

# Hormonal Sensitivity Decreases During the Progression of Flower Senescence in *Lilium longiflorum*

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**Abstract** Although lilies (*Lilium* sp.) are the fourth sold cut flower in the world, much remains to be discovered about the senescence and post-harvest physiology of these ethylene-insensitive flowers. This study investigated the hormonal regulation of flower senescence in *Lilium longiflorum* with a focus on the possible changes in sensitivity to phytohormones during the progression of tepal senescence. After characterizing the hormonal profile of *L. longiflorum* ‘White Heaven’ tepals from anthesis to complete wilting, we evaluated the changes in hormonal sensitivity during senescence by application of a combination of gibberellins and cytokinins at various times post anthesis. We observed a progressive loss of sensitivity to the hormonal treatment: from an increase of more than 2 days in flower lifespan when applied one day after anthesis, to a complete loss of vase-life prolongation when treated at the fourth day after anthesis or later. These observations were accompanied by various changes in the underlying hormonal contents, especially in the gibberellins and abscisic acid (ABA) profile, which was

dependent on the application time. The results not only confirm the role of gibberellins and cytokinins as inhibitors, and ABA as a promoter of senescence, but also reveal a progressive loss in hormonal sensitivity and a hormonal cross-talk during the progression of senescence in tepals of *L. longiflorum*. We conclude that both hormonal levels and sensitivity modulate the progression of tepal senescence in *L. longiflorum*.

**Keywords** Abscisic acid · Cytokinins · Flower senescence · Gibberellins · Hormones · *Lilium longiflorum*

## Introduction

Flower senescence is the terminal phase of developmental processes that leads to the end of its life span. The ability to alter the regulation of post-harvest senescence will be beneficial for improving storage life and maintaining flower quality. Hence, it is the key to understand in great detail the physiology of flower senescence and its temporal progression. Flower senescence is a tightly regulated process involving, in most species, wilting of petals (or tepals) accompanied by nutrient remobilization to the developing ovary and terminating in cell death (Rogers 2013). In many species, like *Lilium longiflorum*, this process is ended by organ abscission (Rogers 2006; van Doorn and Woltering 2008). At a cellular level, cell death seems to mimic an autophagic pattern: vesicles accumulate in the cytosol followed by enlargement of the central vacuole and ultimate vacuolar rupture (Battelli and others 2011; Shahir and Tahir 2011).

In leaves, the senescence process proceeds gradually and is defined by three phases: (i) the initiation phase, (ii) the reorganization phase and (iii) the terminal phase (Munné-Bosch and Alegre 2004; Rogers and Munné-Bosch 2016).

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First, the initiation phase is the result of early signalling cascades that lead to changes in gene expression and trigger the induction of the senescence process. These changes in gene expression lead to alterations in the levels of endogenous plant regulators such as hormones and reactive oxygen species (ROS), among others, which will regulate the expression of senescence-associated genes (SAGs) (Li and others 2012). Concurrently, these genes are regulated by transcription factors (TFs). Nowadays, several TFs have been found to have a role in senescence; however, there is little knowledge on how the global regulatory network works (Penfold and Buchanan-Wollaston 2014). Second, the reorganization phase is characterized by metabolic and cell ultrastructural changes, which are linked to nutrient recycling (Shahir and Tahir 2011) and autophagy-like processes (Shibuya and others 2013; Avila-Ospina and others 2014). Ultimately, the terminal stage is characterized by complete cellular integrity loss. This phase starts with the “point of no return”, point at which, once passed, the organ is committed, inevitably, to death. Karagiannis and Pappelis (1994) proposed that this point coincides with the irreversible degeneration of the nuclear matrix, which results in the inability of the cell to synthesize mRNAs and ribosomal subunits to support the protein synthesis required for maintenance. In flowers, petal (or tepal) senescence proceeds similarly but also with important differences (Rogers and Munné-Bosch 2016). First, in many species, pollination is a primary signal for a sharp acceleration in petal senescence, although even in the absence of pollination, petals will eventually senesce and/or abscise. Nutrient remobilization does not provide substantial amounts of sugars or amino acids to the rest of the plant, as senescing leaves do, but to the ovary for fruit development after fertilization (Jones 2013). Furthermore, petals are fairly nutrient poor compared with leaves, and previous experiments indicate that leaves remobilize a greater amount of nutrients than petals do during senescence (Himelblau and Amasino 2001; Verlinden 2003; Chapin and Jones 2007, 2009; Jones 2013). Re-differentiation of senescing chloroplasts and re-greening of senescing tobacco leaves were observed after application of cytokinins (Zavaleta-Mancera and others 1999a, b), thus suggesting leaf senescence is reversible. Interestingly, when too many plastids were lost during advanced senescence, re-greening capacity deteriorated (Zavaleta-Mancera and others 1999b), thus leading to the so-called concept of “point of no return”, which defines the start of the terminal stage of senescence that once surpassed cannot be reversed (van Doorn and Woltering 2004). In many species, pollination leads to an increase in endogenous ethylene production, which becomes autocatalytic and coordinates cellular events within the different floral organs triggering senescence (Stead and van Doorn 1994; Jones and Woodson 1997). In ethylene-insensitive flowers, particularly in those non-pollinated,

senescence is induced but the process occurs more slowly, and although several post-harvest treatments are known to prolong flower lifespan, it is still not known if the process can be fully reversed.

Senescence is modulated by a cross-talk of hormonal repressors and promoters, as well as by other regulators such as reactive oxygen species (ROS). The major hormonal senescence repressors are cytokinins (CKs) and gibberellins (GAs). CKs are considered the major leaf senescence-delaying hormone (Lim and others 2007; Khan and others 2013). Auxins are also considered as senescence repressors (Basu and others 2013), but their mode of action and cross-talk with other senescence-control mechanisms remains vaguely defined (Mueller-Roeber and Balazadeh 2014), although it has recently been demonstrated that there is a clear correlation between auxin levels and abscission timing (Lombardi and others 2015). On the other hand, the main senescence promoters are ethylene and abscisic acid (ABA), although jasmonic acid (JA) and salicylic acid (SA) may also play a role (Khan and others 2013). In lilies, ABA presumably induces senescence independently of ethylene (Lay-Yee and others 1992; Hunter and others 2004), although ethylene insensitivity is not complete, particularly in some cultivars (Miller 2014). In turn, ROS levels can play an important regulatory role together with hormones in the initiation and progression of senescence, as it has been described before (Overmyer and others 2003). However, other studies have shown that the timing of ROS increases seems to be linked to cell death rather than to the initiation of senescence (Rogers 2012). A transient production of ROS that is not counterbalanced by antioxidant defences, that is an oxidative stress, can therefore occur during flower senescence, but timing may strongly differ between species.

