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RESEARCH REPORT

Ovary-derived precursor gibberellin A_9 is essential for female flower development in cucumber

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ABSTRACT

Gibberellins (GAs) are hormones that control many aspects of plant development, including flowering. It is well known that stamen is the source of GAs that regulate male and bisexual flower development. However, little is known about the role of GAs in female flower development. In cucumber, high levels of GA precursors are present in ovaries and high levels of bioactive GA_4 are identified in sepals/petals, reflecting the expression of GA 20-oxidase and 3-oxidase in these organs, respectively. Here, we show that the biologically inactive precursor GA_9 moves from ovaries to sepal/petal tissues where it is converted to the bioactive GA_4 necessary for female flower development. Transient expression of a catabolic GA 2-oxidase from pumpkin in cucumber ovaries decreases GA_9 and GA_4 levels and arrests the development of female flowers, and this can be restored by application of GA_9 to petals thus confirming its function. Given that bioactive GAs can promote sex reversion of female flowers, movement of biologically inactive precursors, instead of the hormone itself, might help to maintain floral organ identity, ensuring fruit and seed production.

KEY WORDS: *Cucumis*, Female flower development, Gibberellin signalling, Gibberellin transport

INTRODUCTION

Gibberellins (GAs) form a large group of diterpenoid tetracyclic carboxylic acids, some of which are phytohormones that regulate many plant developmental processes (Fleet and Sun, 2005; Pimenta Lange and Lange, 2006; Yamaguchi, 2008; Mutasa-Göttgens and Hedden, 2009). In higher plants, the final part of the GA biosynthetic pathway is catalysed by enzymes encoded by small multigene families that belong to the class of 2-oxoglutarate-dependent dioxygenases (2-ODDs) (Hedden and Phillips, 2015). As in other cucurbits, in cucumber two principal pathways exist: a major 'non-hydroxylation' and a minor '13-hydroxylation' pathway (Pimenta Lange et al., 2013). Both pathways require four families of ODDs: GA 7-oxidases (GA7ox), GA 20-oxidases (GA20ox), GA 3-oxidases (GA3ox) and catabolic GA 2-oxidases (GA2ox). In the non-hydroxylation pathway, biologically inactive precursor GA_{12}-aldehyde is oxidised to bioactive GA_4, the major plant hormone in cucumber, by GA7ox, GA20ox and GA3ox. The GA_4 is further oxidised by GA2ox to form biologically inactive GA_{34} (Fig. 1A). The 13-hydroxylation pathway is introduced by an early 13-hydroxylation step, by which GA_{12} is converted to GA_{53}. The GA 13-oxidases involved belong to the class of cytochrome P450 monooxygenases (Magome et al., 2013). GA_{53} is further oxidised to bioactive GA_1 and inactive GA_8, in parallel to the non-hydroxylation pathway (Fig. 1A).

Cucurbits form a large plant family, the members of which mostly develop unisexual flowers. Many are important crops, such as cucumber, that frequently serve as model plants for studying hormonal regulation of reproductive development (Pimenta Lange et al., 2012; Boualem et al., 2015). Sex determination and flower development are largely under the control of the plant hormones ethylene and GA, respectively (Cheng et al., 2004; Griffiths et al., 2006; Achard et al., 2007; van Doorn and Kamdee, 2014). Bioactive GA from stamen of bisexual and male flowers is translocated to, and controls the development of, the other floral organs (Koornneef and van der Veen, 1980; Nester and Zeevaart, 1988; Weiss and Halevy, 1989; Goto and Pharis, 1999; Hu et al., 2008; Pimenta Lange et al., 2012). This implies that hormonal control of floral organ development in female flowers is regulated differently than in male flowers, but surprisingly little is known about this process. To address this question, we investigated GA signalling during female flower development in cucumber (Fig. 1B).

