (1; 50 μg) with the recombinant yeast cultures (50 ml), producing CYP714A1 and CYP714A2, respectively. The acidic ethyl acetate-soluble phase was purified by reversed-phase HPLC using a CAPCELL PAK C18 TYPE SG120Å column (250 × 6 mm i.d.; Shiseido Company, Ltd, Tokyo Japan) eluted with the following methanol–water (including 0.1% acetic acid) gradient at a flow rate of 1.5 ml min⁻¹: 0–5 min, 20% methanol; 5–40 min, 20–80% methanol; and 40–50 min, 80% methanol. Fractions were collected every minute. The column oven temperature was maintained at 40°C. The following fractions were collected: fraction 34/35 (for 12α-hydroxy GA12), 37/38 (for 16-carboxy GA12) and 44/45 (for remaining GA12). The concentrations of 16-carboxy and 12α-hydroxy derivatives were estimated from these conversion rates, based on the assumption that their recoveries were 100%. For quantification of endogenous 16-carboxy GA12 (4) in plants, its 2H-labeled internal standard ([16-2H] 16-carboxy GA12) was prepared by incubating [17,17-²H₂] GA12 with the CYP714A1-producing yeast culture (see Results).
Bioassay of GA metabolites

The ga1-3 mutant was used to test the biological activities of GAs. In the seed germination assay, ga1-3 seeds (about 50 seeds) were washed with 0.02% (w/v) Tween 20 were imbibed in water with or without various GAs at 4°C for 4 d and then germinated on a filter paper (3MM; GE Healthcare UK Ltd, Little Chalfont, UK) soaked with water with or without various GAs at 22°C under continuous light (36 μmol m−2 s−1) for 3 d. Seeds were scored as germinated when radicle protrusion was visible. To evaluate rosette growth, ga1-3 seeds were stratified in the presence of 50 μM GA_4 in the dark at 4°C for 4 d. The seeds were washed with sterile water five times to remove excess GA_4, placed on 1/2 MS agar medium and then incubated for 9 d. The grown seedlings were transplanted onto new 1/2 MS agar media with or without GAs. After 6 d, the diameters of the rosettes were measured (n = 5). The assays were performed using triplicate samples.

Transgenic plant construction

To construct transgenic plants overexpressing CYP714A1 or CYP714A2 (CYP714A1- or CYP714A2-atOE), coding regions of CYP714A1 and CYP714A2 were amplified from cDNA clones using sets of primers (Supplementary Table S8). The PCR products were ligated into pcR2.1 vector plasmid (Life Technologies Corporation, Carlsbad, CA USA). After confirmation of the nucleotide sequence, the plasmid DNAs were digested with BamHI and PstI, and a DNA fragment containing the cloned DNA was inserted between the CaMV 35S promoter and the nopaline synthase terminator of the pCGN vector. Agrobacterium (strain EHA105)-mediated transformation of WT was conducted as described (Magome et al., 2004). Homozygous T3 plants were used for analysis.

Quantification of GAs

For quantitative GA analysis of CYP714A1 or CYP714A2-atOE plants, aerial parts of the overexpressor plants grown on soil were harvested just before bolting and were stored at −80°C. To analyze siliques of cyp714a1, late-stage siliques (stages 5–10) were collected. For analysis of cyp714a2 mutants, early-stage siliques (stages 3–5) as well as the late-stage siliques were collected. Stages of siliques were defined according to Bowman (1994) and Kleindt et al. (2010). The GA content was determined by liquid chromatography-selected reaction monitoring as previously described (Varbanova et al., 2007).

GA and paclobutrazol treatments

For the GA treatment of CYP714A1-atOE plants (Fig. 5B), 6-day-old seedlings grown on 1/2 MS agar plates were transferred onto 1/2 MS agar plates containing various concentrations (0, 0.01, 0.1 or 2 μM) of each GA compound. Rosette radii of 16-day-old seedlings were measured (n = 13–16). To study the effect of PAC treatment on seed germination (Fig. 7C), the seeds were stored at room temperature at 30% humidity for at least 2 months to allow after-ripening. Seeds (60–100) were incubated on wet filter paper (3MM; GE Healthcare UK Ltd, Little Chalfont, UK) containing various concentrations (0, 2, 10, 50 or 100 μM) of PAC. Seeds were scored as germinated when radicle protrusion was visible.

Real time quantitative RT-PCR

Total RNA of siliques was isolated using the RNAqueous RNA Isolation Kit with Plant RNA Isolation Aid (Life Technologies Corporation, Carlsbad, CA USA). Total RNA of other tissues was isolated using the RNeasy extraction kit (Qiagen, Hilden, Germany). Real-time quantitative RT-PCR using an ABI Prism 7700 sequence detection system (Life Technologies Corporation) was described previously (Magome et al., 2004). 18S rRNA was used for normalization. Specific primers and probes used in this study are listed in Supplementary Table S8.

Supplementary data

Supplementary data are available at PCP online.

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Conflicts of interest

No conflicts of interest declared.

References


Identification of endogenous gibberellins in strawberry, including the novel gibberellins GA_{123}, GA_{124} and GA_{125}. Phytochemistry 55: 887–890.


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