In the current study, with the aim of getting new insights into the possible role of hormonal sensitivity in the control of senescence in lilies, we examined the response of *L. longiflorum* flowers to hormonal applications at different time points. We used applications of a combination of CKs and GAs, well-known inhibitors of senescence, as a tool to monitor time-dependent shifts in hormonal sensitivity during the progression of tepal senescence. Emphasis was put on a possible identification of the “point of no return” in flower senescence in lilies.

## Materials and Methods

### Plant Material and Experimental Design

Lily flowers used in this study were *L. longiflorum* var. *White heaven*. Lilies are grouped according to their genetic phylogeny. The main groups for cut flower production are Asiatic hybrids, Oriental hybrids, *L. longiflorum* hybrids

and LA hybrids (van Doorn and Han 2011). Lily is a perennial ornamental bulbous plant with radial symmetry, belonging to subclass *Monocotyledonae* and family *Liliaceae*, being one of the largest monocot families with the exception of *Gramineae*. Lily flowers have two whorls of coloured floral leaves, called tepals, consisting in three tepals each whorl (van Doorn 2011). The androecium is formed by six stamens and the gynoecium by three fused carpels and a superior ovary (Judd and others 2007).

Plant material for the experiments was obtained as follows. Bulbs of *L. longiflorum*—imported from Netherlands—were planted in two batches at the greenhouse of the experimental fields in the Faculty of Biology at the University of Barcelona. A first round of bulbs was transferred to pots, meanwhile the rest of the bulbs were maintained in a cold room at 4 °C. Two weeks later the second round of bulbs was planted. The bulbs grew in a density of two bulbs per 12 cm × 12 cm × 20 cm plant pot in a peat moss soil and were watered automatically with tap water once per night.

Floral senescence was defined according to Arrom and Munné-Bosch (2010), as follows: stage I, closed flower bud, green tepals and immature androecium; stage II, closed flower bud with less green tepals and brown stamens, reproductive organs more mature; stage III, flower at anthesis, with greenish tepals and pollen inside the anthers; stage IV, white, hydrated tepals, with pollen out from anthers; stage V, wilted tepals—due to loss of turgor; stage VI, wilted tepals with highly withered edges (becoming brownish and suffering an extra and slow dehydration, just before abscission occurs).

All experiments were performed in cut flowers. Harvest started on 7th January 2013, when most of the buds were at stage I and at least one of the buds of the same bulb was at stage II. The stem was cut 10–15 cm from the flower base at stage II, and then leaves from the stem were removed to minimize the appearance of pathogens. Next, each flower was transferred to a 50 mL Falcon tubes with distilled water. The water was regularly renewed to be sure it was absolutely clean. All cut flowers during their post-harvest period were exposed to constant and controlled climatic conditions in a long day room: 16 h of light with a photon flux density of 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 8 h of dark, at 20 °C of temperature and a 50% humidity. Sampling was always performed between 9:00 and 11:00 AM (UTC/GMT +1).

Two independent experiments were performed. First, for a study of flower development, the progression of senescence was followed, in a time-course evolution, from anthesis (stage III) to 6 days after anthesis, which corresponded to stage VI. Samplings of tepals were performed once a day. In a second experiment, we tested the effects of hormonal sensitivity on senescence by applying phytohormones at various time points. Treatments consisted of an application of hormones with a spray (but protecting the

gynoecium). Those treatments were performed either at 1, 3 or 5 days after anthesis. This means that each flower was treated just once with one treatment during its entire lifespan. The sprayed solutions were the following: 5.3 mL L<sup>-1</sup> Promalin<sup>®</sup> (Kenogard, Barcelona, Spain) in distilled water, as recommended by the manufacturer, and clean distilled water as a control. Promalin is a commercial product made of a mixture of 50% of cytokinins (benzyladenine, BA) and 50% of gibberellins (GA<sub>4+7</sub>) at a concentration of 1.9% (w/v) for each hormonal type, which has been shown to be very effective in delaying flower senescence (Ranwala and Miller 1998). The applied hormonal concentrations correspond to 0.44 mM cytokinin plus 0.30 mM GAs. Floral longevity was measured with a daily monitoring of each flower placed in the Falcon tubes, counting the days elapsed to reach the different developmental stages (stage III to abscission). For biochemical analysis, samples were taken after 48 h of each treatment, so that the tepals of each flower were collected and immediately frozen in liquid nitrogen. Then, samples were stored at -80 °C. With this second experiment, the effect of hormonal treatment, the time of application and its interaction were evaluated.

### Tepal Hydration and Protein Analysis

Tepal hydration (calculated as [FW-DW]/DW) was measured by estimating the fresh weight (FW) just after sampling, and subsequently oven-drying the tissue at 80 °C for 24 h to obtain the dry weight (DW). For protein analysis, 50 mg of samples was extracted three times in 1.5 mL buffer extraction solution containing Tris-HCL pH 7.4, EDTA 1 mM, DTT 1 mM and Triton X-100 0.1% using ultra-sonication. After 10 min centrifugation at 10,000 rpm at 4 °C, supernatants were taken and pooled. Then we mixed 200  $\mu\text{L}$  of Bradford reagent (Sigma-Aldrich), 5  $\mu\text{L}$  of buffer extraction solution and 5  $\mu\text{L}$  of sample extraction using three technical replicates per sample. Absorbance was read at 590 nm using a spectrophotometer. The calibration curve was measured with the standard protein bovine serum albumin as described (Bradford 1976).