RESULTS AND DISCUSSION

Cucumber female flower development

Under our growth conditions, cucumber female flowers appear approximately 5 weeks after sowing. From the day of appearance until fully open, flower development is divided into four stages (Fig. 1B). Approximately 6 days after appearance, flowers reach developmental stage I, when flower buds contain greenish floral organs. Three days later, petal tissues turn yellowish and the flower reaches stage II. Another 4 days later, at stage III, the rapid growth phase starts. One day later, at stage IV, the corolla is fully open.

Endogenous GA precursors accumulate in ovaries and bioactive GAs in sepals/petals

In order to unravel GA status during female flower development, we analysed endogenous GA levels of different floral parts at the four developmental stages by combined gas chromatography-mass spectrometry (GC-MS) (Fig. 1C, Table S1). At stage I the full flower was analysed, and from stage II the flowers were dissected into pedicel, ovary, sepals together with petals (designated sepals/petals), and stigma tissues. In flower buds at stage I, GA levels are lower than in stages II and III. As in other cucurbits, in cucumber female flowers the levels of bioactive and catabolic GAs of the 13-hydroxylation pathway (GA_1, GA_8 and GA_{29}) are very low, although some GA precursors of this pathway accumulate (e.g. GA_{19} in sepals/petals and in stigma tissues), which might disturb 20-oxidation due to substrate competition (Table S1).

GA levels of the non-hydroxylation pathway are particularly high at stage II and III before the flower opens (Fig. 1C, Table S1). Highest levels of GA precursors (GA_{12}, GA_{15} and GA_9) are found in

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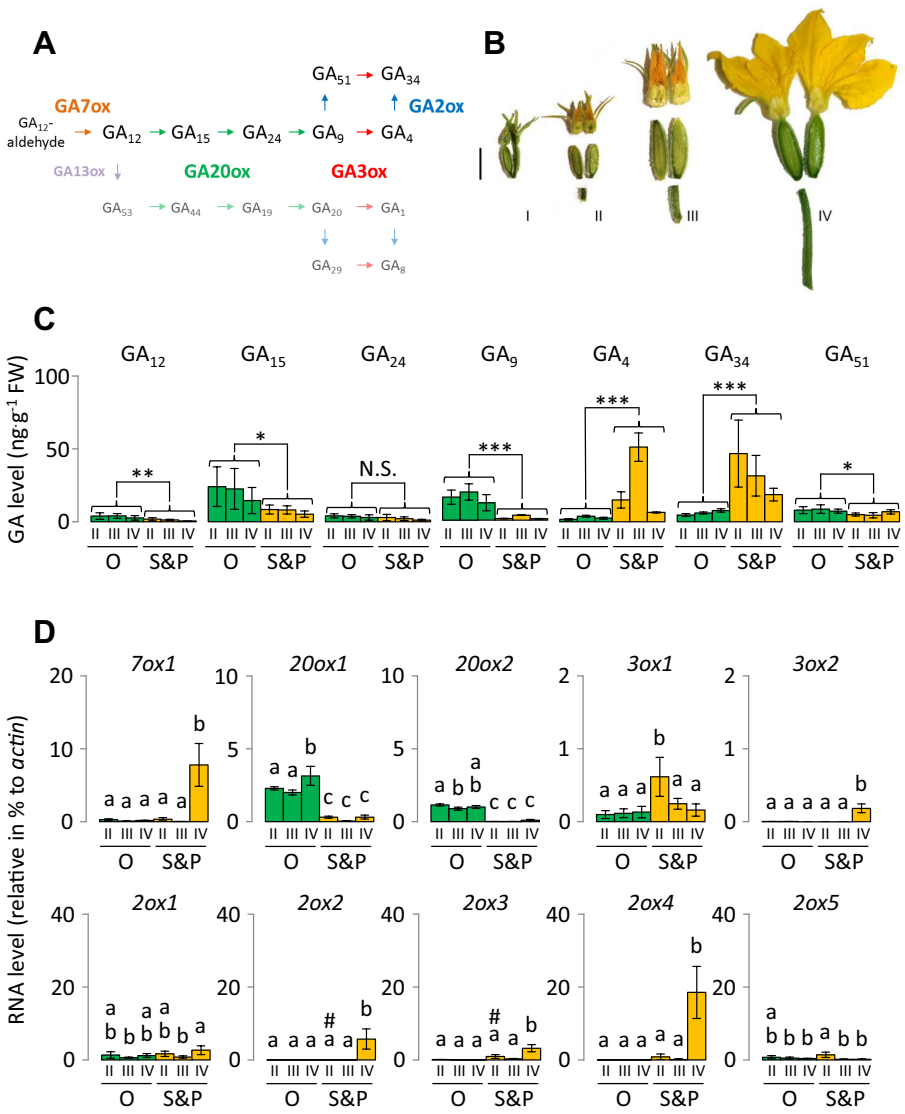

