J Plant Growth Regul (2010) 29:194–209
DOI 10.1007/s00344-009-9124-5

Endogenous Gibberellin Profile During Christmas Rose (*Helleborus niger* L.) Flower and Fruit Development

Belay T. Ayele · Volker Magnus · Snježana Mihaljević · Tatjana Prebeg · Rozelinda Čož-Rakovac · Jocelyn A. Ozga · Dennis M. Reinecke · Lewis N. Mander · Yuji Kamiya · Shinjiro Yamaguchi · Branka Salopek-Sondi

Received: 29 June 2009 / Accepted: 9 October 2009 / Published online: 3 December 2009

Abstract Gibberellins (GAs) were identified and quantified during flower and fruit development in the Christmas rose (*Helleborus niger* L.), a native of southeastern Europe with a long international horticultural tradition. Physiologically, the plant differs from popular model species in two major respects: (1) following anthesis, the initially white or rose perianth (formed in this species by the sepals) turns green and persists until fruit ripening, and (2) the seed is shed with an immature embryo, a miniature endosperm, and a prominent perisperm as the main storage tissue. GA_1 and GA_4 were identified by full-scan mass spectra as the major bioactive GAs in sepals and fruit. LC-MS/MS system in accord with previously verified protocols also afforded analytical data on 12 precursors and metabolites of GAs. In the fruit, GA_4 peaked during rapid pericarp growth and embryo development and GA_1 peaked during the subsequent period of rapid nutrient accumulation in the seeds and continued pericarp enlargement. In the sepals, the flux through the GA biosynthetic pathway was highest prior to the light green stage when the photosynthetic system was induced. Unfertilized, depistillated, and deseeded flowers became less green than the seed-bearing controls; chlorophyll accumulation could be restored by applying GA_1, GA_4, and, less efficiently, GA_3 to the deseeded fruit. The sepals of unfertilized and depistillated flowers indeed contained very low levels of GA_4 and gradually decreasing levels of GA_1. However, the concentrations of their precursors and metabolites were less affected. These data suggest that a signal(s) from the fruit stimulates GA biosynthesis in the sepals resulting in greening. The fruit-derived GAs appear to be mainly involved in pericarp growth and seed development.

Keywords Christmas rose · Fruit and seed development · Gibberellin identification and quantification · *Helleborus niger* L. · Perianth greening

We wish to dedicate this article to the memory of the late Dr. Volker Magnus, our dear colleague and friend, who was greatly involved in this project. Dr. Volker Magnus passed away on July 30, 2009.

Electronic supplementary material The online version of this article (doi:10.1007/s00344-009-9124-5) contains supplementary material, which is available to authorized users.

B. T. Ayele · Y. Kamiya · S. Yamaguchi
RIKEN Plant Science Center, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

V. Magnus · S. Mihaljević · T. Prebeg · R. Čož-Rakovac · B. Salopek-Sondi
Rudjer Bošković Institute, P.O. Box 180, 10002 Zagreb, Croatia

J. A. Ozga · D. M. Reinecke
Department of Agricultural, Food, and Nutritional Science, University of Alberta, Edmonton, AB T6G 2P5, Canada

L. N. Mander
Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia

B. T. Ayele
Department of Plant Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

B. Salopek-Sondi (✉)
Department of Molecular Biology, Ruđer Bošković Institute, Bijenička cesta 54, 10 000 Zagreb, Croatia
e-mail: salopek@irb.hr

Introduction

Except for the model plant *Arabidopsis thaliana*, studies on the metabolism and mode of action of gibberellins (GAs) (Hedden and Kamiya 1997; Hedden and others 2002;

MacMillan 2002; Sponsel and Hedden 2004; Yamaguchi 2008) have been focused mostly on crop species to improve performance and yields (Hedden and Phillips 2000; Hedden 2003). Ornamentals have been treated with exogenous GAs to improve marketability (Halevy 1986), and growth retardants have been applied to manipulate GA biosynthesis and plant size (Rademacher 2000), but endogenous GA physiology has received little attention (Greyson and Tepfer 1967; Greyson and Raman 1975; Weiss and others 1995). This is unfortunate because understanding the biochemical background of an aesthetically or economically important trait will help to minimize the input necessary to make an ornamental perform to market requirements.

The role of GAs in reproductive development (Pharis and King 1985) has been explored in species such as pea (Rodrigo and others 1997; Swain and others 1997; Ozga and Reinecke 2003; Ozga and others 2003), tomato (Rebers and others 1999), tobacco (Itoh and others 1999), rice (Kaneko and others 2003), and *Arabidopsis thaliana* (Fei and others 2004; Hu and others 2008). Work with male-sterile mutants (reviewed by Sawhney and Shukla 1994) and gene expression studies identified the tapetum and pollen grains in the anthers as a major source of GAs directing early flower development (Itoh and others 1999; Kaneko and others 2003). Particularly detailed experiments in *Arabidopsis* (Hu and others 2008) related flower and fruit development to the differential expression of the four genes of GA 3-oxidase that exist in this species.

Here we investigate flower and fruit development in the Christmas rose (*Helleborus niger* L.) which has been classified as a basic dicot (Finch-Savage and Leubner-Metzger 2006) by taxonomists. The genus *Helleborus* comprises about 20 species of Old World perennials with large (3 cm and above), showy spring flowers in various shades of purple, green, and white (Schiffner 1891; Damboldt and Zimmermann 1965; Mathew 1989). Both the original species and the hybrid cultivars are becoming increasingly popular as ornamentals (Burrell and Tyler 2006). Most of them retain the perianth (formed in this species by the sepals) until seed ripening, with its color usually changing to a greenish hue. *H. niger* has an exceptionally long flowering period (2-6 months starting around Christmas) and particularly large flowers (6–13 cm), which change color from white to intensely green, both mixed with purple in sun-exposed locations (Salopek-Sondi and Magnus 2007). The accumulation of chlorophyll in the perianth is linked to the presence of developing fruit, which makes the fertilized Christmas rose flower an interesting model for the induction of photosynthetic activity by an assimilate requiring "sink" tissue. The greening process in the perianth of unfertilized or depistillated flowers stalls at an early stage but could be stimulated to completion by treating the sepals with gibberellins and cytokinins (Salopek-Sondi and others 2002). Here we show that sepal greening can be induced by applying GA_1, GA_3, or GA_4 to the deseeded ovaries. GA_1 and GA_4 were also identified as the major physiologically active, endogenous GAs in the ovaries and other floral tissues. Their levels, along with those of their precursors and catabolites, were quantified in different floral tissues and fruit at different stages of development and in depistillated and unfertilized flowers. Their concentration dynamics are discussed in terms of sepal greening and pericarp and seed development.

Materials and Methods

Plant Material

Flowers of the Christmas rose (*Helleborus niger* L. ssp. *niger* sensu B. Mathew 1989) were collected at two locations in four different growth seasons. Habitat 1 was a natural mountain forest at Gorski kotar, Croatia (altitude: 800 m, dominant tree species: *Abies alba* Mill., *Fagus silvatica* L., *Picea abies* L.). Habitat 2 was a woodlot in the botanical garden of the Faculty of Pharmacy and Biochemistry of the University of Zagreb. The material collected to gain insight into GA dynamics during the whole life cycle of a Christmas rose flower is listed in Table 1 and comprises a series of six developmental stages sampled in 2008 and replicates for most of these stages from 2002, 2003, or 2004 (all samples from habitat 1). Two stages of anthesis were recognized. After bud opening, the (proterogynous) flowers first passed through their 'Female' phase, during which the stigmas were receptive, while the immature anthers were arranged in a ring at the base of the carpels. The 'Male' phase began with the elongation of the filaments and ended with anther abscission. Developing fruits were sampled at four stages that were distinguished by the degree of chlorophyll accumulation in the sepals (which persisted until fruit maturity): 'Light green' (the first greenish tinge), 'Green' (greening well advanced), 'Half ripe' (seeds half ripe, sepal greening reaching a stationary level), and 'Almost ripe' (seeds almost ripe, sepals at the approximate end of the stationary phase after which chlorophyll levels started to decline). To define the developmental stages sampled in a more quantitative fashion, they were also characterized by the weights of the sepals and the pistils or the developing fruit, by fruit lengths, and by chlorophyll meter readings (Table 1).

Unfertilized flowers were harvested in habitat 1, following weather conditions that excluded pollinating insects during the period of stigma receptivity. They were also "produced" (habitat 2) by enclosing emasculated flowers

Table 1 Developmental stages of Christmas rose flowers used for quantitative GA analysis

Developmental stage	Pistil/fruit cluster[a]			Perianth[a]		
	FW (g)	DW (g)	Length[b] (mm)	FW (g)	DW (g)	Chlorophyll meter reading
'Female' phase, 2008	0.12	0.016	Body: 10 Beak: 8	0.81	0.081	0.9
'Female' phase, 2003	0.17	0.027	nd[c]	1.01	0.131	0.9
'Male' phase, 2008	0.24	0.032	Body: 11 Beak: 10	1.64	0.181	0.9
'Male' phase, 2004	0.23	0.033	nd[c]	1.76	0.176	0.9
'Light green,' 2008	0.58	0.080	Body: 15 Beak: 10	1.45	0.193	1.9
'Light green,' 2002	0.75	0.130	Body: 16 Beak: 11	1.33	0.201	nd[c]
'Green,' 2008	1.08	0.158	Body: 17 Beak: 11	1.43	0.205	3.0
'Half ripe,' 2008	2.56	0.321	Body: 25 Beak: 13	1.23	0.208	4.1
'Half ripe,' 2002	3.47	0.471	Body: 26 Beak: 13	1.39	0.187	nd[c]
'Almost ripe,' 2008	3.83	0.589	Body: 27 Beak: 13	1.22	0.186	4.1

[a] The FW and DW values refer to the pistil/fruit cluster, and the entire perianth (all sepals taken together) of a single flower. The values are averages of FW and DW from 6 to 23 flowers

[b] The body of the pistils/fruit corresponds to the main part of the ovary containing the ovules/seeds. The beak is the very top of the ovary narrowing into the style and the stigma

[c] Not specifically determined; the values may be assumed to be similar to those for the 2008 samples

in sacks of gauze to prevent cross-pollination. In depistillated flowers, pistils and anthers were surgically removed shortly before bud opening.

