Ephemerality of a spring ephemeral *Gagea lutea* (L.) is attributable to shoot senescence induced by free linolenic acid

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Running title: Linolenic acid induces shoot senescence

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Abbreviations: Aq, aqueous: BuOH, butanol; EI-MS, electron impact mass spectrometry; EtOAc, ethyl acetate; EtOH, ethanol; JA,jasmonic aicd; JA-Me, methyl jasmonate; MeOH, methanol; NMR, nuclear magnetic resonance; ROS, reactive oxygen species;

(Abatract)
Spring ephemerals are a group of herbaceous plants that fulfill their life cycle on the floor of deciduous forests in temperate and boreal regions during a short period of time between snowmelt and closure of the tree canopy. Near the closure, these plants' shoots senesce rapidly and the plants disappear from the floor. Since the major role of the synchronous senescence is thought to be the recycling of nutrients from vegetative organs to seeds or storage organs, some endogenous compound that is capable of promoting senescence must be involved in the timely senescence. Strong senescence-promoting activity was found in extracts of shoots of a spring ephemeral, *Gagea lutea* (Liliaceae), and the activity in basal leaves reached a maximum just before the commencement of senescence. The active compound was identified as α-linolenic acid. The level, very low one week before flowering, increased rapidly with time and reached a maximum one week after flowering. Senescence was readily observed thereafter. The maximum amount of linolenic acid was more than 1 mmol kg FW^{-1} and could fully induce senescence of the leaves. The results suggest that the ephemerality of the plant, or in other words, short longevity of shoots, is brought about by the accumulation of linolenic acid. Programmed senescence, which can mitigate the cost of survival and reproduction, enables the plant to occupy a narrow niche on the forest floor.

Key words: Spring ephemeral, *Gagea lutea*, Linolenic acid, Shoot senescence,

Introduction

Senescence of a plant after maturation of fruit or storage organs is a crucial developmental process that occurs at the final stage of plant development, and seems to have been acquired during evolution to minimize costs. Too early senescence would reduce total yield, whereas too late senescence would impose the cost of maintaining old and inefficient assimilation organs, and interfere with remobilization of nutrients remaining in vegetative organs to reproductive organs. Therefore the onset of senescence has to be exactly regulated by some endogenous signal. The plant hormones JA and ethylene are associated with the onset of leaf senescence (Grbic and Bleecker 1995, He et al. 2002). However, their implications in the senescence of whole shoots or plants has not been elucidated. The ephemerality of spring ephemerals is brought about by early and synchronous senescence of the shoots. Elucidation of the mechanism of the senescence may supply clues to understand shoot senescence of various Liliaceae plants, such as lily, onion, garlic, and tulip that occur near maturation of the bulbs.

Growth of spring ephemerals in early spring involves some risks such as late frost, but the risks are offset by the advantages of both strong sunlight and weak competition for pollinators (Schemske et al. 1978). In addition, soil moisture is high due to snowmelt, and nutrients are plentiful because of the decomposition of leaves that fell the previous autumn. *Gagea lutea* Ker-Gaul. (Liliaceae), distributed widely in northern Eurasia, is one of the most common spring ephemerals in Hokkaido, Japan. The life cycle and appearance of the plants is shown in Fig. 1. A typical time course of the change in the appearance of the colony is presented in supplementary Fig. S1. The plant reproduces through seeds and division of bulbs. After disappearance of above-ground shoots, the bulbs and seeds lie dormant until the next spring. The species has a unique reproductive strategy affecting the allocation of photosynthetic product to seeds or bulbs (Sunmonu et al. 2013). The earlier the snowmelt, the easier the seed production (Nishikawa 2009).

The species is spreading from the original habitat, forest floors, to lawns and other open spaces. The plant can thrive under human disturbances, such as mowing and foot traffic, and thus the colonies at our botanic garden are spreading to the point of becoming a nuisance. We hypothesize that the plants produce an endogenous senescence-promoting compound responsible for their rapid senescence. The present study was carried out to elucidate the causal compound. For detection of senescence-promoting activity in *Gagea* plant extracts, a bioassay using the leaves of the plant would be ideal. However, it is very difficult to obtain the leaves at any time of year. For convenience, the senescence-promoting activity was mostly assayed by a wheat-leaf test and was sometimes confirmed by an assay using *Gagea* leaves.

Results

Senescence in a forest and in an open field

In the forest, senescence may occur passively from the deficiency of light due to canopy closure. The progress of senescence of forest-grown and open field-grown plants with time were compared by monitoring the change in chlorophyll content in the basal leaves. In the forest, synchronous senescence became observable two weeks after flowering and the shoots of the plants had completely dried after five weeks (supplementary Fig. S2). In the open field, senescence began and proceeded slightly faster than in the forest-grown plants, indicating that the senescence was induced actively by some senescence-promoting compound rather than induced passively by a deficiency of light.