Fig. 1. Gibberellin precursors and GA20ox transcript levels are high in ovary, whereas bioactive GA_4 and GA3ox transcript levels are high in sepals and petals. (A) Final part of the GA biosynthetic pathway in cucumber. Gibberellins (GAs) of the major, 'non-hydroxylation' pathway are in black letters. The '13-hydroxylation' pathway is in grey letters. Enzymes of the pathway are in bold. (B) Longitudinal sections of female flowers showing ovary, style and stigma, and sepals and petals at developmental stages I-IV as described in the text. Scale bar: 1 cm. (C,D) Endogenous GA levels (C) and transcript levels of GA-oxidase genes (D) in ovary (O) and sepals/petals (S&P). Stages of floral organ development as described in B. N.S., not significant; **P*<0.05, ***P*<0.01, ****P*<0.005, *t*-test. Different letters above bars indicate significant differences (*P*<0.05). Average with s.d. of three (or two where indicated by #) biological replicates. FW, fresh weight.

ovaries, and of bioactive GA_4 and catabolic GA_{34} in sepal/petal tissues. Compared with the floral organs, the pedicel contains relatively low GA levels, but GA_{34} and GA_{51} levels are high, which indicates GA catabolism taking place in this organ (Table S1). In stigma, both GA precursors and bioactive GAs are present.

Taken together, these results suggest that ovary and sepals/petals are a rich source of GA precursors and GA hormone, respectively. To further unravel the involvement of the individual floral organs during female flower development, transcripts encoding elements of the GA signalling pathway were quantified.

Ovaries express GA 20-oxidases, whereas GA 3-oxidases are expressed in sepals/petals

The cucumber ovary has high transcript levels for two GA 20-oxidase genes, namely *GA20ox1* and *GA20ox2*, and relatively low levels of GA 3-oxidases and catabolic GA 2-oxidases, which correlates with the accumulation of GA precursors within this organ (Fig. 1D, Table S2). In sepals/petals the transcript levels of one GA 3-oxidase gene, *GA3ox1*, are particularly high at stage II, which correlates with its high bioactive GA_4 synthesis capacity (Fig. 1C,D). By contrast, transcripts of genes that encode GA 20-oxidases, which are important for GA precursor synthesis, are low in sepals/petals (Fig. 1D, Table S2), suggesting GA precursor import from other tissues, such as the ovary. In sepals/petals of the mature flower (stage IV), the levels of transcripts that encode biosynthetic GA7ox1 and GA3ox2 and catabolic GA 2-oxidases (GA2ox2, GA2ox3 and GA2ox4) are high, the latter of which might explain the reduction of biosynthetic GA_4 in mature sepal/petal tissues. Transcripts for other putative GA-oxidases (putative GA3ox5, putative GA3ox6 and putative GA2ox6) recently identified in the cucumber genome (Huang et al., 2015) are absent or at very low levels in ovaries and sepals/petals (Table S3). As a consequence, the distribution of GA-oxidase transcripts suggests split GA biosynthetic pathways between ovary and sepals/petals.