In the flowers collected for hormone analysis, the sepals were separated from the cluster of pistils or developing fruit (follicles), and the stamens and nectaries were discarded. Some nearly ripe fruits were separated into seeds and pericarps. The plant material was stored at -80°C. The samples selected for GA analysis were freeze-dried and ground to a powder to facilitate shipping.

Microscopy

Paraplast-embedded material was prepared according to Ruzin (1999). Carpels with seeds were collected, fixed immediately in FAA (50% ethanol, 10% formalin, 5% glacial acetic acid) overnight (16 h) at 4°C, processed through a dehydration series of ethanol and xylene, embedded in paraplast (Fluka), and then sectioned with a rotary microtome (Leica RM 2255). The 7-µm-thick sections were deparaffinized, rehydrated, and stained with hematoxylin. The sections were observed under an Olympus BX51 light microscope.

For histological and ultrastructural studies, tissue was fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 30 min at 4°C, and postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C. After dehydration in a graded series of ethanol, the tissue was embedded in Spurr's resin. Semithin sections (1 µm thick) of the tissue were stained with a mixture of 2% toluidine blue and 2% borax (1:1) and examined using an Olympus BX51 light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Zeiss EM10A electron microscope (operating at 60 kV accelerating voltage).

GA Treatment of Cut Christmas Rose Flowers

Christmas rose flowers in the 'Male' phase of anthesis were harvested in habitat 1 by cutting their scapes at ground level. They were supplied with distilled water as needed and kept at 5°C (which is around the average temperature at which the flowers develop under natural conditions), under fluorescent light ($\sim$ 40 µmol m^{-2} s^{-1} PAR), at a day/night cycle of 10/14 h. The top 0.5 mm of the ovaries, and the attached styles and stigmas were removed, and the

ovules were extracted using a Kerr dental needle. Cotton plugs were inserted into the cavities of the ovaries and immediately moistened with 30 µl of water. Aqueous solutions (30 µl per cluster of ovaries) containing GA_1, GA_3, or GA_4 (20, 50, or 100 µM) were then applied daily for 40 days. Distilled water was used in the controls. Fertilized cut flowers that received no hormone treatment were also kept under the same conditions. Sepal greening was monitored *in situ* using a Chlorophyll Content Meter (CCM-200, Opti-Sciences, Inc., Hudson, NH, USA) which afforded chlorophyll levels in arbitrary units based on the difference of the absorbances at 600-700 nm (attributed to chlorophyll a + b) and at 900-1000 nm (attributed to light scattering by cell structures and nonspecific absorbance). Measurements were made for each sepal separately in its subterminal part. Evaluations were based on the mean chlorophyll levels of the three greenest adjacent sepals. The number of flower replicates included in the treatments varied depending on availability and is stated in the descriptions of the individual experiments.

Analysis of Endogenous GAs

To assess the dynamics of the GA status during the perianth and fruit development of the Christmas rose, endogenous GA levels (precursors, bioactive GAs, and their catabolites) were determined in the different tissues by LC-MS/MS [consisting of a quadrupole/time-of-flight tandem mass spectrometer (Q-Tof Premier; Waters, Milford, MA, USA) and an Acquity Ultra Performance Liquid Chromatography (Waters) equipped with a reversed-phase column (Acquity UPLC BEH-C18; Waters)] using $^2H_2$-labeled GAs as internal standards, as described previously (Varbanova and others 2007). Briefly, the lyophilized tissues (200 mg DW per sample) were homogenized with 80% acetone (5 ml/100 mg DW), and 500 pg of each [17, 17-$^2H_2$]GA was added to each extract. The acetone extract was incubated at -20°C for about 12 h, filtered through a Bond Elut reservoir (Varian, Palo Alto, CA, USA), and then evaporated to dryness. The extract residue was dissolved in 4 ml of 50% aqueous acetonitrile and partitioned three times against *n*-hexane (2 ml). The aqueous phase was evaporated to complete dryness. The residue was dissolved in 1 M potassium phosphate buffer (1 ml), loaded onto a Bond Elut reservoir, which had a head of about 3 ml of insoluble polyvinylpyrrolidone suspended in water, and then eluted with 1 M potassium phosphate buffer. The pH of this eluate was adjusted to 3 with 6 N HCl loaded onto a reversed phase cartridge (Oasis HLB, 60 mg; Waters), which was preconditioned with 100% acetonitrile followed by 2% aqueous formic acid. The column was then washed with 2% aqueous formic acid and eluted with 80% acetonitrile containing 1% formic acid. The eluate was dried completely using a Speedvac concentrator, dissolved in methanol, and loaded onto an ion-exchange column (Bond Elut DEA, 500 mg; Varian), which was then washed with 100% methanol before elution of the GAs with methanol containing 0.5% acetic acid. The eluate was evaporated to dryness. The residue was dissolved in chloroform:ethylacetate (1:1 v/v) containing 1% acetic acid, loaded onto a Bond Elut SI cartridge (100 mg; Varian), preconditioned with ethyl acetate and chloroform:ethylacetate (1:1 v/v) containing 1% acetic acid, and eluted with chloroform:ethylacetate (1:1 v/v) containing 1% acetic acid. The GA-containing eluate was evaporated and the residue was dissolved in 20 µl of 2% aqueous formic acid prior to LC-MS/MS analysis.

The samples were eluted at 200 µl min^{-1} using the following linear gradient of water (solvent A) and acetonitrile containing 0.05% acetic acid (v/v) (solvent B): 3% solvent B for 0.1 min, gradient to 65% solvent B in 20 min, and isocratic 98% solvent B for 2 min. GAs were identified by comparing the LC retention times, and the MS/MS prominent ions (both molecular and fragment ions) of the endogenous GAs with their respective internal standards. Endogenous GA levels were calculated by generating calibration curves from serially diluted unlabeled GAs against fixed concentrations of ^2H-labeled GAs.

Results and Discussion

Flower and Fruit Development

Depending on the weather (mainly temperature and snow cover), the life cycle of a Christmas rose flower takes from about 2 to almost 6 months. Rather than by a time scale, the developmental stages used for endogenous GAs levels analyses were thus classified by morphological characteristics, as shown in Table 1. Bud opening is followed by a period during which only the pistils are receptive ('Female' phase). Depending on ambient temperatures, the anthers usually mature 1-2 weeks later ('Male' phase), ideally after cross-pollination has been accomplished. In the fruit, a "body" and a "beak" could be distinguished (Table 1; Fig. 1A, C). The former arose from the main (lower) part of the ovary and contained the seeds. Its length increased about threefold during ripening. The beak is formed from the narrowing tip of the ovary, the style, and the stigma. Its length approximately doubled during ripening. Overall fruit growth proceeded in concert with sepal greening. Most fresh weight (FW) and dry weight (DW) accumulation in the fruit (6- to 7-fold from the 'Light Green' to the 'Almost Ripe' stage; Table 1) occurred when chlorophyll accumulation in the sepals was well advanced.

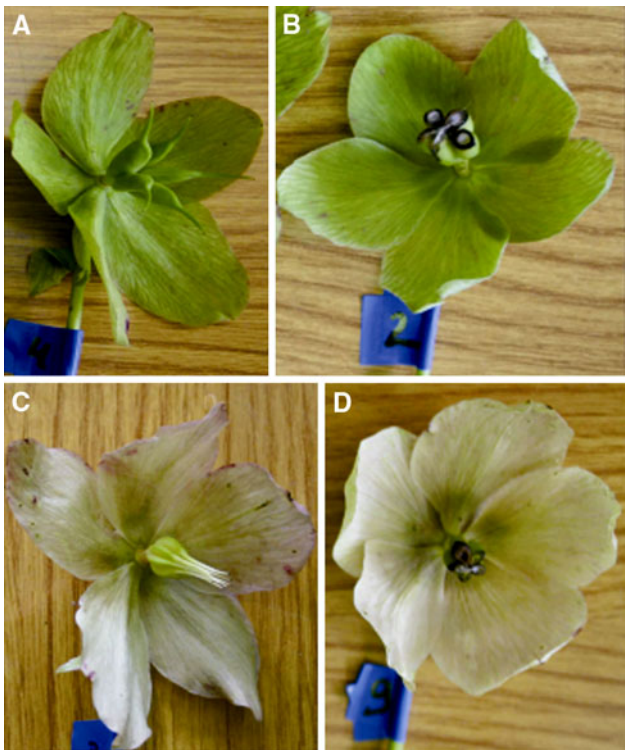

Fig. 1 Effect of deseeding and hormone treatment on sepal greening. At the end of the male phase of anthesis, the tops of the pistils were cut off and the (possibly fertilized) ovules were removed. Cotton plugs were inserted in their place and moistened daily with 30 µl of 50 µM GA_3 (**B**) or with the same volume of water (**D**). For comparison, a flower with normally developing fruit is shown in panel **A** and an unfertilized flower in panel **C**. All four flowers were collected (and deseeded if applicable) on the same day and kept with their scapes in distilled water, at +5°C, under fluorescent light. The photographs were taken after 30 days of treatment

The histological, ultrastructural, and biochemical changes in the greening sepals have already been described in detail (Salopek-Sondi and others 2000, 2002). In brief, during anthesis, only the guard cells contain chloroplasts, resulting in overall chlorophyll levels below 1 µg g FW^{-1}. After fertilization, chloroplasts develop in the entire sepal, apparently by metamorphosis of leucoplasts. The 'Light Green' and 'Almost Ripe' stages in Table 1 correspond to chlorophyll levels of 50 and 350 µg g FW^{-1}. The metamorphosis of the sepals includes growth based on cell enlargement accompanied by substantial expansion of intercellular spaces (Salopek-Sondi and others 2002). The differences in sepal weight beyond the 'Green' phase (Table 1) reflect interindividual variability in the source population.

Selected stages of seed development are shown in Fig. 2. While the stigmas were receptive ('Female' phase) (Fig. 2A, C), the ovule was still differentiating; the embryo sac with egg apparatus became clearly visible while the flower was morphologically in its 'Male' phase (Fig. 2B, D, E, F). After fertilization, embryo development proceeded to reach the heart stage when the seeds were about half ripe (Fig. 2G). Even at seed ripening (Fig. 2H), the embryo was only in an early cotyledonary stage (Tarkowski and others 2006). About 3 months of after-ripening at room temperature and 2 months at 4°C were required for the embryos to differentiate clearly into cotyledons and hypocotyl-radicle (Fig. 2I), thus enabling the seeds to germinate.