### Hormonal Profiling

Hormonal profiling, including CKs, auxin, GAs, ABA, the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), SA and JA were determined by UPLC-MS/MS as described (Müller and Munné-Bosch 2011). One hundred mg of samples was extracted in 2 mL Eppendorf tubes using 0.2 mL 50:49:1 methanol:isopropanol:acetic acid (v/v/v) and ultra-sonication at 4 °C. After centrifugation at 10,000 rpm for 10 min at 4 °C, the supernatant was collected and the pellet was re-extracted. Both mixed

supernatants were filtered with a PTFE filter (Waters, Milford, MA, USA) afterwards and samples injected into the UPLC-MS/MS system. Quantification of each compound was performed using the MRM quantification method and recovery rates calculated for every sample using deuterated standards, as described (Müller and Munné-Bosch 2011).

### Tocopherol Analysis

Tocopherol levels were determined by HPLC as described in Cela and others (2011). In short, 50 mg of sample was extracted with 1.5 mL ice-cold methanol using a Branson 2510 Ultrasonic Cleaner (Branson) for 45 min. Tocopherols from the extract were separated using normal-phase HPLC. The Jasco-HPLC device was equipped with an Inertsil 100A (250 mm × 3 mm, 5 µm, GL Sciences Inc., USA) column operating at room temperature and a FP-1520 fluorescence detector (Jasco, UK). Tocopherols were identified by co-elution with authentic tocopherol standards purchased from Sigma-Aldrich (USA) and were quantified using appropriate calibration curves made from the same standards.

### MDA Assay—Measurement of Lipid Peroxidation

The extent of lipid peroxidation was estimated from the amount of malondialdehyde (MDA) equivalents. Malondialdehyde levels were measured spectrophotometrically following the method described by Hodges and others (1999), which takes into account the possible influence of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay.

### Statistical Analysis

Statistical analyses were performed with the SPSS 15.0 software (SPSS, Chicago, IL, USA). Time-course evolution differences in the studied parameters, as well as differences between treatments and time of application were evaluated using the analysis of variance (ANOVA), followed by the Tukey-B post hoc test and were considered significant at a probability level of  $P < 0.05$ . Student's *t* tests were used to evaluate possible differences between Promalin treatment and control at specific time points of treatment.

## Results

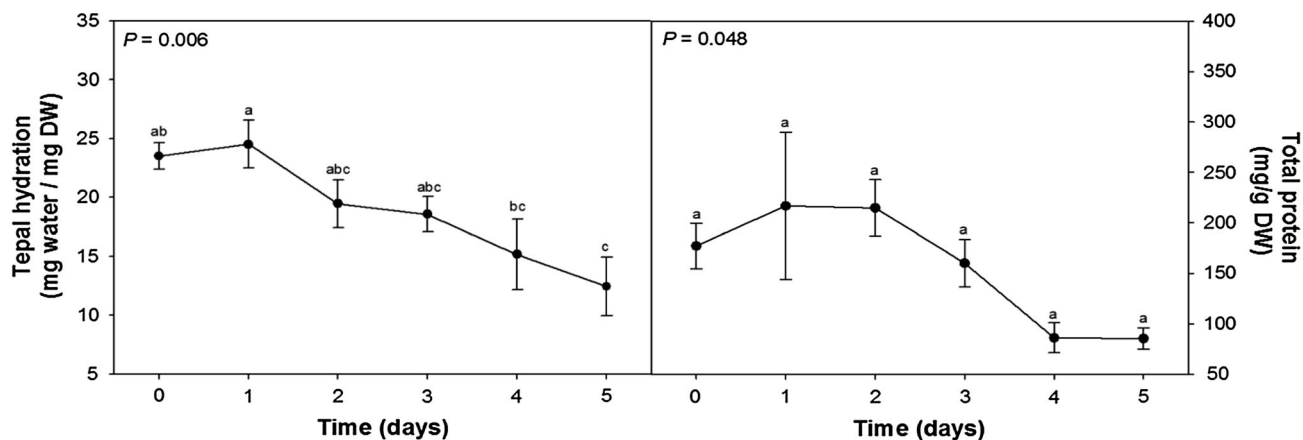
### Time-Course Evolution of Tepal Senescence: A Major Role for Cytokinins and ABA

Tepal senescence in cut flowers was characterized by a progressive tepal dehydration and protein degradation in *L.*

*longiflorum* (Fig. 1). Note that changes were small from day to day, due to the daily monitoring of these parameters during the study, but time-course evolution differences were significant (ANOVA,  $P < 0.05$ ). Comparing both senescence markers, it appears that tepal hydration indicated better than protein levels the daily progression of tepal senescence, reflecting tepal wilting (Fig. 1). Among the studied cytokinins, IPA was the only one showing a significant decline in endogenous levels during the progression of senescence. Neither *trans*-zeatin, its riboside, nor 2-isopentenyladenine showed significant differences during senescence (Fig. 2). Importantly, however, IPA levels decreased progressively, but very substantially, from 0.55 µg g<sup>-1</sup> DW to 0.03 µg g<sup>-1</sup> DW from anthesis to 5 days after anthesis, respectively, thus reflecting a loss of an active cytokinin by 95% during tepal senescence. Regarding the GA profile, no significant differences were observed for either GA investigated, except for GA<sub>24</sub>, which increased the third day after anthesis to decrease later (Fig. 3). No time-dependent variations in the levels of either GA<sub>4</sub>, its precursor GA<sub>9</sub> (Fig. 3) or GA<sub>7</sub> (data not shown) were observed. Among hormonal senescence inhibitors, it appears therefore that an active cytokinin, IPA in particular, decreases markedly during the senescing process, whereas among GAs, only a GA<sub>4</sub> precursor, GA<sub>24</sub> in particular, shows a biphasic response, with increases up to 3 days after anthesis followed by a later decline, suggesting changes in GA metabolism. These changes, however, do not lead to reductions in endogenous concentrations of active GAs during tepal senescence (Fig. 3).