In pedicels, transcript levels for one GA 20-oxidase (GA20ox1), one GA 3-oxidase (GA3ox1) and, particularly, two GA 2-oxidases (GA2ox1 and GA2ox5) are high compared with the floral organs, which might explain the accumulation of GA catabolites in the pedicel (Table S1). In stigma tissues, transcript levels of GA-oxidase-encoding genes are not particularly high, with the exception of biosynthetic GA 7-oxidase (GA7ox1) and three catabolic GA 2-oxidases (GA2ox2, GA2ox3 and GA2ox4) in the mature flower (developmental stage IV, Table S2). Similar to sepals/petals, the GA 2-oxidases might account for the decrease in

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bioactive and the increase in catabolic GAs in the stigma at this stage (Table S1). However, relatively high levels of GA precursors, as found in stigma tissues from flowers at stage II and III, and of bioactive GA_4, as found at stage III, indicate import from other floral organs, such as ovary and the sepals/petals, respectively.

Transcripts encoding GA receptors (GID1a, GID1b) and DELLA growth repressors (DELLA1, DELLA2, DELLA3, DELLA4) are highly expressed in all floral parts at all developmental stages analysed (Table S4), the significance of which remains obscure in terms of the regulation of female flower development. We conclude that GA concentration is the primary means of regulating cucumber flower development.

Precursor d2-GA_9 moves from ovaries and accumulates as bioactive d2-GA_4 in sepals/petals

Our data suggest the translocation of GAs between ovary and sepal/petal tissues. Local and cell-to-cell transport of bioactive GAs within plants has been observed previously (Hu et al., 2008; Pimenta Lange et al., 2012; Shani et al., 2013) and bioactive GAs can be translocated even over long distances (Katsumi et al., 1983; Eriksson et al., 2006; Hu et al., 2008). However, recently it has been shown that the biologically inactive precursor GA_{12}, a GA precursor emerging earlier in the biosynthetic pathway (Fig. 1A), is the major GA that is mobile over long distances in *Arabidopsis* (Regnault et al., 2015).

To investigate the translocation of GAs, deuterated (17,17-d2) GAs were injected into the centre of ovaries of female flowers at developmental stage III. One day later, at stage IV, flowers were dissected into the different floral organs and GAs were analysed by determining the ratio of characteristic deuterium-labelled to unlabelled ions for each GA by combined GC-MS (Table 1, Table S5). GA_{12} isolated from the ovaries is highly 17,17-d2 labelled, but the deuterium label is weak in GA_{12} extracted from pedicel, sepals/petals and stigma, suggesting that most of the 17,17-d2-GA_{12} stays at the site of injections and only a little moves from the ovary to the other floral organs. Incorporation of the deuterium label into other GAs of the pathway is low, which might indicate that little of the injected 17,17-d2-GA_{12} reaches the site of 20-oxidation in floral organs, including the ovary, and the pedicel.

After injection of 17,17-d2-GA_9 into the ovary, the deuterium label was strong in GA_9, GA_4 and GA_{51} extracted from all floral organs, except in GAs extracted from the pedicel (Table 1, Table S5). These results indicate an efficient translocation of GA_9 (and possibly GA_4 and GA_{51}) from the ovary into the other floral organs, but not into the pedicel. After injection of 17,17-d2-GA_4 into the ovary, GA_4 extracted from this organ was highly 17,17-d2 labelled (Table 1), as endogenous GA_4 levels are low in this organ (Table 1). Also, GA_4 extracted from sepals/petals was deuterium labelled, indicating translocation of bioactive GA_4 from the ovary into these organs in a manner that could be similar to that of GA_9 (Table 1). However, given that the ovary contains high levels of endogenous GA_9 and low levels of endogenous GA_4 (Fig. 1C), bioactive GA_4 translocation from this organ is likely to be of limited importance for female flower development.