GA Effects in Deseeded Flowers

We previously induced an *in planta* greening response in the perianths of depistillated Christmas rose flowers by applying GA-containing lanolin pastes to the sepals (Salopek-Sondi and others 2002). To test for possible GA transport from the developing fruit to the sepals, the ovaries were opened at their tips and the ovules were replaced by cotton plugs which were moistened daily with a GA solution or with water as a control. To exclude interference by signaling compounds imported from the mother plant, the flowers were separated from the latter and kept in distilled water at 5°C under fluorescent light. In general, cut Christmas rose flowers survived for extraordinarily long periods of time under these conditions, depending on the stage at which they were picked and on the experimental treatment they received. For instance, a flower depistillated at the bud stage showed no obvious signs of senescence for more than 6 months.

An initial deseeding experiment was started at the end of the 'Male' phase of anthesis (plant material collected in habitat 1), when the anthers had just abscised and the sepals were still white. The treatments included (1) 50 µM GA_3 ($n = 5$) and (2) water ($n = 10$). A set of intact cut flowers was kept for comparison, but only some ($n = 3$) proved to be fully fertilized, as judged by the growth rates of the follicles (fertilized flowers were not clearly recognizable at the end of the 'Male' phase, when the plant material for the experiment was collected). The response for four typical examples after 30 days of treatment is shown in Fig. 1. The mean chlorophyll meter readings for the three greenest adjacent sepals at the time when the photographs were taken were 3.4 ± 0.3 for the cut fertilized flowers (Fig. 1A), 2.1 ± 0.5 for the unfertilized ones (Fig. 1C), 3.6 ± 0.3 for the deseeded GA_3-treated ones (Fig. 1B), and 2.0 ± 0.2 for the deseeded water controls (Fig. 1D). A set of 12 green flowers freshly collected at habitat 1, at about the same time, gave a mean chlorophyll meter reading of 3.3 ± 0.2. GA_3 application to the deseeded follicles could thus replace the effect of developing seeds on sepal greening (Fig. 1A, B).

A more detailed deseeding experiment was started in the 'Male' phase of anthesis (plant material collected in

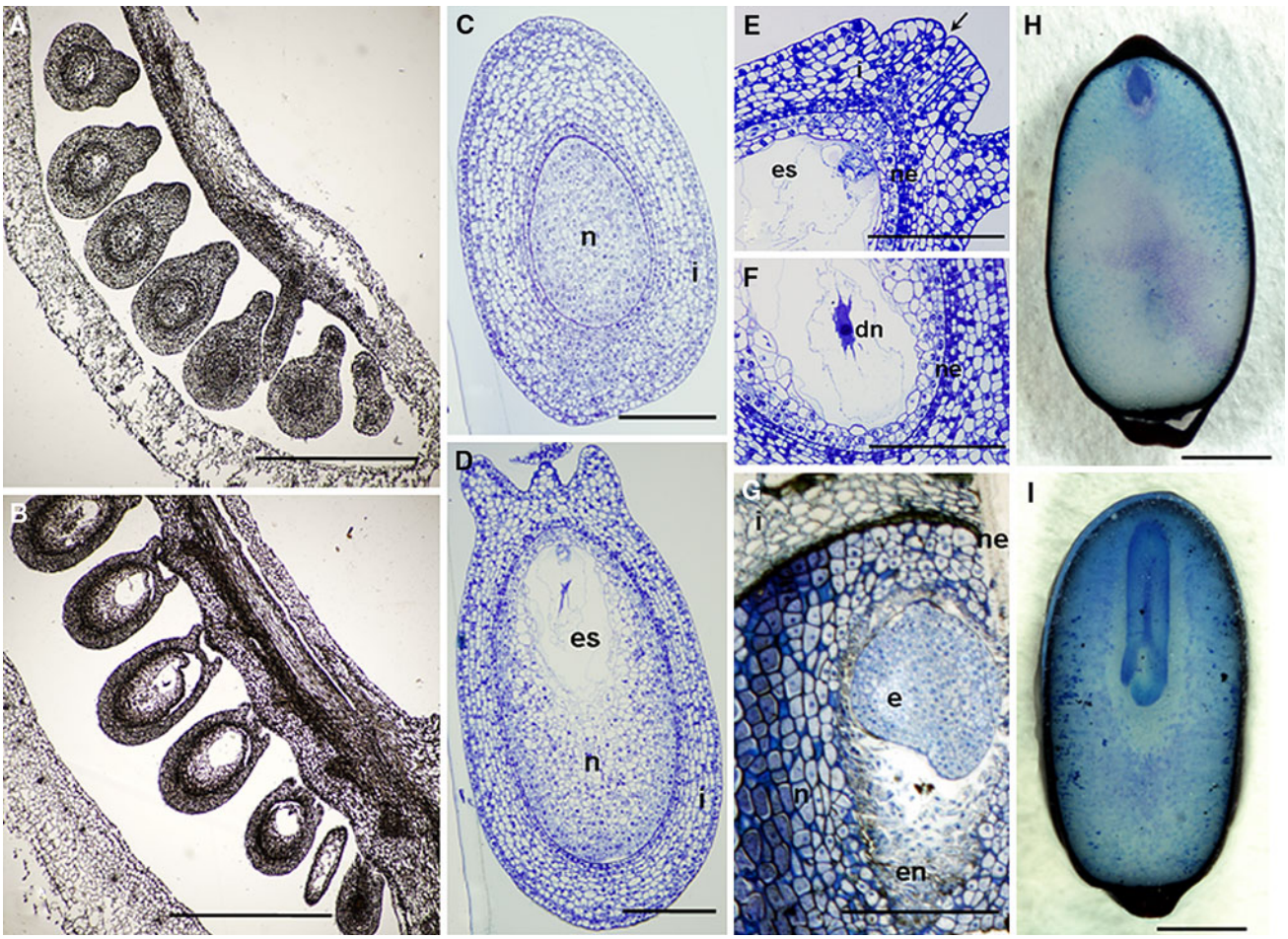

Fig. 2 Approximately median longitudinal sections through fruit (multiovular carpels), ovules, and seeds of *Helleborus niger* L. (*Ranunculaceae*) at different developmental stages. **A** Part of a carpel in the 'Female' phase. **B** Part of a carpel in the 'Male' phase. **C** Ovule in the 'Female' phase showing the nucellus without embryo sac. **D** Ovule in the 'Male' phase showing the nucellus containing the young embryo sac. **E** Same stage as D, enlarged view of the egg apparatus (arrow points to micropyle). **F** Same stage as D, enlarged view of the definitive nucleus (formed after conjugation of the polar nuclei). **G** Partial view of half ripe seed showing a small embryo at the heart stage, enclosed in a small cellular endosperm, all embedded in the large nucellus which is to become the main storage tissue (perisperm). **H** Hand-free section of a ripe seed with underdeveloped embryo (early cotyledonary stage) at its upper tip. **I** Seed with embryo differentiated into cotyledon and hypocotyl-radicle after 5 months of after-ripening (see text for details). Scale bars: A, B, H, I = 1000 μm, C, D, E, F, G = 200 μm. dn, definitive nucleus; e, embryo; en, endosperm; es, embryo sac; i, integuments; n, nucellus; ne, nucellar epidermis

habitat 1), when the anthers were still viable. The hormone treatments included GA_1, GA_3, and GA_4 at concentrations of 20, 50, and 100 μM. As shown in Fig. 3, the response to GA_1 and GA_4 was approximately the same at all three concentrations tested, increasing in a near-linear fashion for the first 20 days and then starting to level off. At 20 μM, the effect of GA_3 was barely distinguishable from the water control; at 100 μM, all three GAs induced about the same greening response. The latter response in deseeded GA-treated flowers resulted in chlorophyll meter readings as high as 2.7, which is only slightly below the level found in seed-bearing flowers from habitat 1 of about the same age (3.3 ± 0.2).

At the cellular level, the observed differences in chlorophyll accumulation were reflected by chloroplast abundance and morphology. Typical electron micrographs are shown in Fig. 4. In deseeded flowers treated with 50 μM GA_3 (Fig. 4B) for 30 days, the sepals contained the same kind of lens-shaped chloroplasts as in intact flowers in their 'Green' stage (Fig. 4A) collected at habitat 1; the system of grana thylakoids was even slightly better developed. The other GA treatments tested (except for treatment with 20 μM GA_3, whose effect resembled the control) had a similar effect (data not shown). Although strictly quantitative comparisons were not attempted, chloroplasts were obviously less abundant in water-treated deseeded flowers.

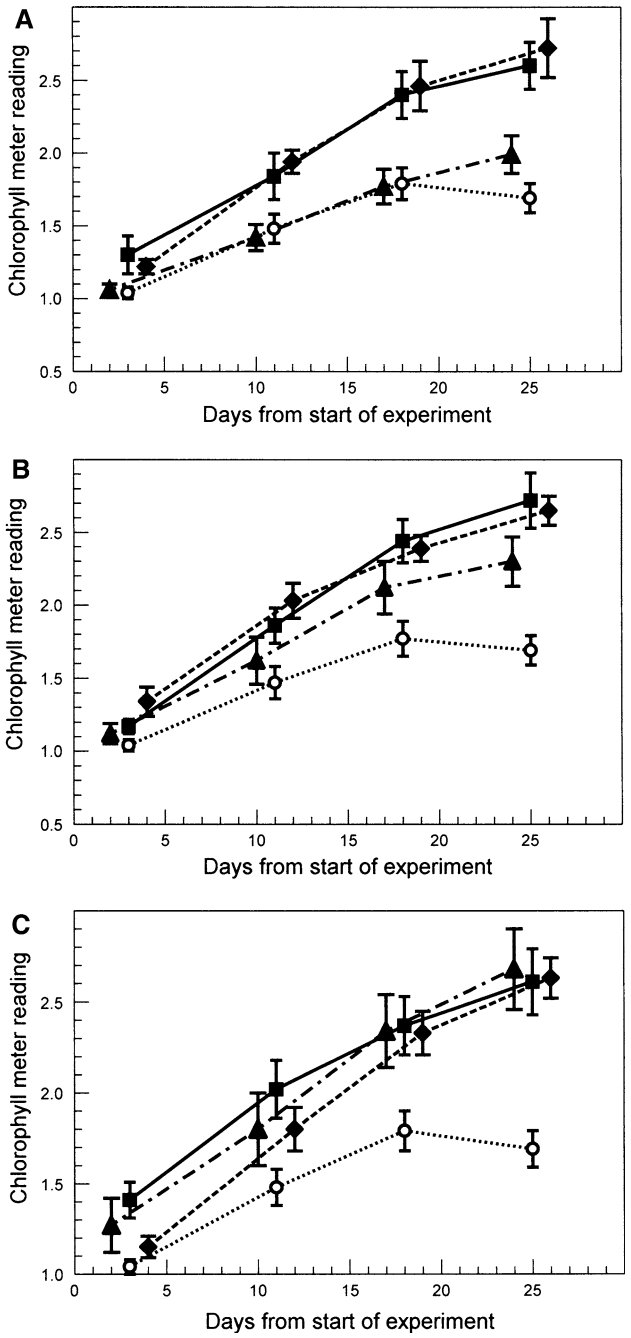

Fig. 3 Greening response of deseeded Christmas rose flowers treated with aqueous solutions of GA_1 (black squares), GA_3 (black triangles), or GA_4 (black diamonds), or with water (plain circles). The GA concentrations were 20 μM (**A**), 50 μM (**B**), and 100 μM (**C**)

Also, these chloroplasts were more flat and the thylakoid system was less abundant (Fig. 4C), all in accord with the interpretation that the greening process [previously shown to involve the metamorphosis of leucoplasts into chloroplasts (Salopek-Sondi and others 2000)] had stopped at an early stage due to the lack of one or more signaling compound(s) normally supplied by the developing seeds.

