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Cucumber gibberellin 1-oxidase/desaturase initiates novel gibberellin catabolic pathways

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Bioactive gibberellins (GAs) are central regulators of plant growth and development, including seed development. GA homeostasis is achieved via complex biosynthetic and catabolic pathways, whose exact activities remain to be elucidated. Here, we isolated two cDNAs from mature or imbibed cucumber seeds with high sequence similarity to known GA 3-oxidases. We found that one enzyme (designated here CsGA3ox5) has GA 3-oxidation activity. However, the second enzyme (designated CsGA1ox/ds) performed multiple reactions, including 1β-oxidation and 9,11-desaturation of GAs, but was lacking the 3-oxidation activity. CsGA1ox/ds overexpression in *Arabidopsis* plants resulted in severely dwarfed plants that could be rescued by the exogenous application of bioactive GA_4, confirming that CsGA1ox/ds catabolizes GAs. Substitution of three amino acids in CsGA1ox/ds, Phe^{93}, Pro^{106}, and Ser^{202}, with those typically conserved among GA 3-oxidases, Tyr^{93}, Met^{106}, and Thr^{202}, respectively, conferred GA 3-oxidase activity to CsGA1ox/ds and thereby augmented its potential to form bioactive GAs in addition to catabolic products. Accordingly, overexpression of this amino acid–modified GA1ox/ds variant in *Arabidopsis* accelerated plant growth and development, indicating that this enzyme variant can produce bioactive GAs *in planta*. Furthermore, a genetically modified GA3ox5 variant in which these three canonical GA 3-oxidase amino acids were changed to the ones present in CsGA1ox/ds was unable to convert GA_9 to GA_4, highlighting the importance of these three conserved amino acids for GA 3-oxidase activity.

Gibberellins (GAs) are biosynthesized *via* complex pathways to regulate plant growth and development and to respond to the environment (1–4). In cucurbits, as in *Arabidopsis*, GA_4 is the predominant bioactive GA during most phases of development (for molecular structure; Fig. 1). For GA homeostasis in plants, regulation of GA biosynthesis as well as GA deactivation is important. GA biosynthesis involves several classes of enzymes, including terpene cyclases, cytochrome P450 monooxygenases, and, as the final steps, 2-oxoglutarate–dependent dioxygenases (2-ODDs). GA catabolism involves also several classes of enzymes, including GA-methyltransferases, cytochrome P450-monooxygenases, and, again, 2-ODDs (2).

Two families of 2-ODDs biosynthesize the bioactive GAs. For GA_4 from GA_{12} (molecular structure; Fig. 1), GA 20-oxidases oxidize and then remove the C-20 of GA_{12} to form the C-4–C-10 γ-lactone of the C_{19}-GA, GA_9. GA 3-oxidases introduce a 3β-hydroxyl group into GA_9 to form bioactive GA_4. Further families of 2-ODDs consist of GA-inactivating enzymes. The first identified and most prominent ones are GA 2-oxidases, many of which have important functions in plant stress responses (3, 4). Additional GA-deactivation reactions catalyzed by 2-ODDs are present in cucurbits. In developing pumpkin seeds, GA 20-oxidases (CmGA20ox1 and CmGA20ox2) catalyze the oxidation of C-20 of GA_{12} to form a tricarboxylic acid C_{20}-GA_{25} (not shown in Fig. 1) (5, 6). Plants overexpressing pumpkin CmGA20ox1 develop a dwarf phenotype, which demonstrates the catabolic function of this enzyme *in planta* (7–9) and indicates neofunctionalization within the GA 20-oxidase gene family.

Here, we report the isolation and characterization of two new cDNA molecules from cucumber seeds that encode proteins with amino acid sequences highly similar to members of the GA 3-oxidase family (Fig. 2). One of them adds to the four already known GA 3-oxidases and was therefore designated CsGA3ox5. This enzyme codes for a new cucumber GA 3-oxidase that converts the GA_9 to bioactive GA_4 (Fig. 1, *red arrow*). In contrast, the second one, designated CsGA1ox/ds, has no GA 3-oxidase activity but instead produces several metabolites (Fig. 1, *blue arrows*). One of the CsGA1ox/ds main products, GA_{61}, is formed from the substrate GA_9 similar to what was recently found for a GA 1-oxidase from wheat (10). However, in addition, CsGA1ox/ds produces GA_{88} (9,11-desaturated GA_4) from GA_4 (Fig. 1). We further demonstrate CsGA1ox/ds catabolic function *in planta*. When overexpressed in *Arabidopsis*, the plants develop a dwarf phenotype that can be rescued by exogenous application of bioactive GA_4. By exchanging three of its amino acids, Phe^{93}, Pro^{106}, and Ser^{202}, for Tyr^{93}, Met^{106}, and Thr^{202}, respectively, engineered GA1ox/ds gains GA 3-oxidase activity and partly loses is catabolic function. Accordingly, *Arabidopsis* plants overexpressing the engineered GA1ox/ds(Y^{93},M^{106},T^{202}) show accelerated growth and development. In addition, by replacing the amino acids Tyr^{128}, Met^{141}, and Thr^{237} of CsGA3ox5 with Phe^{128}, Pro^{141}, and Thr^{237}, respectively, the GA 3-oxidase activity of the engineered enzyme is completely abolished. Our results indicate that these three conserved amino acids among GA

This article contains supporting information.