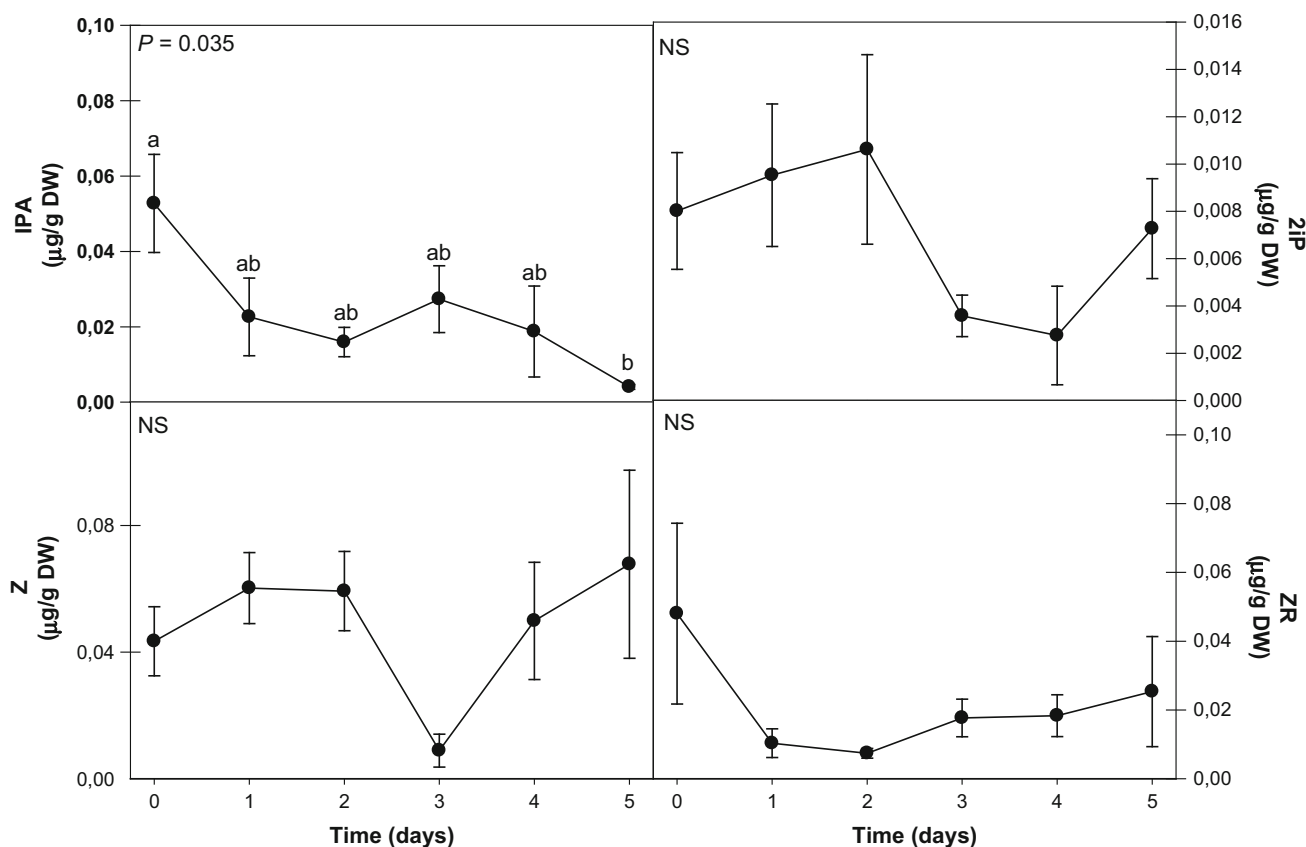
Among potential senescence promoters, ABA and SA were investigated. *L. longiflorum* is considered an ethylene-insensitive flower, so ethylene might not play a role. The levels of ACC—the ethylene precursor—were measured, obtaining very low amounts and with no changes along the time points (data not shown). This, together with the 2.2-fold increases of ABA levels during tepal senescence (Fig. 4), confirms that ABA is the major promoter of senescence in these ethylene-insensitive flowers. Interestingly, however, variations in ABA levels were subject to a strong variation, and concentrations did not clearly increase until the latest stages of senescence (Fig. 4). In contrast, SA levels decreased during the progression of tepal senescence (Fig. 4), whereas those of JA did not show any significant differences (Fig. S1). Taken together, the hormonal profile suggests that cytokinins, IPA in particular, is the most important inhibitor of tepal senescence in *L. longiflorum*, and IPA may act in concert with ABA during the latest stages of the process.

Hormonal profiling variations were evaluated together with those of tocopherols (vitamin E) and MDA, an indicator of the extent of lipid peroxidation. MDA and α-tocopherol levels were not altered during the progression



**Fig. 1** Time-course evolution (expressed as days after anthesis) in hydration and protein levels during natural development in tepals of *L. longiflorum*. Data correspond to the mean  $\pm$  SE of  $n = 4$ . Results

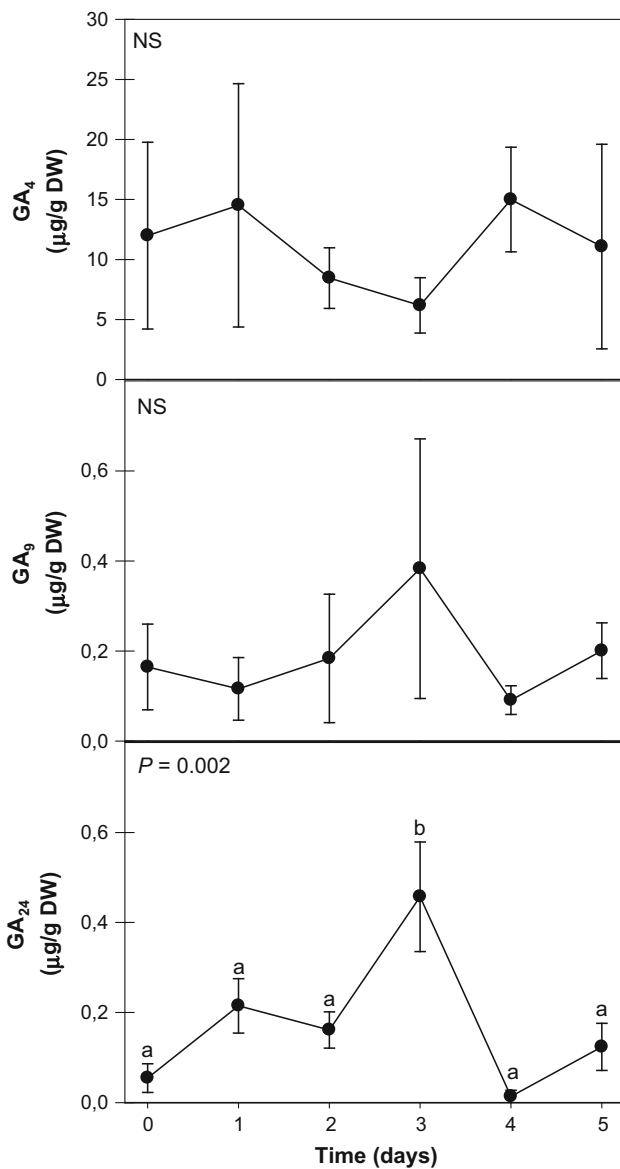
of statistics are shown inside the panels (ANOVA,  $P < 0.05$ ). When significant, *different letters* indicate differences between days as indicated by the Tukey post hoc test. DW dry weight