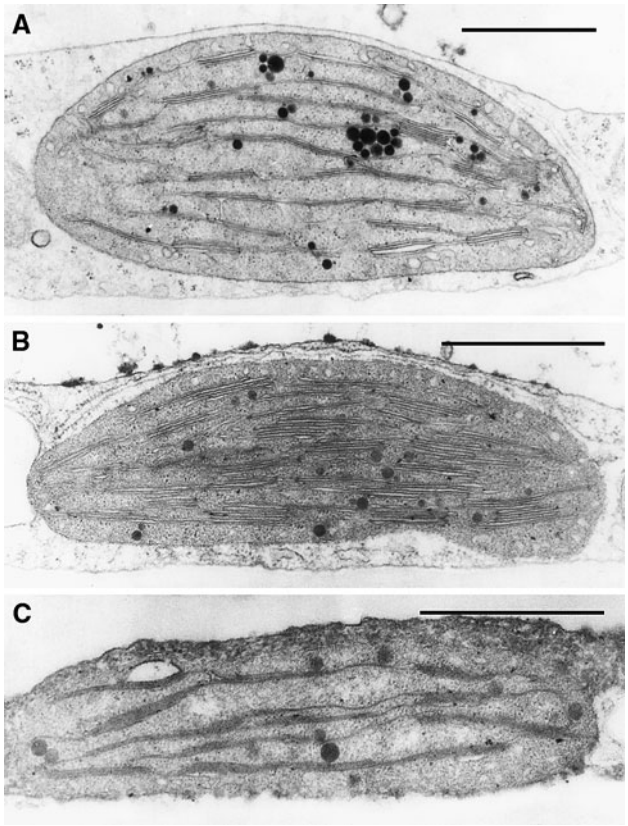

Fig. 4 Electron micrographs of mesophyll chloroplasts from the sepals of (**A**) a flower in the 'Green' stage collected at its natural habitat, (**B**) a deseeded flower treated with 50 μM GA_3 as described in the Materials and Methods section, and (**C**) a deseeded flower treated with water as a control. Scale bars = 1 μm

GAs also stimulated chlorophyll formation in other experimental systems such as regreening orange fruit (Coggins and others 1960) and dormant cocklebur cotyledons (Esashi and others 1977), and arrested chlorophyll loss, for instance, in detached lettuce leaves (Aharoni and Richmond 1978) and Alstroemeria shoots (van Doorn and van Lieburg 1993; Kappers and others 1998), apparently by promoting the expression of protochlorophyllide oxidoreductases that catalyze the key step in chlorophyll biosynthesis (Kuroda and others 1996), and by suppressing chlorophyllase formation (Trebitsh and others 1993).

Identification of Endogenous GA_1 and GA_4

Because GA_1 and GA_4 were equally effective (and more effective than GA_3) in inducing and maintaining sepal greening in deseeded Christmas rose flowers, they were suspected to play endogenous developmental roles. Indeed, the identity and presence of GA_1 and GA_4 in both the sepals and the developing fruit were confirmed by LC-MS/MS. This involved full scanning and monitoring of the fragments derived from the parental pseudomolecular ions

([M - H]$^-$). The major fragmentation pathways appear to be based on successive elimination of H_2O, CO_2, and $CH_3CH = C=O$ (methylketene). The full mass spectra are shown in Supplementary Figs. S1 and S2. The identities of a number of prominent fragment ions are listed in Supplementary Table S1. Thus, in *H. niger*, both the non-13-hydroxylation (leading to GA_4) and the early 13-hydroxylation (leading to GA_1) GA biosynthesis pathways appear to be functional (Fig. 5). Comparable order-of-magnitude levels of GA_1 and GA_4 (MacMillan 2002) were also observed in immature bean seeds (Yamane and others 1977) and, specifically, in the suspensors isolated from such bean seeds (Picciarelli and Alpi 1986), somatic cell embryos of carrot and anise (Noma and others 1979, 1982), and pollinated and unpollinated walnut ovaries (Tadeo and others 1994).

GA Dynamics in Pistils and Developing Fruit

In addition to endogenous GA_1 and GA_4, a number of their precursors and catabolites that are reported to occur in the GA metabolic pathways of different plant species (Fig. 5; Yamaguchi 2008) were quantified. The identity of these GAs in Christmas rose tissues was verified by full-scan LC-MS/MS (data not shown), and then prominent molecular and fragment ions were selected for ^{2}H-isotope dilution based quantification (Varbanova and others 2007; Hu and others 2008). The results for fruit and sepals of the Christmas rose are presented in Figs. 6 and 7 for the data from the 2008 growth season. The data for different growth seasons (Supplementary Tables S2 and S3) generally showed the same tendencies. Strict numerical agreement might not be expected because (1) the developmental stages could be defined only by morphological parameters that, in some cases, changed more slowly than GA patterns, and (2) in flowers collected outdoors, development was dependent on environmental factors such as temperature, rainfall, light quality, and photoperiod, all of which are known to influence GA biosynthesis (Yamaguchi 2008).

The GA_4 level in developing fruit rose to a broad maximum, peaking when sepal greening was complete ('Green' stage), at a concentration about 100 times above that in the pistils in their 'Female' phase (Fig. 6). This pattern overlaps with rapid pericarp growth (Table 1; Tarkowski and others 2006) and embryo and endosperm differentiation, which passes through its initial stages during the 'Light Green' phase to yield, at seed maturation, a miniature embryo in its early cotyledonary stage, enclosed in a just slightly larger endosperm, all enveloped by a prominent perisperm as the major storage tissue (Niimi and others 2006; Tarkowski and others 2006). Bioactive GAs accumulate during rapid pericarp growth (pea; Rodrigo and others 1997) and during early embryo and endosperm growth (Pharis and King 1985; Rodrigo and others 1997; Yamaguchi 2008). Particularly detailed *in vitro* studies in carrot and anise (Noma and others 1979, 1982; Mitsuhashi and others 2003) indicated increased GA_4 biosynthesis during the differentiation of somatic embryos. Developing *Helleborus* embryos pass through a similar sequence of stages. Inspection of the overall pattern of the occurrence of non-13-hydroxylated GAs in Christmas rose fruit (Fig. 6) suggests a regulatory role for transient activation of GA 20-oxidase(s) (GA_9 increases), GA 3-oxidase(s) (GA_4 increases), and a specific GA 2-oxidase for the catabolism of GA_4 to GA_{34}. The peak in GA_4 level at the 'Green' stage (51 ng g DW^{-1}) was accompanied by that of its immediate precursor, GA_9, in concert with a large drop in the concentration of the GA_9 catabolite GA_{51}. The levels of the upstream precursors GA_{12}, GA_{15}, and GA_{24} passed through minor transient peaks when GA_4 started to decrease ('Half ripe' stage). A large flux through GA_4 to GA_{34} is indicated during this stage since the levels of the catabolite GA_{34} increased up to 169 ng g DW^{-1} (Fig. 6A).

The early 13-hydroxylation pathway to bioactive GA_1 showed a different profile, with GA_1 low from the 'Female' to the 'Light green' stages, but peaking at 17 ng g DW^{-1} by the 'Half ripe' stage when the fruit was rapidly increasing in weight (Table 1; Fig. 6B). During later phases of fruit development, it is likely that the bioactive GA_1 and GA_4 are involved in pericarp growth and early seed development, similar to that observed during pea fruit development (Rodrigo and others 1997; Ozga and others 2009).

For quantitative data for the GA distribution between these two fruit compartments, we analyzed a sample collected at habitat 2 at the late 'Almost ripe' stage, about a week beyond the time span covered by Figs. 6 and 7. The results are shown in Fig. 8. Interestingly, the pericarps contained about six times more GA_1 than the seeds, whereas the GA_4 concentrations were equivalent in both tissues. Pericarps had high levels of the GA_4 catabolite GA_{34}, indicating high flux through GA_4 during earlier developmental stages, whereas seeds and pericarps had significant levels of GA_8, indicating that both tissues had GA_1-synthesizing activity with subsequent catabolism. Measuring the GA profiles in seeds and pericarps at earlier stages of development will help clarify tissue localization of the transient GA_1 and GA_4 peaks observed in the fruit (Fig. 6).

Other species such as pea (Ozga and others 2003), citrus (Ben-Cheikh and others 1997), and walnut (Tadeo and others 1994) also showed a short transient increase in GA levels immediately following pollination or fertilization. The number of time points at which Christmas rose flowers were analyzed was too small to detect such rapid fluctuations.