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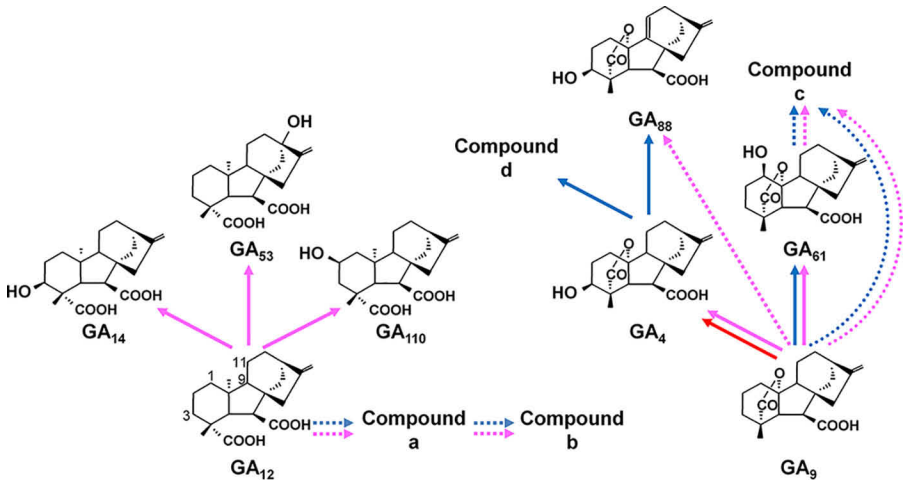

Figure 1. Catalytic properties of recombinant GA oxidases. GA 1-oxidase/desaturase (CsGA1ox/ds) (*blue arrows*), engineered GA1ox/ds(Y^{93},M^{106},T^{202}) (*pink arrows*), and GA 3-oxidase5 (CsGA3ox5) (*red arrow*) are shown. Compound a is putative didehydro-GA_{12}; compound b is putative didehydro-hydroxy-GA_{12}; compound c is putative hydroxy-GA_9 diacidene; and compound d is putative C/D rearranged dihydroxy-GA_4. *Dotted lines* indicate putative pathways.

3-oxidases are a fundamental prerequisite for GA 3-oxidase activity.

Results

Cloning of GA 3-oxidase–like genes from cucumber

Two GA 3-oxidase–like cDNA molecules were cloned from mature or imbibed cucumber seeds. Both proteins harbor the conserved amino acid sequences characteristic for 2-oxoglutarate– dependent dioxygenases including 2-oxoglutarate and iron-binding sites and are phylogenetically closely related to known GA 3-oxidases (Fig. 2 and Fig. S1). One of the GA oxidases (designated CsGA3ox5) harbors the typical domains as found in other GA 3-oxidases (11) (Fig. S1). The other one (designated CsGA1ox/ds) is closely related to CsGA3ox5 (55% amino acid identity) and differs from the conserved domains of the other GA 3-oxidases in only a few amino acids, including Phe^{93}, Pro^{106}, and Ser^{202} (Fig. S1). A homologous gene of *CsGA1ox/ds* is also expressed in melon seeds (*CumGA1ox/ds-like*, 77% amino acid identity; Fig. 2 and Fig. S1).

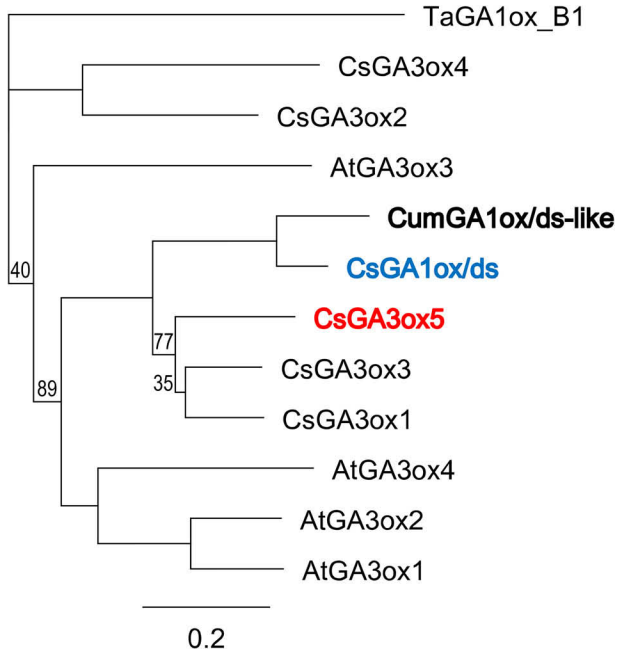

Figure 2. Phylogenetic tree of the GA 3-oxidase–like family. GA 3-oxidases from *C. sativus* and *A. thaliana*, GA 1-oxidases/desaturase from *C. sativus*, GA 1-oxidase/desaturase-like from *C. melo*, and GA 1-oxidase from *Triticum aestivum* are shown. The amino acid sequences of the GA oxidases were aligned in MUSCLE, with the Jukes–Cantor genetic distant model, and the Neighbor-joining tree build method in Geneious Prime (Biomatters Ltd.). The unrooted tree was drawn, and only bootstrap values of less than 95% are shown using Geneious Prime; the *scale bar* represents the number of substitutions per site.

