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Functional characterization of gibberellin oxidases from cucumber, *Cucumis sativus* L.

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ABSTRACT

Cucurbits have been used widely to elucidate gibberellin (GA) biosynthesis. With the recent availability of the genome sequence for the economically important cucurbit *Cucumis sativus*, sequence data became available for all genes potentially involved in GA biosynthesis for this species. Sixteen cDNAs were cloned from root and shoot of 3-d to 7-d old seedlings and from mature seeds of *C. sativus*. Two cDNAs code for GA 7-oxidases (CsGA7ox1, and -2), five for GA 20-oxidases (CsGA20ox1, -2, -3, -4, and -5), four for GA 3-oxidases (CsGA3ox1, -2, -3, and -4), and another five for GA 2-oxidases (CsGA2ox1, -2, -3, -4, and -5). Their enzymatic activities were investigated by heterologous expression of the cDNAs in *Escherichia coli* and incubation of the cell lysates with ^{14}C-labelled, D2-labelled, or unlabelled GA-substrates. The two GA 7-oxidases converted GA_{12}-aldehyde to GA_{12} efficiently. CsGA7ox1 converted GA_{12} to GA_{14}, to 15α-hydroxyGA_{12}, and further to 15α-hydroxyGA_{14}. CsGA7ox2 converted GA_{12} to its 12α-hydroxylated analogue GA_{111}. All five GA 20-oxidases converted GA_{12} to GA_9 as a major product, and to GA_{25} as a minor product. The four GA 3-oxidases oxidized the C_{19}-GA GA_9 to GA_4 as the only product. In addition, three of them (CsGA3ox2, -3, and -4) converted the C_{20}-GA GA_{12} to GA_{14}. The GA 2-oxidases CsGA2ox1, -2, -3, and -4 oxidized the C_{19}-GAs GA_9 and GA_4 to GA_{34} and GA_{51}, respectively. CsGA2ox2, -3, and -4 converted GA_{51} and GA_{34} further to respective GA-catabolites. In addition to C_{19}-GAs, CsGA2ox4 also converted the C_{20}-GA GA_{12} to GA_{110}. In contrast, CsGA2ox5 oxidized only the C_{20} GA_{12} to GA_{110} as the sole product. As shown for CsGA20ox1 and CsGA3ox1, similar reactions were catalysed with 13-hydroxylated GAs as substrates. It is likely that these enzymes are also responsible for the biosynthesis of 13-hydroxylated GAs *in vivo* that occur at low levels in cucumber.

1. Introduction

Gibberellins (GAs) are signalling molecules that are major regulators of plant growth and developmental. The cucumber, *Cucumis sativus* L., is an important crop plant of the cucurbit family that has been widely used in GA research. It played an important role in the early studies on alteration of sex expression by GAs (Malepszy and Niemirowicz-Szczytt, 1991; Fuchs et al., 1977). Furthermore, GA control of flowering, tendril growth and development (Ameha et al., 1998), fruit growth (Goodwin, 1978; Ogawa and Aoki, 1977), and parthenocarpy (Ogawa et al., 1989) was investigated utilizing this species. More recently, in cucumber sites of GA synthesis and action were studied identifying the role of cotyledons for maintaining GA levels in the hypocotyl (Asahina et al., 2002, 2007).

Members of the cucurbit family have also added substantially to our knowledge of key components of the GA biosynthetic pathways (Graebe, 1987; Lange, 1998). GA biosynthesis is complex, the final part of the pathway is catalysed by oxidases that belong to the class of 2-oxoglutarate-dependent dioxygenases (designated GA oxidases; for recent reviews see Yamaguchi (2008) and Hedden and Thomas (2012)). Recently, this part of the pathway has been identified as primarily responsible for the regulation of bioactive GA synthesis (Hedden and Thomas, 2012). However, for cucumber, information on GA biosynthesis is still limited. So far, only one cDNA has been isolated, that codes for an enzyme of an early step of GA biosynthetic pathway, a putative *ent*-kaurene synthase (Shirai et al., 2002). Also a GA responsive *lh* mutant was found in cucumber (Lopez-Juez et al., 1995). Two parallel GA biosynthetic pathways lead to bioactive GAs in plants, a "non-hydroxylation" and an "early-13-hydroxylation" pathway (Hedden and Thomas, 2012). There is considerable evidence that in cucumber, like in pumpkin, the "non-hydroxylation" pathway is the major one (see Scheme 1), although GAs of the "early-13-hydroxylation" pathway were also identified at low levels in this species (Hemphill et al., 1972; Nakayama et al., 1989, 1991; Smith et al., 1991).