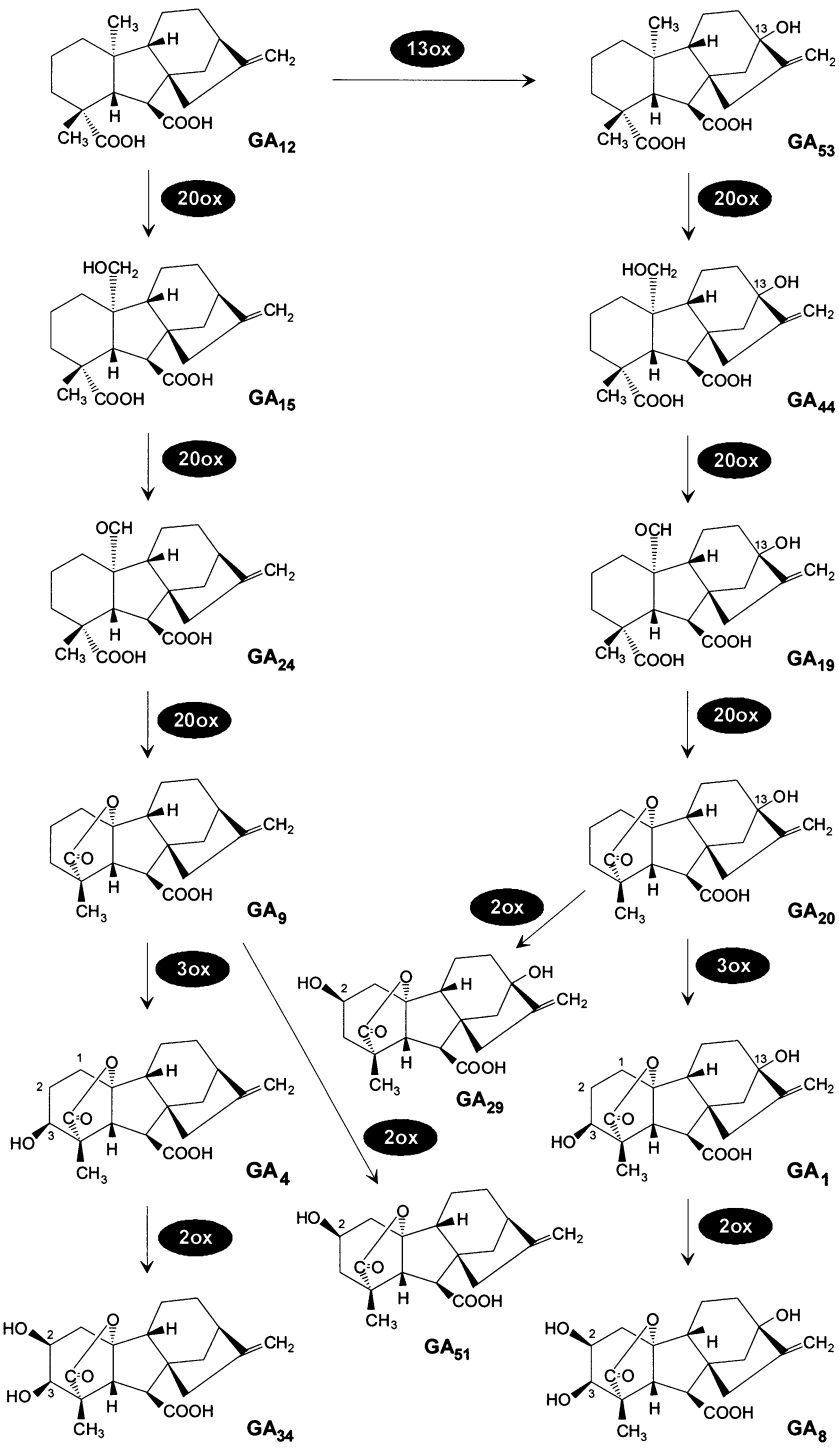

Fig. 5 GAs analyzed in reproductive tissues of the Christmas rose. Based on our analyses, both the non-13-hydroxylation GA pathway (left) and the early 13-hydroxylation pathway (right) are proposed to operate in this species. The enzymes involved are shown using the following abbreviations: 2ox, GA 2-oxidase; 3ox, GA 3-oxidase; 13ox, GA 13-oxidase; 20ox, GA 20-oxidase

GA Dynamics in the Sepals

GA concentrations in the sepals ranged from 2 to 50 times lower than those in the fruit tissues and generally dropped during development from their maximum at the 'Female' stage (Figs. 6, 7). GA_4 increased 5.7-fold by the 'Light green' stage (Fig. 7A), when sepal greening was initiated and remained generally elevated (compared to the level at anthesis, 'Male' phase). The levels of GA_4 precursors, GA_{12}-GA_{24}, and its catabolite, the GA_{34} were elevated prior to the 'Light green' stage, indicating that metabolism to GA_4, and further to GA_{34}, was high during early development. GA_1 levels were steady throughout development but increased 2.5-fold by the 'Almost ripe' stage

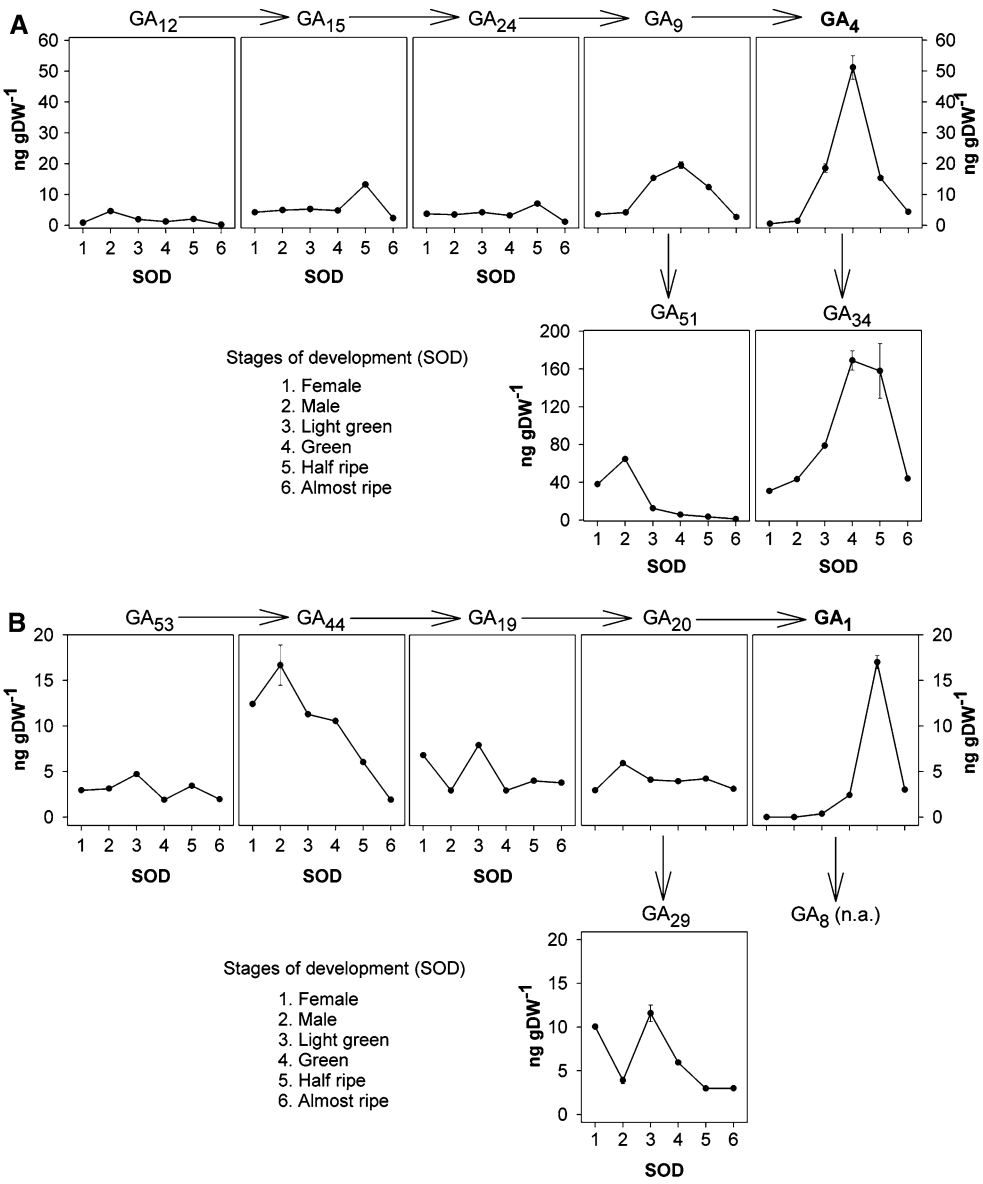

Fig. 6 Endogenous GA levels in pistils and developing fruit of *Helleborus niger* during anthesis and fruit ripening. The GAs presented include those in the non-13-hydroxylation (**A**) and the early 13-hydroxylation (**B**) pathways. The developmental stages analyzed were the "Female' (1) and the 'Male' (2) phases (two stages of anthesis), the 'Light green' (3) and the 'Green' (4) stages (increasing chlorophyll accumulation in the sepals), and the 'Half ripe' (5) and 'Almost ripe' (6) stages (two later stages of fruit development). Data are means ± SE; $n = 2$ except for all 'Female' stage GAs and the 'Light green' stage GA_{34}, where $n = 1$ (n is the number of replicate analyses of the same plant sample). In the presented series of experiments, endogenous GA_8 and its internal standard were not recovered following purification; this GA was thus not quantified (n.a. = data not available)

(Fig. 7B). All of the GA metabolites and catabolites were present in the fruit as well as in the sepals, suggesting that GA metabolism takes place in both tissues.

Experiments with a number of species appear to imply that perianth development in the growing bud depends on GAs supplied by the anthers (Pharis and King 1985; Weiss and others 1995; Yamaguchi 2008). It thus seems logical to suspect that the greening response of the Christmas rose perianth (sepals), after the anthers have abscised, continues to depend on remote GA sources, among which the GA-rich developing fruit would be a likely candidate. This assumption would also agree with the above experiments that showed that GAs supplied through the deseeded pistils can stimulate sepal greening (Figs. 1, 3). The analytical data on GA dynamics (Fig. 7; Supplementary Table S3) through the life cycle of the perianth indicate, however, that the situation is not so simple.

Obvious parallels between the concentration dynamics in the sepals with that in the fruit were not observed. It is thus unlikely that the fruit are the only source of GAs for the sepals. The data suggest that the sepals contain GA-synthesizing and -metabolizing enzymes.