Catalytic properties of closely related CsGA 1-oxidase/desaturase and CsGA 3-oxidase 5

The catalytic properties of the recombinant CsGA1ox/ds and CsGA3ox5 were investigated by expression of the respective coding sequence in pET101/D-TOPO® in *Escherichia coli* BL21 and incubation of cell lysates with ^{14}C-labeled GA substrates. As expected, recombinant CsGA3ox5 converts the C_{19}-GA precursor GA_9 to the GA plant hormone GA_4 (Fig. 1 and Table 1). The C_{20}-GA precursor GA_{12} is not converted by this enzyme (data not shown). However, cucumber CsGA1ox/ds converts GA_{12} to two main compounds with unknown identities. From their mass spectra, compound a represents monounsaturated GA_{12}, and compound b represents monounsaturated hydroxy GA_{12} (Fig. 1 and Table 1). This enzyme also hydroxylates GA_9 to 1β-hydroxy GA_9 (GA_{61}) and to a compound c with unknown identity (Table 1) that, from its mass spectrum, represents monounsaturated hydroxy GA_9 diacid. Incubations with increasing CsGA1ox/ds lysate concentrations indicate that compound c is produced first followed by GA_{61} (Fig. S2, *A–D*). Compound c was also found endogenously in mature cucumber seeds together with several newly identified 2β-hydroxylated GAs 2β-OH GA_{15}, GA_{46}, GA_{43}, GA_{51}, and GA_{34}, and 1,2-ene,3β-GAs, GA_{30}, and GA_7 diacid 9,10-ene (Table S1). Additionally, CsGA1ox/ds converts the plant hormone GA_4 to monounsaturated GA_4 (GA_{88}) and compound d with unknown identity (Table 1). From its mass spectrum compound d has similarity to a C/D rearranged dihydroxy GA_4. The ratio between GA_{88} and compound d does not change in incubations with increasing CsGA1ox/ds lysate concentrations

Table 1

Identification of incubation products of recombinant GA oxidases

Mass spectra and Kovats retention indices (KRI) of products of the methyl ester trimethylsilylether derivatives (30) from incubations of selected GAs with cell lysates from *E. coli* expressing recombinant GA3ox5 and GA1ox/ds from cucumber and with engineered GA1ox/ds(Y^{93},M^{106},T^{202}). Compound a is putative [1-,7-,12-,18-^{14}C]didehydro-GA_{12}; compound b is putative[1-,7-,12-,18-^{14}C]didehydro-hydroxy-GA_{12}; compound c is putative [17-^{14}C]hydroxyGA_9 diacid-ene; and compound d is putative [17-^{14}C]C/D rearranged dihydroxy-GA_4.