Table 1. Deuterated precursor GA_9 moves from ovary to sepals/petals

	17,17-d2-GA_{12}		17,17-d2-GA_9		17,17-d2-GA_4	
d2-GA/GA ratio	Ovary	Sepals/petals	Ovary	Sepals/petals	Ovary	Sepals/petals
GA_{12}	8.7±3.1	1.4±0.8				
GA_{15}	0.3±0.1	0.3±0.1				
GA_{24}	0.1±0.0	0.1±0.0				
GA_9	0.1±0.0	0.1±0.0	3.6±0.7	4.6±0.6		
GA_4	0.2±0.1	0.2±0.1	2.6±0.7	3.9±1.3	97±1.4	5.9±1.3
GA_{34}	0.2±0.0	0.2±0.0	0.5±0.1	0.4±0.0	5.5±1.3	0.8±0.1
GA_{51}	0.1±0.0	0.1±0.0	2.1±0.1	2.1±0.7		

Deuterated 17,17-d2-GA_{12}, -GA_9 or -GA_4 (100 ng each) were injected into ovaries. d2-GA/GA ratio refers to the intensity of characteristic 17,17-d2-labelled relative to unlabelled ions (e.g. d2-GA_{12}/GA_{12}), shown as the average with s.d. of three biological replicates.

Injection of labelled GAs into the cucumber ovary and the distribution of endogenous GAs in the female flower thus revealed that the biologically inactive precursor GA_9 is the major transported GA from ovary to sepals/petals in cucumber female flowers. Similarly, Proebsting et al. (1992) reported that the biologically inactive precursor GA_{20}, a product of the GA 20-oxidase, is the major transported GA from leaves to apices in pea.

Transient expression of pumpkin GA 2-oxidase injected into ovaries arrests cucumber female flower development

Because no efficient protocol for cucumber transformation is available to confirm the function of specific GAs for female flower development, transient expression of pumpkin catabolic *CmGA2ox1* (Radi et al., 2006) was performed in cucumber ovaries. This catabolic pumpkin enzyme acts on both GA_9 and GA_4 (Frisse et al., 2003) (Fig. 1A). *Agrobacterium tumefaciens* harbouring a *CmGA2ox1* construct, either in antisense or sense orientation, was injected into the ovaries at developmental stage II. Five days later, at developmental stage IV, flowers expressing *CmGA2ox1* in antisense orientation develop normally, whereas the development of flowers expressing *CmGA2ox1* in sense orientation is arrested at stage II (Fig. 2A,B) and abort 1 week after the treatment (Fig. 2C). *A. tumefaciens* infection was localised to the ovary without spreading to sepals/petals (Table S6). In flowers, expressing the antisense construct, levels of the four GAs (GA_9, GA_4, GA_{34} and GA_{51}) analysed are high. However, in the arrested flowers expressing *CmGA2ox1* in sense orientation, precursor GA_9 and bioactive GA_4 levels are very low and undetectable, respectively (Table 2).

Application of 17,17-d2-GA_{12} to the petals of flowers expressing the *CmGA2ox1* sense construct in ovaries does not restore flower development (Fig. 2D). However, application of 17,17-d2-GA_9 to the petals fully restores flower development (Fig. 2E), indicating that GA_9 is essential and sufficient for normal female flower development. Only very little of the deuterated GAs were translocated from the sepals/petals/stigma to the ovary and the 17,17-d2 label of GA_{12} was hardly incorporated into other GAs of the biosynthetic pathway (Table 3). These results might indicate a lack of 20-oxidase activity in sepal/petal tissues, as expected on the basis of transcript analysis (Fig. 1D, Table S2). In addition, similar to the situation in ovaries (see above), the exogenously applied GA_{12} might not reach the site of GA 20-oxidation within the petals. However, the 17,17-d2 label of GA_9 was incorporated into bioactive GA_4 and into catabolic GA_{34} and GA_{51}, reflecting high expression of GA 3-oxidase and GA 2-oxidases, as found for sepal/petal tissues at developmental stage IV (Fig. 1D, Table 3).

The absolute requirement of GAs for cucumber female flower development resembles what has been described for bisexual flower development (Goto and Pharis, 1999; Griffiths et al., 2006). Moreover, our results suggest a limited importance of GAs for early ovary development. Two other hormones, ethylene and auxin, are known to control this process (Zhang and O'Neil, 1993) and their translocation from other floral organs might be necessary for ovary development.