Detection of the senescence-promoting activity and its time course change

To detect activity of any senescence-promoting compounds in the plants, solvent fractions were obtained from plants harvested one week after flowering, just before the commencement of senescence, and tested in a wheat-leaf assay. Strong activity was found in EtOAc fractions of the floral

shoots and the basal leaves (Fig. 2). Considerable activity was also found in BuOH fractions. The activity in floral shoots at a concentration of 1 g FW equivalent ml^{-1} was comparable to that of 0.1 mM JA-Me. On the other hand, no appreciable activity was found in any of the fractions obtained from the bulbs. The senescence-promoting compound in EtOAc fraction was further purified with a silica gel column. The fraction was dissolved in toluene and passed through a silica gel column. After washing the column with toluene, the compounds adsorbing to the column were eluted sequentially with 10%, 20%, 40%, 80% EtOAc in toluene and finally with EtOAc. The strongest activity was found in the 10% EtOAc fraction (Fig. 3). This fraction was capable of inducing senescence of *Gagea* leaves (supplementary Fig. S3). The time course changes in the senescence-promoting activity in the leaves and floral shoots were examined using the 10%EtOAc fraction with wheat leaves. Almost no activity was found in this fraction from basal leaves until the commencement of flowering (Fig. 4). Thereafter the activity continued to increase, reaching a maximum two weeks after flowering. Then the activity gradually decreased and almost disappeared after five weeks. Senescence became visible two weeks after flowering. In extracts of floral shoots, a similar activity profile was observed except that there were two peaks of activity. These results suggest that the senescence-promoting compound detected here is responsible for the senescence.

Isolation and identification of the senescence-promoting compound

An attempt at isolation of the active compound was made utilizing the assay for senescence-promoting activity according to the procedure summarized in Fig. 5. From 1.0 kg of fresh basal leaves harvested one week after flowering, 120 mg of active compound was obtained. The recovery of the compound by the procedure was estimated by the standard addition method as 72 ± 5% (±SD, n=3). The EI mass spectrum revealed that the compound had a molecular ion peak at *m/z* 278 and the fragmentation pattern was identical to that of linolenic acid (Fig. 6). From the ^{1}H and ^{13}C NMR spectral data, this compound was identified as 9*Z*,12*Z*,15*Z*-octadecatrienoic acid

(α-linolenic acid). Linolenic acid had strong senescence-promoting activity with *Gagea* leaves as well as wheat leaves (Fig. 7). The activity at a concentration of 3 mM was stronger than that of JA-Me at 0.1 mM.

The time course change in the level of linolenic acid

The time course change in the level of linolenic acid during the growth of the plants was examined the following year. The fluctuations in the level of linolenic acid in basal leaves and floral shoots during the growth of the plants were very similar (Fig. 8). In the basal leaves, a sharp peak in the amount of the compound was found one week after flowering, just before the commencement of senescence. In the floral shoots, the largest peak was found at the same time as in the basal leaves, and an additional small peak was found four weeks after flowering.

Discussion

The results presented herein strongly suggest that the ephemerality of *Gagea* plants is caused by accumulation of linolenic acid. Maintenance of shoots on the dim forest floor after canopy closure must cause a deficit of storage compounds. To avoid such a cost, spring ephemerals have developed programmed senescence in response to an endogenous senescence-promoting compound, in this case linolenic acid, and thereby can exploit a narrow niche on the forest floor. Time course changes in the senescence-promoting activity detected by the bioassay (Fig. 4) were slightly different from that of the levels of linolenic acid (Fig. 8). The senescence-promoting activity expressed as per unit fresh weight of the sample decreased after every step in the purification procedure. When all of the fractions obtained by a chromatography were recombined, almost all the activity was recovered, suggesting the presence of synergists that enhance the activity of linolenic acid. We have tried to isolate a synergist, but have failed due to the low reproducibility of their ancillary activity. The differences in the results shown in Figures 4 and 8 seem to be due to removal of these synergistic compounds, as well as year-to-year variation.