Enzyme	Substrate	Compounds formed	KRI	Mass spectrum *m*/*z* (relative intensity)
CsGA3ox5	[17-^{14}C]GA_9	[17-^{14}C]GA_4	2564	M^+ 420 (17), 388 (14), 360 (8), 330 (26), 291 (38), 286 (100), 270 (23), 263 (24), 227 (89), 226 (98), 203 (39), 181 (35), 175 (31), 129 (84)
CsGA1ox/ds	[1-,7-,12-,18-^{14}C] GA_{12}	Compound a	2341	M^+ 366 (9), 334 (76), 319 (12), 304 (100), 291 (42), 257 (10), 245 (51), 229 (29), 215 (16), 203 (26), 187 (32), 157 (34), 145 (37)
		Compound b	2594	M^+ 454 (0), 334 (27), 306 (24), 275 (6), 245 (43), 229 (6), 212 (100), 201 (11), 184 (29), 180 (44), 161 (11), 154 (26)
	[17-^{14}C]GA_9	[17-^{14}C]GA_{61}	2460	M^+ 420 (19), 405 (9), 388 (30), 361 (30), 360 (22), 349 (12), 298 (100), 286 (25), 243 (16), 227 (28)
		Compound c	2332	M^+ 434 (13), 374 (31), 359 (6), 344 (6), 315 (16), 312 (40), 289 (13), 284 (100), 252 (18), 225 (64), 209 (8), 197 (17), 167 (17), 155 (20)
	[17-^{14}C]GA_4	[17-^{14}C]GA_{88}	2566	M^+ 418 (13), 387 (12), 362 (39), 328 (13), 289 (67), 284 (100), 261 (29), 225 (51), 199 (40), 181 (56), 143 (49)
		Compound d	2735	M^+ 524 (100), 434 (16), 342 (13), 330 (17), 271 (11), 257 (37), 243 (34), 229 (11), 215 (21), 211 (17), 195 (22), 171 (31)
GA1ox/ds(Y^{93},M^{106},T^{202})	[17-^{14}C] GA_{12}	Compound a	2332	M^+ 360 (17), 328 (93), 313 (13), 300 (100), 285 (48), 253 (14), 241 (56), 225 (32), 209 (12), 197 (23), 185 (16), 155 (30), 143 (24)
		Compound b	2594	M^+ 448 (0), 360 (2), 328 (29), 300 (34), 269 (5), 241 (52), 225 (8), 210 (100), 199 (5), 182 (31), 178 (50), 154 (38)
		[17-^{14}C]GA_{14}	2538	M^+ 450 (0), 435 (5), 418 (32), 390 (16), 328 (16), 300 (55), 289 (57), 285 (33), 261 (41), 260 (36), 241 (43), 233 (64), 201 (33), 173 (41), 129 (100)
		[17-^{14}C]GA_{53}	2545	M^+ 450 (33), 435 (3), 418 (7), 391 (18), 375 (8), 359 (5), 331 (10), 301 (4), 277 (5), 253 (24), 241 (34), 237 (21),210 (83), 209 (100), 195 (35), 181 (82)
		[17-^{14}C]GA_{110}	2577	M^+ 450 (3), 418 (21), 390 (35), 375 (3), 318 (9), 300 (67), 285 (52), 274 (35), 260 (20), 259 (22), 241 (100), 225 (40), 197 (18), 173 (20), 171 (22), 145 (46)
	[17-^{14}C]GA_9	[17-^{14}C]GA_4	2566	M^+ 420 (24), 388 (17), 360 (9), 330 (30), 291 (38), 286 (100), 270 (23), 263 (23), 227 (60), 226 (97), 203 (29), 181 (23), 175 (22), 129 (55)
		[17-^{14}C]GA_{88}	2565	M^+ 418 (12), 387 (11), 362 (37), 328 (24), 289 (53), 284 (100), 261 (20), 225 (65), 199 (35), 181 (24), 143 (28)
		[17-^{14}C]GA_{61}	2459	M^+ 420 (27), 405 (13), 388 (41), 361 (38), 360 (54), 349 (15), 298 (100), 286 (30), 270 (19), 243 (19), 227 (36)
		Compound c	2327	M^+ 434 (12), 374 (25), 359 (5), 344 (5), 315 (11), 312 (31), 289 (4), 284 (100), 252 (13), 225 (36), 209 (8), 197 (8), 167 (9), 155 (10)

(Fig. S2, *E* and *F*) or at limiting iron supply (Fig. S3, *F* and *I*), indicating that both products might be catalyzed directly from GA_4. CsGA1ox/ds has cofactors requirements typical for 2-ODDs; without adding cofactors to the incubation mixtures, none of the substrates is converted, including [^{14}C]GA_{12}, [^{14}C]GA_9, and [^{14}C]GA_4 (Fig. S3, *A–F*). CsGA1ox/ds has absolute requirements for 2-oxoglutarate and ascorbate, and its activity is stimulated in the presence of Fe^{2+} (Fig. S3, *G–I*). However, after chelating the iron by EDTA, the requirement for Fe^{2+} becomes absolute (Fig. S3*J*). Taken together, this multifunctional 2-ODD is best described as GA 1-oxidase/desaturase.

Engineered GA1ox/ds gains GA 3-oxidase activity, and engineered GA3ox5 loses GA 3-oxidase function

As revealed by amino acid sequence alignments CsGA1ox/ds protein is closely related to the GA 3-oxidase protein family (Fig. S1). However, GA1ox/ds differs within the predicted conserved domain region in two amino acids (Phe^{93} and Pro^{106}) from GA 3-oxidases (11) (Fig. S1, underlined in *black*). Moreover, one amino acid (Ser^{202}) of GA1ox/ds is different within the putative iron-binding sites of GA 3-oxidases (Fig. S1, *red boxes*). To clarify their function, we employed two strategies.