**Fig. 2** Time-course evolution (expressed as days after anthesis) in the endogenous concentration of cytokinins levels during natural development in tepals of *L. longiflorum*. Data correspond to the mean  $\pm$  SE of  $n = 4$ . Results of statistics are shown inside the panels

(ANOVA,  $P < 0.05$ ). NS not significant. When significant, *different small letters* indicate differences between days as indicated by the Tukey post hoc test. DW dry weight, 2-*iP* isopentenyladenine, IPA isopentenyladenosine, Z *trans*-zeatin, ZR *trans*-zeatin riboside



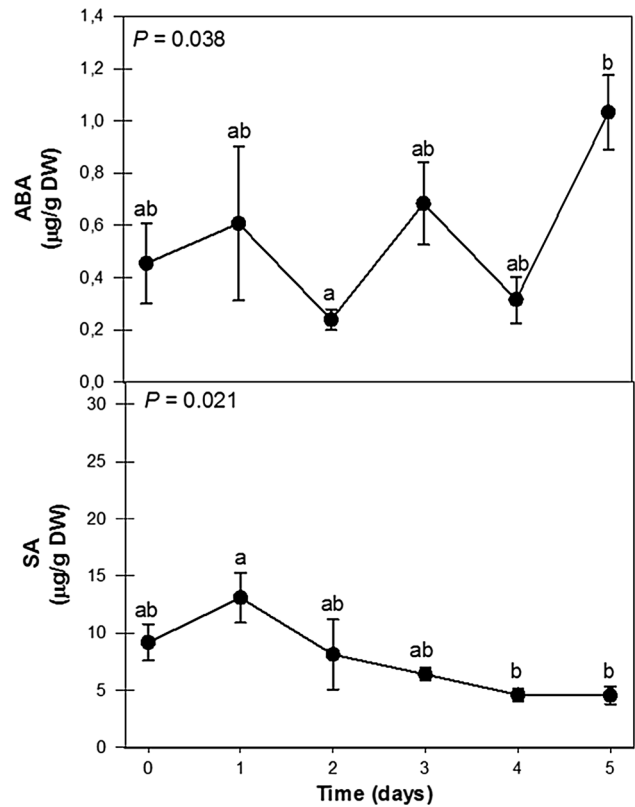


**Fig. 3** Time-course evolution (expressed as days after anthesis) in the endogenous concentration of gibberellins levels, including GA<sub>4</sub> and its precursors, GA<sub>9</sub> and GA<sub>24</sub> during natural development in tepals of *L. longiflorum*. Data correspond to the mean ± SE of *n* = 4. Results of statistics are shown inside the panels (ANOVA, *P* < 0.05). *NS* not significant. When significant, *different small letters* indicate differences between days as indicated by the Tukey post hoc test. *DW* dry weight, *GA* gibberellin

of senescence, whereas those of α-tocopherol, the precursor of α-tocopherol, increased during senescence, particularly at the latest stages (Fig. S2).