The mechanism by which linolenic acid is produced in temporal relation to the induction of shoot senescence remains unknown. Lapointe (2001) suggested that leaf senescence of a spring ephemeral is caused by a decrease in sink activity of underground storage organs after filling with carbohydrates. It is probable that overflow of the carbohydrates triggers linolenic acid synthesis. To obtain *Gagea* leaves for bioassay in this study, the plants were raised from bulbs in a growth chamber. After the excision of leaves, leaves were recovered from residual underground parts within two weeks, and as a result the senescence of the plants was delayed ca. three weeks compared to the senescence of intact plants. It is likely that the delay in senescence of defoliated plants occurs in compensation for loss of carbohydrates caused by the removal of the leaves. A high tolerance of the plants to mowing and foot traffic seems to be due to this characteristic. The shoots senescence of various Liliaceae plants, such as lily, onion and garlic, near maturation of bulbs seems to be caused by an accumulation of linolenic acid.

In both *Gagea* and wheat leaves, the senescence-promoting activity of linolenic acid increased with an increase in concentration, and considerable activity was found at a concentration of 3 mM (Fig. 7). Even at this concentration in the wheat assay, the fatty acid applied to the filter paper did not dispersed into the assay medium and not smeared the leaf surface, indicating that the senescence was not induced by inhibition of respiration. The compound seems to be incorporated into the leaf tissue in a concentration-dependent manner irrespective of its low solubility in water. But when wheat leaves are placed on 3 mM linolenic acid for one day, net increase in the concentration of linolenic acid in the leaves was only 0.15 mM, indicating that the rate of uptake is low. The maximum level of endogenous linolenic acid in *Gagea* leaves and the floral shoots was almost 1 mmol and 2 mmol kg FW^{-1}, respectively, when the values were corrected based on the recovery. These levels would seem to be fully sufficient to induce shoot senescence.

Linolenic acid is a major constituent of the glycolipids in the plastid

membrane, and the fatty acid released from the membrane is the precursor of JA synthesis (Vick and Zimmerman 1984, Weber et al. 1997). We have to examine whether linolenic acid itself has senescence-promoting activity or exerts the activity after conversion to JA or JA-Me. On the other hand, linolenic acid has antibacterial activity (Andrew 2008), and one of the suggestions for a mechanism behind its activity is through the production of ROS (Wang et al. 1992). It is also possible that linolenic acid exerts senescence-promoting activity by an increase in the level of ROS. Furthermore, linolenic acid (18:3) is a known precursor of various biologically active compounds such as 9-hydroperoxy-linolenic acid (anti-fungal activity) (Ohta et al. 1990) and α-ketol linolenic acid (flower-inducing activity) (Suzuki et al. 2003). Linolenic acid may exert senescence-promoting activity after conversion to these unstable metabolites. We have found that hexadecatrienoic acid (16:3) monoglyceride is involved in the maintenance of the leaf rosette of radish plants as an anti-bolting compound (Yoshida et al. 2010). Free fatty acids and their derivatives appear to play important roles as endogenous chemical signals that control various developmental events in plants.

Materials and Methods

Plant materials

The experimental site was a colony (ca. 20 m^2) of *Gagea lutea* in a deciduous forest of the Botanic Garden of Hokkaido University. Flowering of the plants begins in late April. The canopy of overstory trees closes completely early in June. During growth of the plants, a specific species of *Synchytorium* fungus often infects them and induces wilt. To prevent infection, a fungicide (benomyl) diluted one thousand times was sprayed on the plants every two weeks after flowering. Spraying strongly inhibited infection of the leaves with no effect on the growth and development of the plants. The plants were harvested once a week from the middle of April (one week before flowering) until late May (almost complete senescence). The harvested plants were

divided into bulbs, basal leaves, and floral shoots with flowers or fruits (Fig. 1). To detect and isolate the senescence-promoting compound, bulbs with roots, basal leaves and floral shoots with flowers were harvested one week after flowering. Similar experiments were repeated three times in three different years. The results were reproducible. To obtain field-grown plants, 200 bulbs had been transferred from the forest floor to the experimental farm of our university.

Estimation of the senescence of the plants

The degree of senescence was estimated by chlorophyll content in 20 randomly selected basal leaves. The chlorophyll content was measured by a SPAD-502 meter (Konica Minolta, Japan) ca. 3 cm below the leaf tip.

Assay for senescence-promoting activity by wheat leaves

Wheat seeds (*Triticum aestivum* L. cv. Haruyutaka) were surface-sterilized for 40 min with 1% sodium hypochlorite solution and rinsed thoroughly. After immersion in water for 2 h, they were sown on a wet vermiculite surface embryo-side up and grown under a 16-h photoperiod at 25ºC. Illumination (2.0 mW cm^{-2}) was provided by white fluorescent lamps. After one week, 5 cm apical segments were excised from the first leaves and used for the assay. Each sample to be tested was dissolved in MeOH and applied to a 9-cm Petri dish containing a sheet of filter paper. After completely drying the sample, 5 ml McIlvain's citrate-phosphate buffer (pH 4.7, diluted tenfold with water and containing 0.2% Tween 20 and 10^{-6} M benzyl adenine) was added to the dish. Benzyl adenine was used to prevent spontaneous senescence of the leaves after excision. Ten leaf segments were placed in the dish with the adaxial leaf surface positioned upward. To check the efficacy of this assay method, JA-Me was used as a standard senescence-promoting compound (Koda 1992, Ueda et al. 1981) at concentrations between 10^{-6} M and 10^{-4} M. After incubation for 4 days at 25ºC in the dark, the remaining chlorophyll in the leaf segments was extracted with 5 ml boiling 80% ethanol. After immediate cooling and