First, we changed these amino acids in GA1ox/ds to the respective ones conserved among GA 3-oxidases producing the following three engineered GA1ox/ds constructs, GA1ox/ds(T^{202}), GA1ox/ds(Y^{93},M^{106}), and GA1ox/ds(Y^{93},M^{106},T^{202}). GA1ox/ds(T^{202}) product formation resembles that of native CsGA1ox/ds but GA_{88} that was not formed by the engineered enzyme (Table 1 and Table S2). The catalytic properties of GA1ox/ds(Y^{93},M^{106}) and GA1ox/ds(Y^{93},M^{106},T^{202}) are quite similar. Both engineered enzymes also produce GAs previously identified with CsGA1ox/ds, compound a and b from GA_{12}, but additionally GA_{14} (3β-hydroxy GA_{12}), GA_{110} (2β-hydroxylation), and GA_{53} (13-hydroxylation; Table 1, Table S2, and Fig. S4). Contrarily to the original CsGA1ox/ds, the two engineered GA1ox/ds(Y^{93},M^{106}) and GA1ox/ds(Y^{93},M^{106},T^{202}) produce mainly GA_4 (3β-hydroxylation) and a small amount of GA_{88} (3β-hydroxylation and 9,11-desaturation) from its precursors GA_9 (Table 1, Fig. S4*D*, and Table S2). Like CsGA1ox/ds, the two engineered enzymes also produce compound c and GA_{61} from GA_9, but at much lower yield (Fig. S4*D* and Table S2). In addition, GA1ox/ds(Y^{93},M^{106}) produces GA_1 and, like the WT CsGA1ox/ds, GA_{88} from GA_4. However, in contrast to CsGA1ox/ds, GA1ox/ds(Y^{93},M^{106},T^{202}) does not convert GA_4, which is similar to other GA 3-oxidases including the newly identified CsGA3ox5 (Fig. S4*E*). As for CsGA1ox/ds, the new catalytic properties of GA1ox/ds(Y^{93},M^{106},T^{202}), including 2-, 3-, and 13-oxidation, all require cofactors typical for 2-ODDs (Fig. S4). Thus, engineered GA1ox/ds(Y^{93},M^{106},T^{202}) gains GA 3-oxidase activity producing GA_4 and loses, at least partly, GA 1-oxidase/desaturase activity.

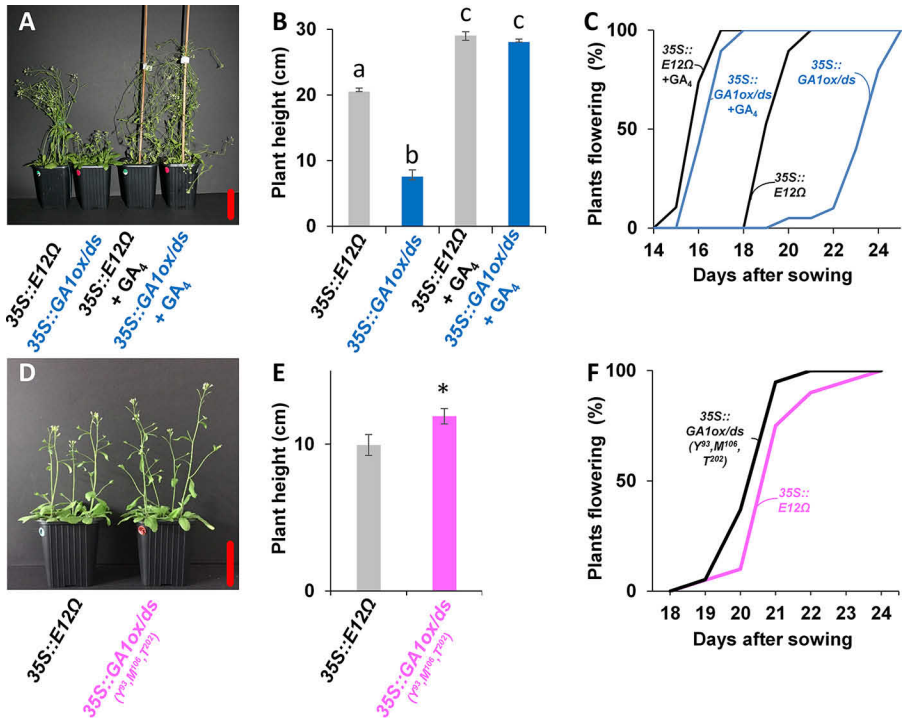

Figure 3. Phenotypical characterization of *Arabidopsis* overexpressing CsGA1ox/ds or engineered GA1ox/ds(Y^{93},M^{106},T^{202}). *A*, phenotype of 28-day-old plants. From *left* to *right*, *35S::E12Ω* (empty vector), *35S::CsGA1ox/ds*, line 2251, *35S::E12Ω* treated with GA_4, and *35S::CsGA1ox/ds*, line 2251 treated with GA_4. *B*, average plant height. The means ± S.E. were obtained from at least 19 independent plants. The *letters* over the *bars* indicate significant differences between experiments ($p < 0.001$). *C*, percentage of plants flowering over time. *D*, phenotype of 25-day-old plants, overexpressing either the empty vector or engineered GA1ox/ds(Y^{93},M^{106},T^{202}) in line 15-4. *E*, average plant height. The means ± S.E. were obtained from 15 independent plants. The *asterisk* over the *bar* indicates a significant difference ($p < 0.05$, Student's *t* test). *F*, percentage of plants flowering over time. *Red bars* represent 5 cm.