Recently, the genome sequence of cucumber (*C. sativus*) became public (Huang et al., 2009) which simplifies comprehensive investigations of genes involved in GA biosynthesis in this species. We identified 16 genes in the cucumber genome database that show

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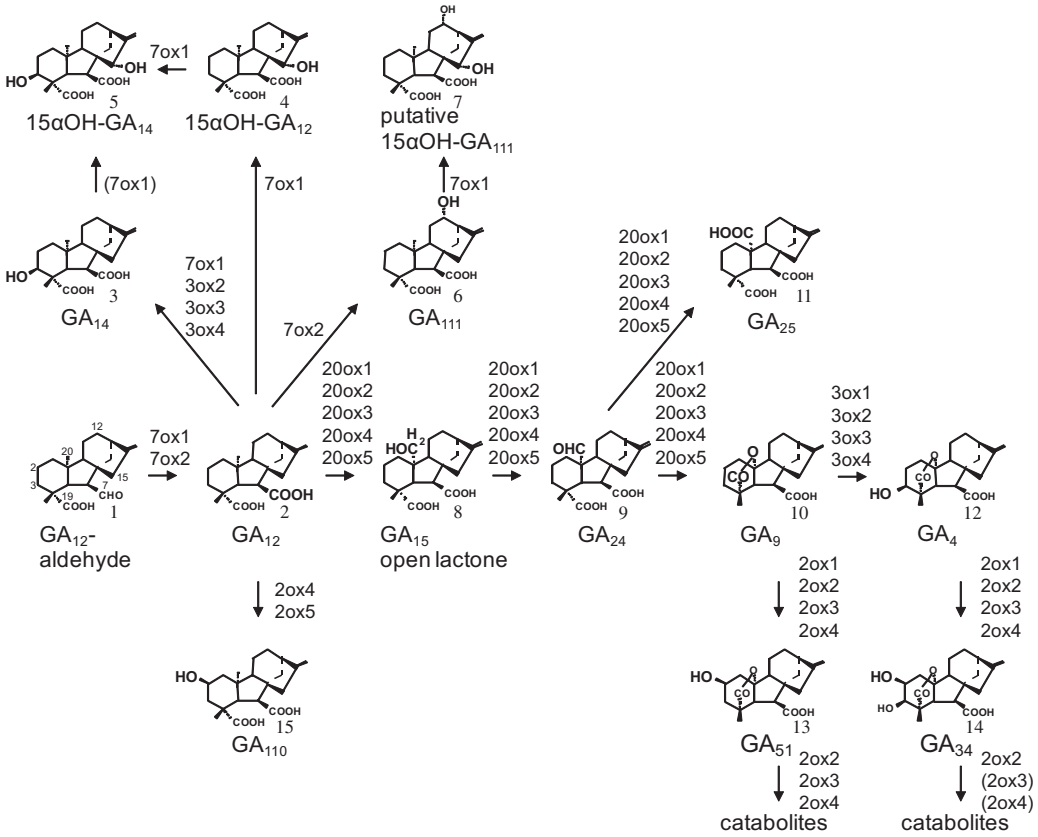

Scheme 1. Reactions catalysed by recombinant GA-oxidases from *Cucumis sativus*. Reaction steps in brackets are deduced from respective incubation products as identified in Tables 2–5.

Table 1
Cucumis sativus GA-oxidases, accession numbers, and respective Csa putative gene codes (Cucumber genome version 2 CuGenDB).

GA-oxidases	Cs cloned genes	GenBank Accession No.	Csa putative GA-oxidases codes	Comments
7-Oxidases	*CsGA7ox1*	HE582622	Csa1M033050.1	
	CsGA7ox1	HE582623	Csa1M033040.1	
20-Oxidases	*CsGA20ox1*	FR720083	Csa5M172270.1	
	CsGA20ox2	HE582624	Csa6M476070.1	
	CsGA20ox3	HE582625	Csa6M075200.1	Extra amino acid sequence at position 53: SGDPVALSETCHLLDQV
	CsGA20ox4	HE582626	Csa3M179110.1	At position 107: F is exchanged to L
	CsGA20ox5	HE680065	Csa6M351370.1	
3-Oxidases	*CsGA3ox1*	FR720085	Csa7M434970.1	
	CsGA3ox2	HE582627	Csa2M379300.1	
	CsGA3ox3	HE582628	Csa7M435500.1	
	CsGA3ox4	HE680066	Csa2M379320.1	
2-Oxidases	*CsGA2ox1*	HE582629	Csa4M075200.1	
	CsGA2ox2	HE582630	Csa7M413380.1	At position 34: I is exchanged to V; at position 227: R is exchanged to Q
	CsGA2ox3	HE582631	Csa1M439830.1	
	CsGA2ox4	HE680067	Csa6M523390.1	Extra amino acid sequence at position 98: KNIGS
	CsGA2ox5	HE983622	Csa1M064730.1	

considerable similarities to pumpkin GA-oxidases (Table 1, Fig. 1), cloned their respective cDNA molecules by a PCR based approach, and characterized enzymatic activities of their encoded recombinant proteins by heterologous expression in *Escherichia coli*.

2. Results and discussion

2.1. Cloning C. sativus GA-oxidase cDNA molecules and their heterologous expression in E. coli

The amino acid sequences of known pumpkin GA-oxidases were used to identify 16 putative GA-oxidase genes in the cucumber genome (http://www.icugi.org/; Huang et al., 2009; Pimenta Lange et al., 2012). For this study, total RNA was used, derived from cucumber seedlings and mature seeds, that was reverse transcribed. cDNA molecules were amplified by a PCR-based method using suitable specific primer pairs for each of the 16 putative GA-oxidases that cover the ORF of each of the genes. The cDNA molecules were cloned into pET101/D-TOPO® expression vector and sequenced on both strands. Considerable sequence differences to the cucumber genome data base were found for CsGA20ox3 and CsGA2ox4, harbouring 17 and 5 extra amino acids, respectively (http://www.icugi.org/; Huang et al., 2009; Table 1). For CsGA20ox4 one and for CsGA2ox2 two amino acids were changed.