Taken together, our results show that ovary-derived GA precursor GA_9, although biologically inactive, moves to sepals/petals, where

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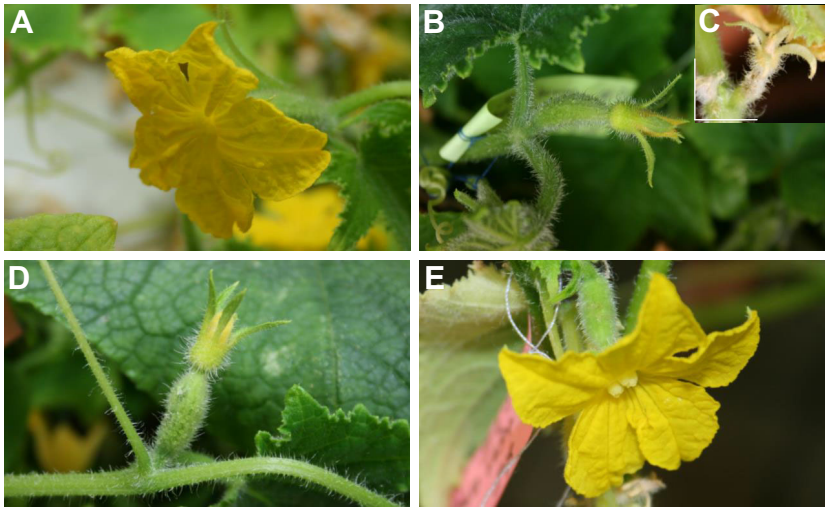

Fig. 2. Transient expression of catabolic pumpkin *CmGA2ox1* in cucumber ovaries arrests female flower development, and this is rescued by deuterated GA_9 applied to the petals. (A) Representative cucumber female flowers expressing *CmGA2ox1* antisense (100% fully open, *n*=8, corresponding to developmental stage IV, see Fig. 1B) or (B,C) *CmGA2ox1* sense (83% arrested at stage II, *n*=24) expressed in ovaries. See Table 2. (D) *CmGA2ox1* sense expressed in ovaries with an additional 50 ng 17,17-d2-GA_{12} (83% arrested at stage II, *n*=6) or (E) 17,17-d2-GA_9 (92% fully open, stage IV, *n*=13) added to the petals. See Table 3. Shown at 5 (A,B,D,E) or 7 (C) days after the treatment.

it is converted to bioactive GA_4 just before the rapid growth phase to promote anthesis of the female flower. Our findings also imply that female organs have a more prominent, yet underestimated, role in GA control of flower growth and development. Bioactive GA is necessary for stamen development in early flowers (Hu et al., 2008) and also promotes sex reversion of female flowers (Peterson and Anhder, 1960; Galun, 1961; Shifriss and George, 1964). Translocation of the GA precursor instead of the hormone has recently been proposed for regulating sex expression of fern (Tanaka et al., 2014). In a similar manner, translocation of GA as a biologically inactive precursor might help to maintain ovary identity, while stamen development is suppressed, realising site-specific GA-regulated sepal/petal development in female flowers.

MATERIALS AND METHODS

Plant material and growth conditions

Cucumber (*Cucumis sativus* var. 'Hokus') seeds (Botanischer Garten der Technischen Universität Braunschweig) were imbibed for 2 h and sown in soil:vermiculite (2:1 v/v). Germination and growth conditions were as described for pumpkin (Lange et al., 2005).

Translocation of deuterated GAs

Deuterated GAs (5 µl containing 100 ng 17,17-d2-GA_{12}, -GA_9 or -GA_4 in 0.5% Tween-20) were injected into the centre of ovaries of *C. sativus* female flowers at development stage III (Fig. 2, ovaries 1.1-1.3 cm length). Flowers of each treatment were harvested 1 day later at stage IV, separated into different parts (pedicel, ovary, petals plus sepals, and stigma) and frozen immediately in liquid nitrogen.