In a second strategy, we changed the GA 3-oxidase conserved amino acids in CsGA3ox5(Y^{128},M^{141},T^{237}) to the respective ones of GA1ox/ds. The engineered GA3ox5(F^{128},P^{141},S^{237}) enzyme does not 3β-hydroxylate GA_9 to GA_4 as has been identified for the native CsGA3ox5 (Fig. S5 and Table 1). Taken together, our results indicate that the GA 3-oxidase conserved amino acids Tyr and Met are important for GA 3-oxidase activity, with Thr having a minor function.

Overexpression of CsGA1ox/ds in Arabidopsis reveals its catabolic function in planta

Both genes, *CsGA3ox5* and *CsGA1ox/ds*, are expressed specifically during cucumber mid-seed development when catabolic GA 2-oxidases are not expressed (Fig. S6; RRID: SCR_018401). The presence of endogenous compound c in mature seeds additionally indicates a function of CsGA1ox/ds during seed development (Table S1). In addition to the previously described endogenous GAs from mature seeds of *Cucumis sativus* (12), we identified several 2β-hydroxylated gibberellins in mature seeds; this correlates with the GA 2-oxidase expression at the late stages of seed development (Fig. S6).

Gibberellin 3-oxidases are well-characterized enzymes and have an important function for plant development. They catalyze the last step of GA biosynthesis leading to the GA plant hormone (13). However, a function of GA 1-oxidases/desaturases for regulation of the gibberellin hormone pool *in planta* is not known. Overexpression of CsGA1ox/ds in *Arabidopsis* results in transgenic plants with a phenotype similar to *Arabidopsis* plants overexpressing catabolic GA 2-oxidases or the catabolic pumpkin GA 20-oxidase (CmGA20ox1) (7). Such a phenotype (extreme dwarfism, late flowering, dark green leaves) is typical for GA-deficient plants and reveals the catabolic function of CsGA1ox/ds for GA homeostasis (Fig. 3, *A–C*, Fig. S7, and Table S3). In fact, levels of all GA, including precursors GA_{12}, GA_{15}, GA_{24}, bioactive GA_4, and the catabolite GA_{34}, all were extremely low in the CsGA1ox/ds overexpressor line when compared with the control plants overexpressing the empty vector (Table S4). Moreover, the gibberellin-like compound a, compound c, and compound d that are products of recombinant CsGA1ox/ds were identified as endogenous constituents in the transgenic *35S::GA1ox/ds* plants (Table S5).

Exogenous GA_4 treatment of *Arabidopsis* CsGA1ox/ds overexpressors and respective *35S::E12Ω* control plants results in plants developing similar phenotypes, confirming the GA catabolic function of CsGA1ox/ds *in planta* (Fig. 3, *A–C*). Moreover, engineered GA1ox/ds(Y^{93},M^{106},T^{202}) plants show no dwarfism but instead show an accelerated growth phenotype (Fig. 3, *D–F*) resembling plants with elevated endogenous GA content (7). These results validate the importance of the amino acids Tyr, Met, and Thr for GA 3-oxidase activity.

Discussion

Here we present evidence for novel alternative GA catabolic pathways active in cucumber seeds. We have cloned two genes from mature cucumber seeds: *CsGA1ox/ds* and *CsGA3ox5*. Both genes appear to be highly expressed only during seed development and not at other stages of generative or vegetative development (14) (RRID:SCR_018401). Phylogenetically, both

cluster to the GA 3-oxidase family. Consistently, the catalytic properties of CsGA3ox5 are closely related to ones of typical GA 3-oxidases (15). Recombinant CsGA3ox5 produces bioactive GA_4 from the precursor GA_9 by 3β-hydroxylation at carbon-3. However, CsGA1ox/ds has catabolic function because if overexpressed in *Arabidopsis*, the resulting transgenic plants develop a strong dwarfism, similar to plants overexpressing GA 2-oxidases (7, 16, 17), that can be restored to the phenotype of control plants by exogenous application of the plant hormone GA_4. Moreover, recombinant CsGA1ox/ds lacks GA 3-oxidase activity. Instead, it hydroxylates precursor GA_9 at the 1β position and produces GA_{61}. Recently, another GA 1-oxidase was identified from bread wheat with structural homology to the GA 3-oxidase family (10). Unlike the bread wheat one (TaGA1ox1), CsGA1ox/ds is a multifunctional enzyme; *e.g.* with its 9,11-desaturase activity it produces GA_{88} from GA_4. Moreover, it also accepts C_{20}-GA_{12} as a substrate and produces several unknown gibberellin-like molecules. The presence of endogenous compound c in mature cucumber seeds supports the role of GA1ox/ds for GA catabolism during seed development. Other products of CsGA1ox/ds were not identified endogenously in mature cucumber seeds. Because the *CsGA1ox/ds* gene is exclusively expressed in mid-seed development, CsGA1ox/ds products are likely further metabolized during seed maturation (Fig. S6).