**Sensitivity of Cut Lily Flowers is Quantifiable**

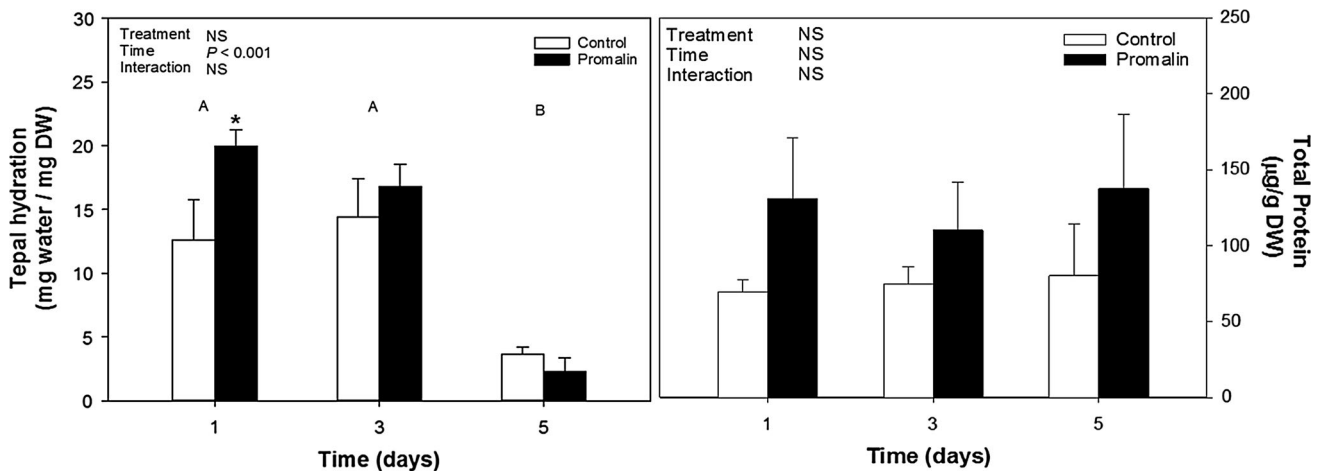
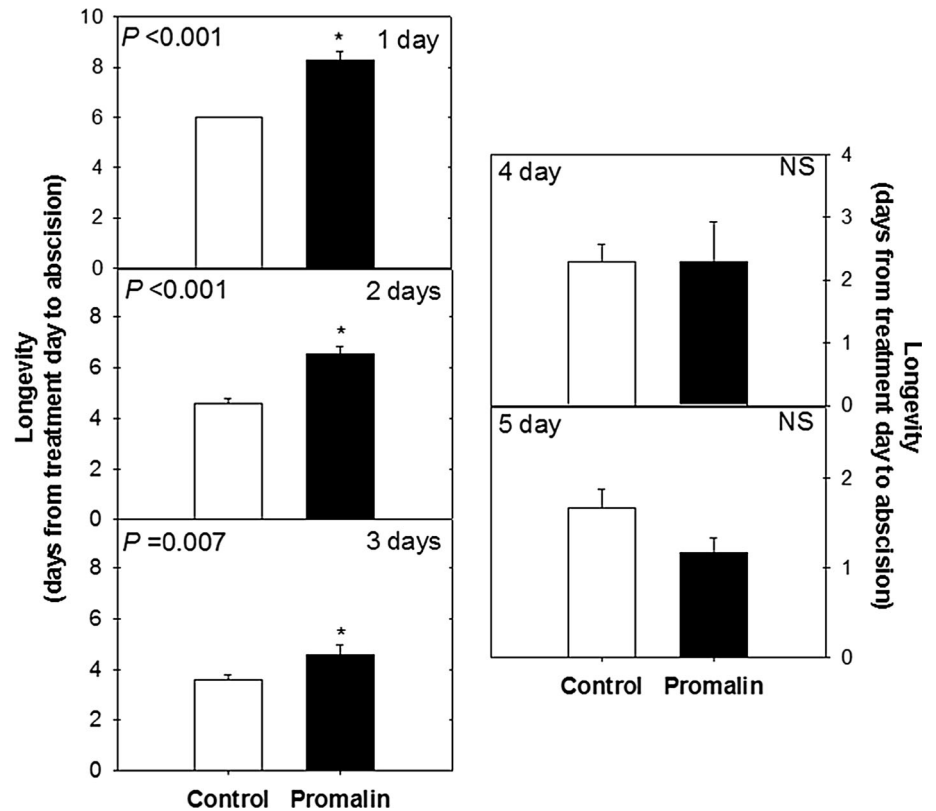
Using Promalin treatments, we could accurately describe a gradual decrease in hormonal sensitivity during the



**Fig. 4** Time-course evolution (expressed as days after anthesis) in the endogenous concentration of hormone senescence promoters levels during natural development in tepals of *L. longiflorum*. Data correspond to the mean ± SE of *n* = 4. Results of statistics are shown inside the panels (ANOVA, *P* < 0.05). *NS* not significant. When significant, *different small letters* indicate differences between days as indicated by the Tukey post hoc test. *DW* dry weight, *ABA* abscisic acid, *SA* salicylic acid

progression of tepal senescence. It resulted to be precisely limited by a point that once passed, the organ was inevitably committed to death (Fig. 5). Significant positive effects in retarding senescence were observed when Promalin was applied 1, 2 or 3 days after anthesis. When applied 1 day after anthesis, the longevity was extended 2.2 days, whereas treatments on days 2 and 3 prolonged flower lifespan by 1.9 and 0.8 days, respectively. Afterwards, there were no differences between the treatments; the application of hormones after the third day was useless. Therefore, sensitivity loss occurred gradually and was completely lost at the fourth day after anthesis (Fig. 5). It is worthy to note that the lifespan of flowers was longer when Promalin was applied 1 day after anthesis than in controls, and was similar when applied 2 days after anthesis compared to controls. The application at day 3 was very positive but was not able to fully reverse the senescence process (Fig. 5).

**Fig. 5** Longevity of control and Promalin-treated *L. longiflorum* flowers. Data correspond to the mean  $\pm$  SE of  $n = 7$ . Results of statistics are shown inside the panels (ANOVA,  $P < 0.05$ ) and asterisks indicate significant differences between treatments at specific time points (Student's *t* test,  $P < 0.05$ ). Control, empty bars; Promalin-treated, solid bars. NS not significant



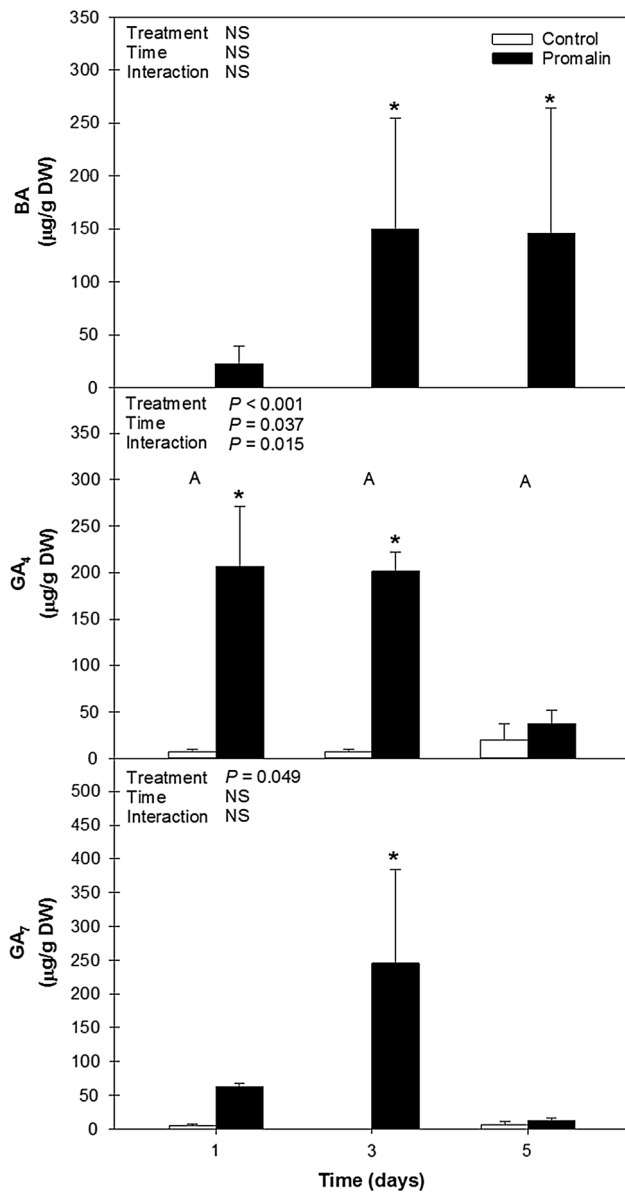
**Fig. 6** Hydration and protein levels in tepals of *L. longiflorum* flowers treated with Promalin or a control solution at 1, 3 and 5 days post-anthesis. Analyses were performed 48 h after treatments. Data correspond to the mean  $\pm$  SE of  $n = 4$ . Results of statistics are shown inside the panels (ANOVA,  $P < 0.05$ ). When significant,