GAs in Unfertilized and Depistillated Flowers

A partially independent capacity for GA metabolism in the sepals is corroborated by comparative analyses of intact, depistillated, and unfertilized flowers. When flowers were depistillated at the bud stage (habitat 1) and analyzed several weeks after anthesis (when the surrounding intact

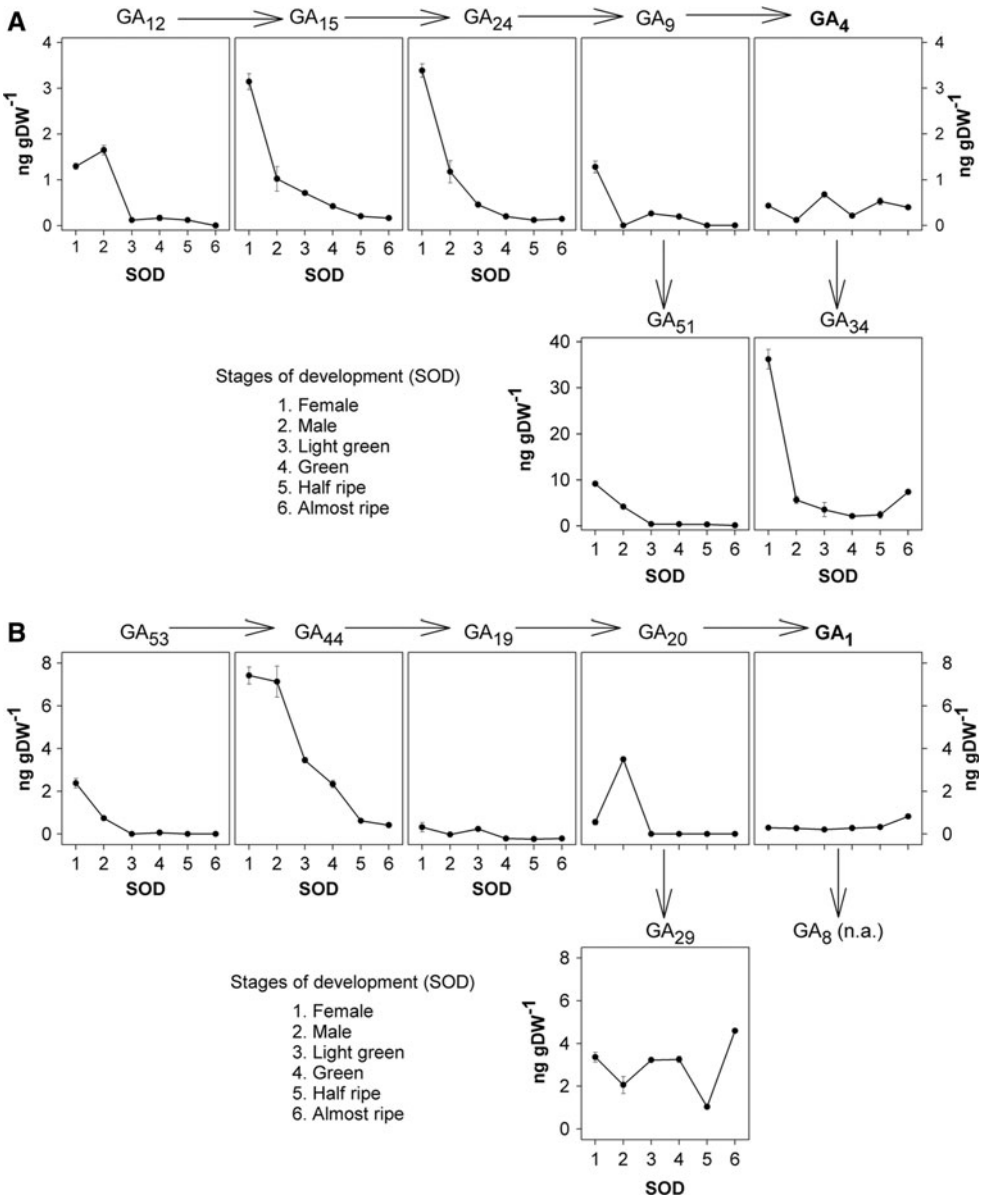

Fig. 7 Endogenous GA levels in the sepals of *Helleborus niger* during anthesis and fruit ripening. The GAs presented include those in the non-13-hydroxylation (**A**) and the early 13-hydroxylation (**B**) pathways. Developmental stages are the same as in Fig. 6. Data are mean ± SE; $n = 2$ for all GAs assayed except the 'Light Green' stage for GA_{19}, GA_1, and GA_{29}, where $n = 1$. Endogenous GA_8 and its internal standard were not recovered following purification; this GA was thus not quantified (n.a. = data not available)

flowers were at the 'Light green' stage), all GA metabolites from the non-13-hydroxylation pathway (Fig. 5) were present but at lower levels than in the sepals of intact flowers except for similar levels of GA_{24} (Fig. 9A). Depistillation resulted in decreased levels of GA_4 to 0.01 ng g DW^{-1} compared to 0.12 ng g DW^{-1} in the sepals of intact flowers. Interestingly, sepals from depistillated and intact flowers had similar GA profiles for the early 13-hydroxylation pathway, and GA_1 remained at 0.2 ng g DW^{-1} (Fig. 9A).

Unfertilized flowers were available at the age equivalent to the 'Half ripe' stage. They contained GAs from both the non-13-hydroxylation and early 13-hydroxylation pathways (except GA_9) (Fig. 9B). However, GA_1 and GA_4 were 2.6 and 1.4-times lower, respectively, in the sepals of unfertilized flowers than in intact flowers. When GA_1 or GA_4 levels were lower in sepals of depistillated and unfertilized flowers, their respective catabolites, GA_{34} and GA_8, were also lower, suggesting that decreased biosynthesis rather than increased catabolism resulted in reduced GA_4 and GA_1 levels.

Flowers at the late 'Almost ripe' stage (closer to seed ripening than the 'Almost ripe' material in Figs. 6, 7) were compared to both unfertilized and depistillated flowers of the same age (habitat 2). As seen in Fig. 10, unfertilized pistils contained neither GA_4 nor GA_1, and low amounts of their precursors and catabolites, except for significant levels of GA_{29}, whereas fruit from fertilized flowers had significant levels of all GAs (except GA_{19}), including GA_1 and GA_4. Although the levels of non-13-hydroxylation

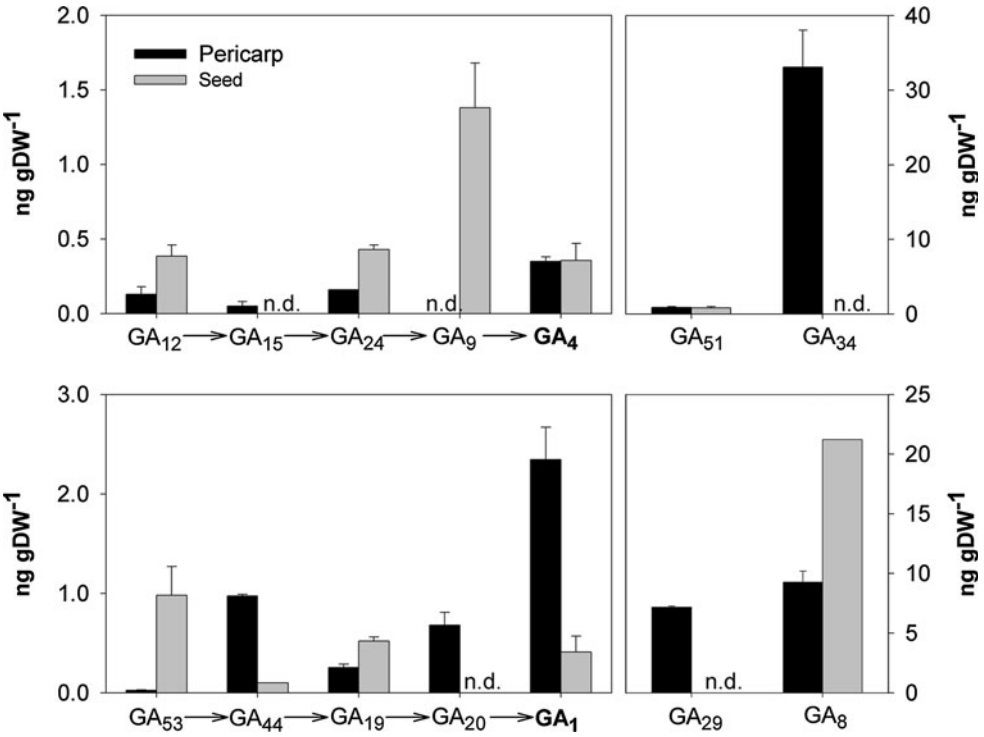

Fig. 8 Comparison of GA levels in the pericarps and the seeds of Christmas rose fruit at the late 'Almost ripe' stage. The overall dry weight (fresh weight) of the pericarps was 1.45 (9.78) g, and that of the seeds was 1.61 (4.90) g. Metabolites of the non-13-hydroxylation pathway (including GA_4) are presented in the top panels and those of the early 13-hydroxylation pathway (including GA_1) in the bottom panels. Data are mean ± SE; $n = 2$ for all samples except for seed GA_{15}, GA_{34}, GA_{44}, GA_{20}, and GA_8, where $n = 1$. n.d. = not detected, where internal standard was detected but endogenous GA was not

GAs remained low in the sepals of unfertilized flowers (Fig. 10A, C), the levels of some early 13-hydroxylated GAs (GA_{20}, GA_8, GA_{29}) remained high (Fig. 10B, D). This may suggest that GA 3-oxidase (converts GA_{20} to GA_1) is downregulated in the absence of seeds.

Although GA_4 is a possible fruit-derived signal candidate for sepal development, our results are most simply explained by assuming that the sepals contain their own set of GA-forming and -metabolizing enzymes, and sepal GA biosynthesis responds to other activation signal(s) from the developing fruit. Furthermore, signaling factors from other organs cannot be excluded, especially under natural conditions where organs like roots and leaves may supply signaling molecules to the perianth.

Conclusions

GAs are known to play important roles in reproductive development, from the induction of flowering, through the concerted development of the individual organs of a flower, to fruit set and maturation (Pharis and King 1985; Finkelstein 2004; Yamaguchi 2008). Popular model dicots in which these processes have been investigated in detail, such as pea (Rodrigo and others 1997; Swain and others 1997; Ozga and Reinecke 2003; Ozga and others 2009), tomato (Rebers and others 1999), and *Arabidopsis* (Fei and others 2004; Hu and others 2008), have seeds dominated by the embryo at maturity. In *Helleborus,* embryo and endosperm remain small (Niimi and others 2006; Tarkowski and others 2006) and their differentiation is already well advanced when nutrient accumulation accelerates in the surrounding perisperm. In this developmental paradigm, the GA biosynthesis profiles suggest that bioactive GAs also appear to be important to pericarp and seed growth and development.