CsGA1ox/ds differs in two amino acids, Phe^{93} and Pro^{106}, if compared with the specific conserved domain of GA 3-oxidases (11). Moreover, a third amino acid within the iron-binding domain of CsGA1ox/ds (Ser^{202}) is also different from the one conserved among GA 3-oxidases. A *GA1ox/ds-like* gene coding for a protein with similar amino acid composition is present in melon seeds (*CumGA1ox/ds-like*; Fig. 2 and Fig. S1), and homologue genes may be present in other plant species. We substituted these three particular amino acids of CsGA1ox/ds with the ones present in the GA 3-oxidase family, Tyr^{93}, Met^{106}, and Thr^{202}, respectively. Overexpression of engineered GA1ox/ds(Y^{93},M^{106},T^{202}) in *Arabidopsis* results in mutant plants that show accelerated growth and development, indicating a loss of catabolic and a gain of anabolic function of the engineered enzyme. Indeed, recombinant GA1ox/ds(Y^{93},M^{106},T^{202}) mainly shows 3β-hydroxylation activity, having, as side reactions, GA 1β-, 2β-, and GA 13-oxidase activities. In previous work, GA 13-oxidase activity was associated with GA 3-oxidases from *Marah macrocarpus* and bread wheat (18, 19), suggesting that these three amino acids are important not only for GA 3-oxidase but also for 13-oxidase activity.

Functional expression in *E. coli* of two additional GA1ox/ds constructs, GA1ox/ds(Y^{93},M^{106}) and GA1ox/ds(T^{202}), indicate that Tyr^{93} and Met^{106} have important functions and Thr^{202} has only a minor function for GA 3-oxidase activity of engineered GA1ox/ds. In contrast, GA 3-oxidase activity of CsGA3ox5 is abolished by replacing the characteristic GA 3-oxidase amino acids (Tyr^{128}, Met^{141}, and Thr^{237}) with the respective CsGA1ox/ds ones (Phe, Pro, and Ser). These results support the view that the GA 3-oxidase conserved amino acids (Tyr, Met, and Thr) are necessary for regulating GA 3-oxidase activity, although these three amino acids important for GA 3-oxidase activity are also present in TaGA1ox1, an enzyme expressing GA 1-oxidase activity only (10). However, TaGA1ox1 is phylogenetically and functionally not very closely related to GA 3-oxidases, suggesting that within this enzyme GA 1-oxidase activity might overrule potential GA 3-oxidase activity.

Recently, it was suggested that GA activity is tightly regulated through the expression of catabolic cytochrome P-450 monooxygenases and GA methyltransferases in mid-development during *Arabidopsis* seed maturation (20–22). This study proposes that cucurbits employ alternative pathways for fine-tuning GA homeostasis during seed development by expressing catabolic CsGA1ox/ds in early and mid-stages during seed maturation when the catabolic GA 2-oxidases are not expressed (Fig. S6). Similarly, in cell-free enzyme systems prepared from developing pumpkin embryos, a catabolic GA 20-oxidase, CmGA20ox1, is expressed that might substitute GA 2-oxidase activities in these tissues (5–7, 23, 24). Moreover, only a few amino acid changes turn the catabolic CsGA1ox/ds to an anabolic GA 3-oxidase function, implying neofunctionalization within the GA 3-oxidase gene family as has been previously found for the GA 20-oxidase family in cucurbits. We conclude that, at least in cucurbits, GA biosynthesis and catabolism are reached by functionally and phylogenetically closely related proteins, indicating evolutionary flexibility to achieve contrasting enzymatic functions. This study opens gateways to further understand evolutionary strategies toward hormonal regulation of seed development.

Materials and methods

Plant material, RNA isolation, and production of cDNAs

Cucumber (*C. sativus* var. Hokus) seeds were obtained from the Botanischer Garten der Technischen Universität Braunschweig. For RNA isolation, seeds were used directly or after 24 h of imbibition in water. Total RNA was isolated with NucleoSpin® RNA plant kit (Macherey–Nagel) following the manufacturer's instructions. To remove genomic DNA, total RNA was treated with DNase I using RapidOut DNA removal kit (Thermo Scientific™) according to the manufacturer's instructions. DNase I–treated RNA (100 ng) was used in 10-μl reverse transcription reactions to produce cDNA molecules using the PCRBIO cDNA synthesis kit (PCRBIOSYSTEMS), according to the manufacturer's indications.

Cloning of GA 1-oxidase/desaturase and GA 3-oxidase from cucumber

The cDNA molecules were used as templates in 10-μl PCRs containing 5 μl of Platinum™ SuperFi™ PCR Master Mix or Phusion High-Fidelity PCR Master Mix (Invitrogen and Thermo Scientific, respectively) and sequence-specific primers designed according to the coding sequence of the cucumber putative GA 3-oxidases. The PCR conditions and lengths of the expected PCR products are listed in Table S4. After reamplification, PCR products were purified by agarose gel electrophoresis (GeneJet™ gel extraction kit; Thermo Scientific) and cloned into a pET101/D-TOPO® expression kit (Invitrogen) following the manufacturer's instructions. Positive clones were identified by PCR, and respective plasmid DNAs were sequenced on both strands.