different capital letters indicate differences between days as indicated by the Tukey post hoc test. NS not significant. Asterisks indicate significant differences between treatments at specific time points (Student's *t* test,  $P < 0.05$ ). DW dry weight

### Promalin Treatment Reveals Hormonal Cross-Talk

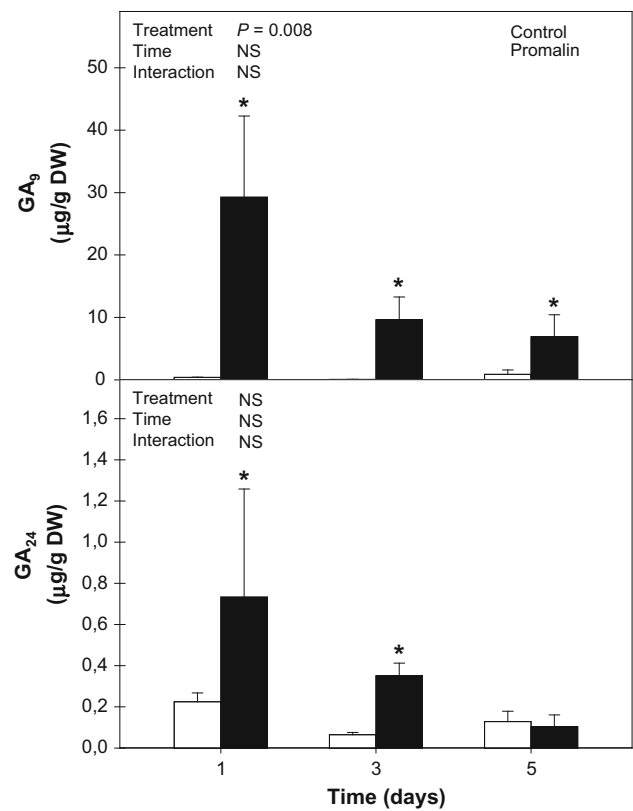
Tepal hydration increased after Promalin applications, but only when it was applied 1 day after anthesis (Fig. 6). Since measurements were performed 48 h after hormonal application, the positive effect was not observed when Promalin was applied 3 days after anthesis. Protein levels

were not significantly altered by Promalin treatments (Fig. 6). Among the hormones measured, not only the levels of benzyladenine and  $GA_4 + GA_7$  increased after Promalin application, as components of this product (Fig. 7), but also those of  $GA_9$  and  $GA_{24}$  (Fig. 8). Interestingly, effects on  $GA_4$  precursors, that is  $GA_9$  and  $GA_{24}$ , were more apparent with the application 1 day after



**Fig. 7** Endogenous concentration of applied hormones in tepals of *L. longiflorum* flowers treated with Promalin or a control solution at 1, 3 and 5 days post-anthesis. Analyses were performed 48 h after treatments. Data correspond to the mean ± SE of *n* = 4. Results of statistics are shown inside the panels (ANOVA, *P* < 0.05). When significant, different capital letters indicate differences between days as indicated by the Tukey post hoc test. NS not significant. Asterisks indicate significant differences between treatments at specific time points (Student’s *t* test, *P* < 0.05). Control, empty bars and Promalin-treated, solid bars. DW dry weight, BA benzyladenine, GA gibberellins

anthesis than 3 or 5 days after anthesis. Furthermore, levels of ABA decreased in Promalin-treated flowers, but only when applied 1 and 3 days after anthesis, not later (Fig. 9), thus clearly indicating a hormonal cross-talk. Interestingly, whereas benzyladenine, as an exogenous hormone, accumulated particularly in tepals that were treated 3 and



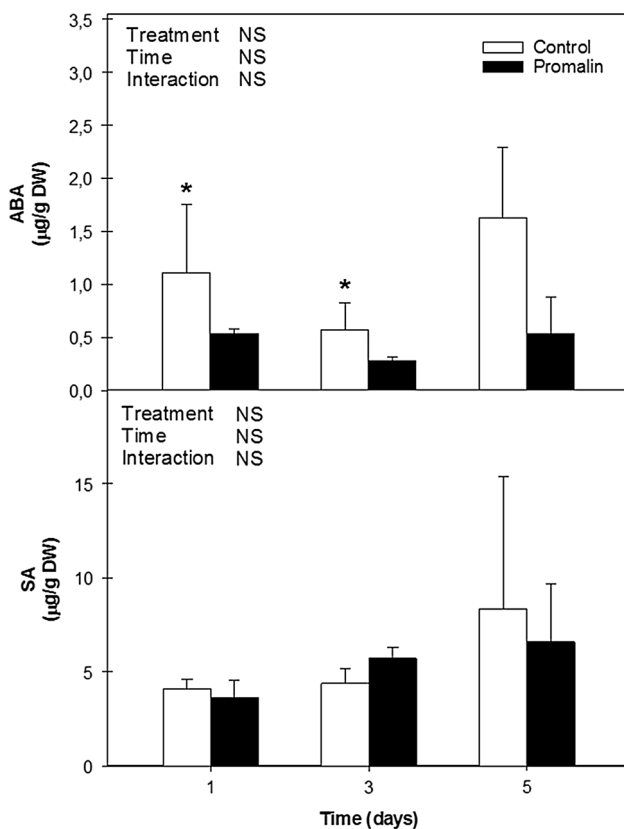
**Fig. 8** Endogenous concentration of the GA<sub>4</sub> precursors, GA<sub>9</sub> and GA<sub>24</sub> in tepals of *L. longiflorum* flowers treated with Promalin or a control solution at 1, 3 and 5 days post-anthesis. Analyses were performed 48 h after treatments. Data correspond to the mean ± SE of *n* = 4. Results of statistics are shown inside the panels (ANOVA, *P* < 0.05). When significant, different capital letters indicate differences between days as indicated by the Tukey post hoc test. NS not significant. Asterisks indicate significant differences between treatments at specific time points (Student’s *t* test, *P* < 0.05). Control, empty bars and Promalin-treated, solid bars. DW dry weight, GA gibberellins