The presence of a persisting perianth that becomes photosynthetically active during seed development may be an ecophysiological adaptation in Christmas roses. Even though the leaves are winter-green, they are frequently covered by snow and debris during early seed development and regularly deteriorate at their later stages, while the new generation of leaves is not yet fully functional. The green sepals are thus a reliable and often the main direct source of photosynthates for the assimilate-requiring fruit.

The developing seeds obviously play a role in the metamorphosis of the sepals because this process remains incomplete in unfertilized, depistillated, and deseeded flowers. In these cases, perianth greening was resumed upon treatment with exogenous GAs. The sepals responded, for instance, to exogenous GA_1 and GA_4 applied to the interior of deseeded fruit, thus indicating that transport of these molecules from the fruit to the perianth is possible. The dynamics of GA metabolism in intact, depistillated, and unfertilized flowers suggested, however, that the sepals can also synthesize and metabolize their own GAs.

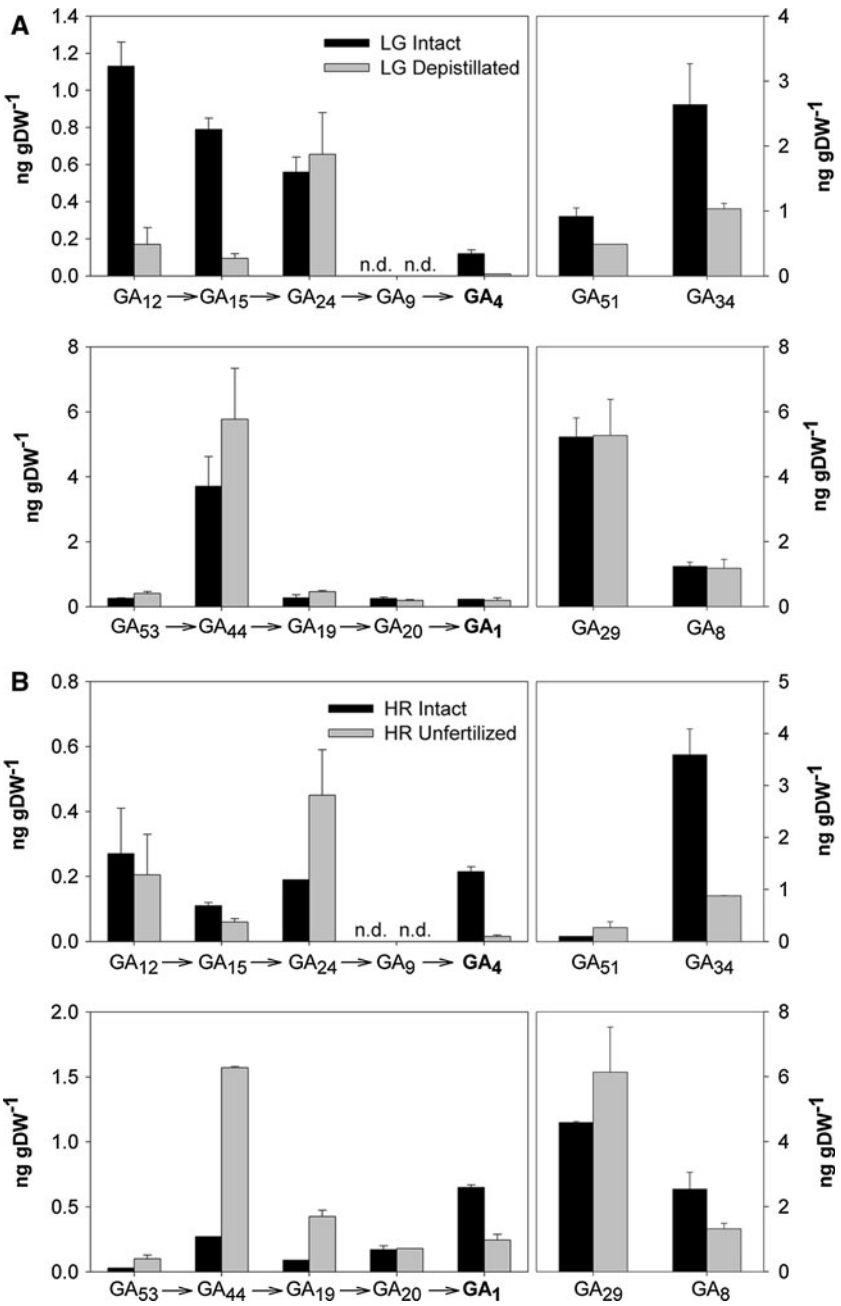

Fig. 9 GAs in the sepals of depistillated (**A**) and unfertilized (**B**) flowers at the 'Light green' (LG) (**A**) and 'Half ripe' (HR) (**B**) developmental stages compared with intact (fertilized) controls. Metabolites of the non-13-hydroxylation pathway (including GA_4) are presented in the top panels of A and B and those of the early 13-hydroxylation pathway (including GA_1) are presented in the bottom panels. Data are mean ± SE; $n = 2$ for all samples. n.d. = not detected, where internal standard was detected but endogenous GA was not

In the pea fruit, pericarp growth, among other things, requires bioactive GA, normally GA_1, which is synthesized in response to auxin supplied by the developing seeds (Ozga and Reinecke 2003; Ozga and others 2009). If they are removed, the pericarp will still grow (and GA_1 will be synthesized) if supplied with 4-chlorindole-3-acetic acid. In *Helleborus*, on the other hand, although treatment of deseeded (see above) and depistillated (Salopek-Sondi and others 2002) flowers with bioactive GAs did trigger sepal greening, auxins showed no effect (Salopek-Sondi and others 2002). It is thus unlikely that in intact flowers auxins released by the developing fruit stimulate GA biosynthesis in the sepals.

If GAs are involved in sepal greening in *Helleborus*, then one might expect that inhibitors of GA biosynthesis, applied to intact flowers, should inhibit chlorophyll accumulation. Paclobutrazol, applied in lanolin to the sepals of fruit-bearing flowers, was inhibitory at high concentrations (10 mM) (Salopek-Sondi 2000; Salopek-Sondi and others 2002). Also, as shown previously (Tarkowski and others 2006), sepal greening in intact fertilized flowers is influenced by cytokinins; this process should therefore only be partially

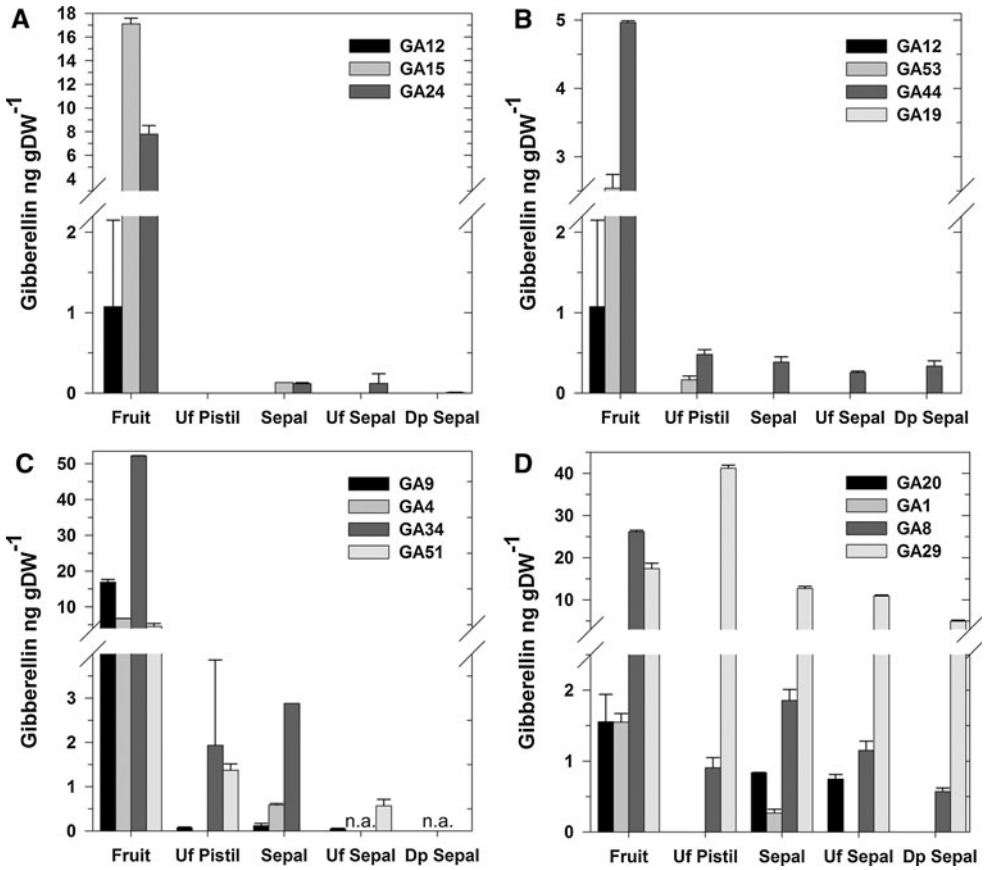

Fig. 10 GAs in flowers from the late 'Almost Ripe' stage (fruit and sepals) compared to unfertilized and depistillated flowers of the same age. Metabolites of the non-13-hydroxylation pathway (including GA_4) are presented in panels **A** and **C**, those of the early 13-hydroxylation pathway (including GA_1) in panels **B** and **D**. Although the sepals of fertilized (intact fruit), unfertilized (Uf), and depistillated (Dp) flowers were roughly comparable in weight, the fresh weight (dry weight) of the ripening fruit was 3.497 (0.470) g per cluster, but only 0.197 (0.033) g per cluster for the unfertilized pistils. Data are mean ± SE, $n = 2$ for all samples except for the intact sepal stage, where $n = 1$. Endogenous GA_{12}, GA_{51}, GA_{53}, and GA_{19} (in intact sepal); GA_{12}, GA_{15}, GA_4, GA_{53}, GA_{19}, and GA_1 (in unfertilized sepal); GA_{12}, GA_{15}, GA_{24}, GA_4, GA_{19}, GA_{20}, and GA_1 (in unfertilized pistil); and GA_{12}, GA_{15}, GA_9, GA_4, GA_{51}, GA_{53}, GA_{19}, GA_{20}, and GA_1 (in unfertilized pistil) were not detected even if their respective internal standards were recovered. GA_{34} was not quantified in the unfertilized sepal and depistillated sepal stages because its internal standard was not recovered following purification (n.a. = data not available)

attenuated by inhibition of GA biosynthesis. Overall, our data suggest that a signal(s) from the fruit stimulates GA biosynthesis in the perianth resulting in greening. Further research will be required to understand the intricate signaling mechanisms that coordinate sepal greening in *Helleborus*.