Heterologous expression of recombinant cucumber GA 1-oxidase/desaturase and GA 3-oxidase

Plasmid DNA of the cloned CsGA1ox/ds, and CsGA3ox5 and of the custom (BioCat) synthesized CsGA1ox/ds(T^{202}), CsGA1ox/ds(Y^{93},M^{106}), CsGA1ox/ds(Y^{93},M^{106},T^{202}), and CsGA3ox5(Y^{128},M^{141},T^{237}) was used to transform BL21 Star™ *E. coli* (Invitrogen), according to the manufacturer's instructions. Production of recombinant GA oxidases and protein induction was described previously (15, 24).

Enzyme assays and analysis of incubation products

^{14}C-Labeled GAs were prepared as described elsewhere (25). 17-^{14}C-Labeled GA_{12} was a gift from Professor L. Mander (Canberra, Australia). If not otherwise indicated, preparations of *E. coli* cell lysates (70 μl) were incubated with 2-oxoglutarate and ascorbate (100 mM each, final concentrations). $FeSO_4$ (0.5 mM), catalase (1 mg/ml), and the ^{14}C-labeled substrate (2 μl in methanol; 0.33 nmol for (1,7,12,18-$^{14}C_4$)-labeled GAs and 1 nmol for (17-^{14}C)-labeled GAs) were added in a total volume of 100 μl and incubated for 4 h at 30 °C. Variations in incubation conditions are specified for particular experiments. Analysis of incubation products by HPLC and GC-MS was done as described previously (25).

Overexpression of CsGA1ox/ds in Arabidopsis

To express CsGA1ox/ds in *Arabidopsis*, cDNA molecules were PCR-amplified using Phusion High-Fidelity PCR Master Mix, as described above, with primers containing restriction sites (Table S6) and cloned at the BamHI, EcoRI sites of a modified pBE2113 vector containing a strong promoter cassette and a translational enhancer (E12–35-Ω) (7, 26). To express the engineered enzyme CsGA1ox/ds(Y^{93},M^{106},T^{202}) in *Arabidopsis*, the cDNA cloned into pET-21a(+) vector at the EcoRI-XhoI sites was isolated with BamHI and XhoI and ligated at the same restriction sites of the modified pBE2113 vector and transformed into TOP10 competent *E. coli* cells (Invitrogen). After sequencing of respective plasmid DNAs, constructs carrying the CsGA1ox/ds and CsGA1ox/ds(Y^{93},M^{106},T^{202}) copies were introduced into *Arabidopsis* WT plants via *Agrobacterium tumefaciens*–mediated transformation using the floral dip method (27). Selection of transgenic lines was done as described previously (7) with reverse gene-specific primers, identical to the ones described for the real-time PCR experiments, and a forward vector-specific primer 5′-CTACAACTACATCTAGAGG-3′ in the PCR experiments. After scoring at T3, three homozygous lines for every gene were taken to generate T4 homozygous plants (Table S3). Line 2251 for *35S::CsGA1ox/ds* and line 15-4 for *35S::GA1ox/ds(Y^{93},M^{106},T^{202})* were chosen for further phenotypical, biochemical, and molecular characterization.

Gene expression analysis

Total RNA extraction has been described above. First-strand cDNA synthesis was done with 300 ng of DNase I–treated total RNA using the SuperScript IV VILO Master Mix in a 5-μl reaction volume according to the manufacturer's protocol (Invitrogen). 1 μl of cDNA was diluted 10 times with water and used for 10-μl quantitative RT-PCRs as described previously (28) but using 5 μl of qPCRBIO SyGreen No-Rox mix (PCR Biosystems) using a two-step cycling according to the manufacturer's protocol. The *Arabidopsis SAND* gene (AT2G28390) was used as the internal reference gene (28). The real-time PCR Miner program was used to calculate the relative expression of each gene (RRID:SCR_018402) (29) as the average of two technical PCR replicates. Technical replicates with a difference from the mean $Ct \geq 0.5$ were excluded. All experiments were done with at least three biological replicates. The samples were tested for the absence of genomic DNA by RT minus quantitative PCR. The primers used for quantitative PCR are listed in Table S7.

Analysis of endogenous gibberellins

For analysis of endogenous GAs, 2 g (fresh weight) of frozen, pulverized plant tissues were extracted and analyzed by full-scan GC-MS (25).

Statistical analysis

For phenotypic characterization experiments, statistical analysis was performed using Student's *t* test as indicated for the individual experiments.

Data availability

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers LT827069 (CsGA1ox/ds) and MK433203 (CsGA3ox5). All other data and materials for this publication are available to be shared upon request by contacting the corresponding authors Theo Lange (theo.lange@tu-bs.de) or Maria João Pimenta Lange (m.pimenta@tu-bs.de).

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Abbreviations—The abbreviations used are: GA, gibberellin; 2-ODD, 2-oxoglutarate– dependent dioxygenase.

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