5 days after anthesis, the exogenous application of Promalin led to reduced levels of natural cytokinins, including not only those of IPA, but also those of *trans*-zeatin and its riboside (Suppl. Fig. S3). Neither tocopherols nor MDA levels were influenced by Promalin treatment (Suppl. Fig. S5).

### Discussion

Flowers play a critical role in angiosperm reproduction and are often pigmented and/or scented to attract pollinators. However, despite its irreplaceable ecological role, the flower is energetically expensive to maintain and that is why its lifespan is tightly limited. As shown in the present study in lilies, tepal dehydration and protein degradation are important events during tepal senescence. In many species, petal wilting is hastened by ethylene, but in





**Fig. 9** Endogenous concentration of abscisic acid (ABA) and salicylic acid (SA) in tepals of *L. longiflorum* flowers treated with Promalin or a control solution at 1, 3 and 5 days post-anthesis. Analyses were performed 48 h after treatments. Data correspond to the mean  $\pm$  SE of  $n = 4$ . Results of statistics are shown inside the panels (ANOVA,  $P < 0.05$ ). When significant, *different capital letters* indicate differences between days as indicated by the Tukey post hoc test. *NS* not significant. *Asterisks* indicate significant differences between treatments at specific time points (Student's *t* test,  $P < 0.05$ ). Control, *empty bars* and Promalin-treated, *solid bars*. *DW* dry weight

numerous others it is not affected or only slightly affected by ethylene treatments, as shown in *L. longiflorum* (van Doorn 2001b). In the present study, it is shown that among endogenous cytokinins, IPA was probably the most active compound inhibiting tepal senescence in lilies, and IPA may act in concert with ABA in modulating tepal senescence. Furthermore, aside from the cross-talk of endogenous hormonal concentrations, hormonal sensitivity may also be a major factor controlling tepal senescence in lilies, being an additional regulatory physiological mechanism that gradually changes over time.

Optimal floral longevity is determined by the interaction of three major factors: daily cost of maintaining a flower relative to the cost of constructing a new one; rate at which pollen is received to fertilize ovules; and rate at which pollen is disseminated (Ashman and Schoen 1994). Based on this, perianth senescence is regulated by pollination.

However, more typically, senescence occurs gradually as part of a temporal programme of flower development that is accelerated by pollination (O'Neill 1997). In addition, the pollination role during floral senescence is generally regulated by endogenous ethylene (van Doorn 2001a). What happens however in lilies, in which (i) pollination does not occur, (ii) flowers are insensitive to ethylene, and (iii) senescence is characterized by tepal de-greening due to chlorophyll degradation? Will these flowers be subject to a potential re-greening, as it happens with leaves? Is it possible to use the concept of “the point of no return” in these flowers? Pollination and ethylene were clearly not the first signals triggering senescence, because neither of them operated in our system using *L. longiflorum*. According to our results, senescence in these flowers responds to a finely defined hormonal sensitivity in tepals, which is gradually lost with time. This appears to be triggered by a cross-talk between cytokinins and ABA, the first decreasing and the latter increasing, which appear to be the main executors of senescence, as it has been suggested previously in ethylene-insensitive flowers (van Doorn and Woltering 1988). These results are indeed in agreement with previous studies obtained in a hybrid of the same species (*L.A. Courier*, which is a *L. longiflorum*  $\times$  Asiatic hybrid, Arrom and Munné-Bosch 2012).

From an applied point of view, we demonstrated when a hormonal treatment should be applied to reach the maximum efficiency in physiological improvement in terms of longevity. It was shown that this short-time period is limited by hormonal sensitivity during floral senescence. In the present study, the point at which sensitivity was completely lost was at the fourth day after anthesis; afterwards it was totally useless to spray tepals with Promalin. Once this point is reached, tepals inevitably undergo senescence. This critical point has been defined before in leaf senescence as ‘the point of no return’ (Karagiannis and Pappelis 1994), that is the point at which, once passed, the leaf is committed to death (Munné-Bosch and Alegre 2004). Here, hormonal sensitivity loss was a quantifiable parameter, as it occurs in leaves (Zavaleta-Mancera and others 1999b), and was between 2 and 3 days after anthesis in the cut flowers used in the present study. Promalin was effective and gave excellent results when applied 1 and 2 days after anthesis, but not after 3 days of anthesis. However, Promalin treatments did not result in re-greening, as happens in leaves, simply because tepals at anthesis are already completely de-greened in this species. Therefore, we suggest here not talking about “re-greening” or “point of no-return” in this case, but about “hormonal sensitivity loss”.

It is concluded from our experiments that during floral senescence in *L. longiflorum*, complete sensitivity loss occurs at the fourth day after anthesis. We found that among the natural cytokinins, IPA plays a major role

during floral senescence together with ABA. Finally, Pro-malin can be used to effectively extend flower lifespan in lilies with applications up to 2 days after anthesis. Further, insights into the complex interplay between ABA, cytokinins and gibberellins are however required to better understand flower senescence in lilies.

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