Acknowledgments This work was supported by a fellowship from the Japan Society for the Promotion of Science to B. T. Ayele; by research grants 098-0982913-2829, 098-0982913-2838, and 098-1782-739-2749 from the Croatian Ministry of Science, Education and Sports; and by a Natural Science and Engineering Research Council of Canada Discovery Grant (OPGO138166) to J. A. Ozga. We are grateful to the staff of the botanical garden of the Faculty of Pharmacy and Biochemistry who made their Christmas rose collections available for our experiments. We thank M. Katunarić from the School of Dental Medicine of the University of Zagreb for supplying Kerr dental needles. We are particularly indebted to Atsushi Hanada for technical assistance in quantifying the GA levels.

References

Aharoni N, Richmond AE (1978) Endogenous gibberellin and abscisic acid content as related to senescence of detached lettuce leaves. Plant Physiol 62:224–228

Ben-Cheikh W, Perez-Botella J, Tadeo FR, Talon M, Primo-Millo E (1997) Pollination increases gibberellin levels in developing ovaries of seeded varieties of citrus. Plant Physiol 114:557–564

Burrell CC, Tyler JK (2006) Hellebores. A comprehensive guide. Timber Press, Portland, OR 296 pp

Coggins CW Jr, Hield HZ, Garber MJ (1960) The influence of potassium gibberellate on Valencia orange trees and fruit. J Am Soc Hort Sci 76:193–198

Damboldt J, Zimmermann W (1965) 289. Helleborus. In: Hegi G (ed) Illustrierte Flora von Mittel-Europa, vol III, part 3. Carl Hanser Verlag, München, pp 91–107

Esashi Y, Katoh H, Hata Y, Gotô N (1977) Dormancy and impotency of cocklebur seeds. VII. Inability of dormant cotyledons to form chlorophyll. Plant Physiol 59:122–125

Fei H, Zhang R, Pharis RP, Sawhney VK (2004) Pleiotropic effects of the *male sterile33* (*ms33*) mutation in *Arabidopsis* are associated with modifications in endogenous gibberellins, indole-3-acetic acid and abscisic acid. Planta 219:649–660

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. New Phytol 171:501–523

Finkelstein RR (2004) The role of hormones during seed development and germination. In: Davies PJ (ed) Plant hormones. Biosynthesis, signal transduction, action. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 513–537

Greyson RI, Raman K (1975) Differential sensitivity of "double" and "single" flowers of *Nigella damascena* (Ranunculaceae) to emasculation and to GA_3. Am J Bot 62:531–536

Greyson RI, Tepfer SS (1967) Emasculation effects on the stamen filaments of *Nigella hispanica* and their partial reversal by gibberellic acid. Am J Bot 54:971–976

Halevy AH (1986) Recent advances in the use of growth substances in ornamental horticulture. In: Bopp M (ed) Plant growth substances 1985. Springer-Verlag, Berlin, pp 391–398

Hedden P (2003) The genes of the green revolution. Trends Genet 19:5–9

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol 48:431–460

Hedden P, Phillips AL (2000) La manipulation des hormones chez les plantes. Biofutur 204:46–48

Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B (2002) Gibberellin biosynthesis in plants and fungi: a case of convergent evolution? J Plant Growth Regul 20:319–331

Hu J, Mitchum MG, Barnaby N, Ayele BT, Ogawa M, Nam E, Lai W-C, Hanada A, Alonso JM, Ecker JR, Swain SM, Yamaguchi S, Kamiya Y, Sun T-P (2008) Potential sites of bioactive gibberellin production during reproductive growth in *Arabidopsis*. Plant Cell 20:320–336

Itoh H, Tanaka-Ueguchi M, Kawaide H, Chen X, Kamiya Y, Matsuoka M (1999) The gene encoding tobacco gibberellin 3β-hydroxylase is expressed at the site of GA action during stem elongation and flower organ development. Plant J 20:15–24

Kaneko M, Itoh H, Inukai Y, Sakamoto T, Ueguchi-Tanaka M, Ashikari M, Matsuoka M (2003) Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? Plant J 35:104–115

Kappers IF, Jordi W, Maas FM, Stoopen GM, van der Plas LHW (1998) Gibberellin and phytochrome control senescence in alstroemeria leaves independently. Physiol Plant 103:91–98

Kuroda H, Masuda T, Ohta H, Shioi Y, Takamiya K-I (1996) Effects of light, developmental age and phytohormones on the expression of the gene encoding NADPH-protochlorophyllide oxidoreductase in *Cucumis sativus*. Plant Physiol Biochem 34:17–22

MacMillan J (2002) Occurrence of gibberellins in vascular plants, fungi, and bacteria. J Plant Growth Regul 20:387–442

Mathew B (1989) Hellebores. Alpine Garden Society, Surrey 180 pp

Mitsuhashi W, Toyomasu T, Masui H, Katho T, Nakaminami K, Kashiwagi Y, Akutsu M, Kenmoku H, Sassa T, Yamaguchi S, Kamiya Y, Kamada H (2003) Gibberellin is essentially required for carrot (*Daucus carota* L.) somatic embryogenesis: dynamic regulation of gibberellin 3-oxidase gene expressions. Biosci Biotechnol Biochem 67:2438–2447

Niimi Y, Han DS, Abe S (2006) Temperatures affecting embryo development and seed germination of Christmas rose (*Helleborus niger*) after sowing. Sci Hortic 107:292–296

Noma M, Huber J, Pharis RP (1979) Occurrence of $\Delta^{1(10)}$ gibberellin A_1 counterpart, GA_1, GA_4 and GA_7 in somatic cell embryo cultures of carrot and anise. Agric Biol Chem 43:1793–1794

Noma M, Huber J, Ernst D, Pharis RP (1982) Quantitation of gibberellins and the metabolism of [^{3}H]gibberellin A_1 during somatic embryogenesis in carrot and anise cell cultures. Planta 155:369–376

Ozga JA, Reinecke DM (2003) Hormonal interactions in fruit development. J Plant Growth Regul 22:73–81

Ozga JA, Yu J, Reinecke DM (2003) Pollination-, development-, and auxin-specific regulation of gibberellin 3β-hydroxylase gene expression in pea fruit and seeds. Plant Physiol 131: 1137–1146

Ozga JA, Reinecke DM, Ayele BT, Ngo P, Nadeau C, Wickramarathna AD (2009) Dvelopmental and hormonal regulation of gibberellin biosynthesis and catabolism in pea fruit. Plant Physiol 150:448–462

Pharis RP, King RW (1985) Gibberellins and reproductive development in seed plants. Annu Rev Plant Physiol 36:517–568

Picciarelli P, Alpi A (1986) Gibberellins in suspensors of *Phaseolus coccineus* L. seeds. Plant Physiol 82:298–300

Rademacher W (2000) Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways. Annu Rev Plant Physiol Plant Mol Biol 51:501–531

Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang Y-Y, Imai R, Sekimoto H, Kamiya Y (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J 17:241–250

Rodrigo MJ, García-Martínez JL, Santes CM, Gaskin P, Hedden P (1997) The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. Planta 201:446–455

Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, Oxford 322 pp

Salopek-Sondi B (2000) Regulation of morphogenesis in the flower of the Christmas rose (*Helleborus niger* L. var. *macranthus* (Freyn) Schiffner), PhD thesis, University of Zagreb, Zagreb, Croatia, 101 pp

Salopek-Sondi B, Magnus V (2007) Developmental studies in the Christmas rose (*Helleborus niger* L.). Int J Plant Dev Biol 1:151–159

Salopek-Sondi B, Kovač M, Ljubešić N, Magnus V (2000) Fruit initiation in *Helleborus niger* L. triggers chloroplast formation and photosynthesis in the perianth. J Plant Physiol 157:357–364

Salopek-Sondi B, Kovač M, Prebeg T, Magnus V (2002) Developing fruit direct post-floral morphogenesis in *Helleborus niger* L. J Exp Bot 53:1949–1957

Sawhney VK, Shukla A (1994) Male sterility in flowering plants: are plant growth substances involved? Am J Bot 81:1640–1647

Schiffner V (1891) Monographia Hellebororum. Kritische Beschreibung aller bisher bekannt gewordenen Formen der Gattung Helleborus. Nova Acta Academiae Cesareae Leopoldino-Carolinae Germanicae Naturae Curiosorum 56:1–198

Sponsel VM, Hedden P (2004) Gibberellin biosynthesis and inactivation. In: Davies PJ (ed) Plant hormones. Biosynthesis, signal transduction, action. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 63–94

Swain SM, Reid JB, Kamiya Y (1997) Gibberellins are required for embryo growth and seed development in pea. Plant J 12:1329–1338

Tadeo FR, Talon M, Germain E, Dosba F (1994) Embryo sac development and endogenous gibberellins in pollinated and unpollinated ovaries of walnut (*Juglans regia*). Physiol Plant 91:37–44

Tarkowski P, Tarkowská D, Novák O, Mihaljević S, Magnus V, Strnad M, Salopek-Sondi B (2006) Cytokinins in the perianth, carpels and developing fruit of *Helleborus niger* L. J Exp Bot 57:2237–2247

Trebitsh T, Goldschmidt EE, Riov J (1993) Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme in *Citrus* fruit peel. Proc Natl Acad Sci USA 90:9441–9445

van Doorn WG, van Lieburg MJ (1993) Interaction between the effects of phytochrome and gibberellic acid on the senescence of *Alstroemeria pelegrina* leaves. Physiol Plant 89:182–186

Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochov R, Yu F, Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Rodermel S, Weiss D, Pichersky E (2007) Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. Plant Cell 19:32–45

Weiss D, van der Luit A, Knegt E, Vermeer E, Mol JNM, Kooter JM (1995) Identification of endogenous gibberellins in petunia flowers. Induction of anthocyanin biosynthetic gene expression and the antagonistic effect of abscisic acid. Plant Physiol 107:695–702

Yamaguchi S (2008) Gibberellin metabolism and its regulation. Annu Rev Plant Biol 59:225–251

Yamane H, Murofushi N, Osada H, Takahashi N (1977) Metabolism of gibberellins in early immature bean seeds. Phytochemistry 16:831–835