Transient expression of *CmGA2ox1*

Cells of *A. tumefaciens* EHA105 expressing sense or antisense copies of *Cucurbita maxima* (*Cm*) *GA2ox1* cDNAs (Radi et al., 2006) grown in LB medium plus antibiotics were harvested and resuspended in 10 mM MES buffer containing 10 mM $MgCl_2$ and 200 µM acetosyringone to a final OD_{600} of 1.0, modified according to Shang et al. (2014). After incubation at room temperature for 2 h, 5 µl of the suspension was injected into the centre of the ovaries of *C. sativus* female flowers at the beginning of developmental stage II (Fig. 2, ovaries 0.6-0.7 cm length). *A. tumefaciens* infection was quantified by real-time PCR using vector- and *CmGA2ox1*-specific primers (Radi et al., 2006). For quantitative analysis of endogenous GAs, 5 days later three full flowers of each treatment were harvested and analysed as described below.

For restoring flower formation, *A. tumefaciens* expressing sense copies of *CmGA2ox1* cDNAs were injected into the ovary as described above and, simultaneously, 17,17-d2-GA_{12} or 17,17-d2-GA_9 (5 µl, 50 ng each in 0.5% Tween-20) was applied onto the petals of each flower. Three flowers were harvested 5 days later, separated into ovary and petals/sepals/stigma and frozen immediately in liquid nitrogen.

Table 2. *CmGA2ox1* in cucumber ovaries reduces endogenous GA levels in the female flowers

GA	*CmGA2ox1* antisense	*CmGA2ox1* sense
GA_9	19±3.5	0.9±1.2**
GA_4	12±4.8	0.0±0.0*
GA_{34}	13±0.3	8.2±2.9
GA_{51}	8.1±0.8	3.2±1.1*

Levels are given as ng/g FW. *P<0.05, **P<0.01, *t*-test. Average with s.d. of two (*CmGA2ox1* antisense) or three (*CmGA2ox1* sense) biological replicates (see Fig. 2A,B).

Gene expression analysis

Total RNA extraction, cDNA synthesis and real-time PCR analysis were performed essentially as described previously (Pimenta Lange and Lange, 2015). *Actin* (AB010922) was used as a reference gene for normalisation of the real-time PCR assays (Wan et al., 2010). The relative expression level of each gene was averaged over two repeats. qRT-PCR analyses were performed on at least two biological replicates. Primers are listed in Table S7.

Quantitative analysis of endogenous GAs

Quantitative analysis of endogenous GA levels by GC-MS was performed as described previously (Lange et al., 2005).

Table 3. Deuterated precursor GA_9 is metabolised in petals

	17,17-d2-GA_{12} petal application		17,17-d2-GA_9 petal application	
d2-GA/GA ratio	Ovary	Sepals/petals/stigma	Ovary	Sepals/petals/stigma
GA_{12}	0.2±0.1	11.7±4.2	–	–
GA_{15}	0.1±0.0	0.1±0.0	–	–
GA_{24}	0.0±0.0	0.0±0.0	–	–
GA_9	0.0±0.0	0.1±0.1	0.0±0.0	1.3±0.6
GA_4	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.1
GA_{34}	0.1±0.0	0.1±0.0	0.3±0.3	0.9±0.4
GA_{51}	0.1±0.0	0.1±0.1	0.2±0.1	5.2±2.0

Deuterated GAs were applied to petals of female flowers expressing transiently CmGA2ox1 in ovaries (see Fig. 2D,E). d2-GA/GA ratio refers to the intensity of characteristic 17,17-d2-labelled relative to unlabelled ions (e.g. d2-GA_{12}/GA_{12}) for GAs from ovary or sepal/petal/stigma tissues. Average with s.d. of three biological replicates.

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Statistics

Statistical analyses were performed using SPSS statistics software (IBM, version 24). For experiments shown in Fig. 1C and Table 2 the significance was determined using Student's *t*-test and in Fig. 1D by analysis of variance (ANOVA) using the least significant difference (LSD).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization, methodology, validation, formal analysis, investigation, writing - original draft preparation, review and editing, visualization and supervision: M.J.P.L. and T.L.

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Supplementary information

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