20-fold dilution with 80% ethanol, the amount of chlorophyll was determined by the absorbance at 665 nm. Senescence-promoting activity was calculated as {100-100(A_{665} of extract obtained from leaves treated with sample)/(A_{665} of extract obtained from control leaves)}. Since practical difficulties prevented sufficient replication of each assay, the activity of JA-Me at a concentration of 10^{-5} was examined for 5 replicates. Each experiment was repeated 3 times and the results were found to be similar. Benomyl, which was sprayed on the plants as a fungicide, had no appreciable senescence-promoting activity.

Assay for senescence-promoting activity by *Gagea* leaves

Bulbs harvested from the colony early in June were washed thoroughly with running tap water, surface-sterilized with 1% sodium hypochlorite solution for 20 min, and rinsed thoroughly. The bulbs were planted in plastic pots filled with vermiculite wetted by Hyponex solution (1/1,000) and stored at 4ºC in the dark for more than 4 months until the bulbs had fully awakened from dormancy. Then the pots were transferred to a growth chamber (16-h photoperiod, 15ºC) with illumination (ca. 2.0 mW cm^{-2}) provided by white fluorescent lamps. After 4 months in storage, the bulbs began to germinate even at 4ºC and the shoots emerged from the vermiculite. When the pots were transferred to the growth chamber, the shoots began to grow rapidly and formed floral shoots. Flowers began to bloom within 2 weeks (supplementary Fig. S4) and the plants senesced completely 7 weeks after transfer. Leaves were collected from the plants just as they began to flower and were surface-sterilized as above for 10 min. After removal of the apical 2 cm of the leaves, 3-cm segments were prepared from the remaining leaves. Each sample to be tested was dissolved in MeOH, transferred to a 20-ml test tube and dried completely. Five ml of buffer solution as above (except that the concentration of Tween 20 was 0.4%) was added and the tube was sonicated for 5 min to disperse water-insoluble compounds in the buffer. To assay each sample, five leaf segments were immersed in the buffer solution per test tube. Because the leaves are very thick (0.773 ± 0.086 mm, ±SD,

n=16) and have a well-developed cuticle layer, the test tubes were kept under reduced pressure (ca. 50 kPa) for 10 min to facilitate incorporation of the test solution into the leaf tissue. After incubation for 5 days at 15°C in the dark, the remaining chlorophyll in the leaf segments was extracted twice with 5 ml boiling 80% EtOH. The samples were cooled immediately and diluted 20-fold with 80% EtOH, and the amount of chlorophyll was determined from the absorbance at 665 nm. Since practical difficulties involved in obtaining sufficient leaves did not permit the replication of each experiment, the activity of JA-Me at a concentration of 10^{-5} was determined by 5 replicates.

Isolation of senescence-promoting compound. The samples were homogenized with a sufficient amount of ethanol to give a final 70% ethanolic extract and then filtered. The filtrate was concentrated and the resultant aqueous residue was partitioned 3 times against *n*-BuOH and then EtOAc. After removal of the solvent by evaporation, each solvent fraction and the remaining aqueous fraction were analyzed for senescence-promoting activity. The senescence-promoting compound was purified by chromatography on columns of silica gel (Wakogel C-300) and on a Sep-Pak C_{18} cartridge (Waters). The Sep-Pak C_{18} cartridge had been washed with MeOH, water and then 70% MeOH. After each step of purification of the senescence-promoting compound, the fractions were evaporated to dryness, sealed with N_2 gas and kept at -20°C until use. The compound was further purified by semi-preparative HPLC (Prep Nova-Pak HR C_{18} column, 25×100 mm, Waters; solvent, 95% MeOH containing 0.1% acetic acid; flow rate 4 ml min^{-1}, retention time 14.0 min). The elution profile was monitored by absorbance at 210 nm. Final purification was carried out by analytical HPLC (Radial Pak Cartridge Nova-Pak C_{18}, 4.6×250 mm, Waters; solvent, 70% acetonitrile containing 0.1% acetic acid; flow rate 1 ml min^{-1}, retention time 25.8 min). The final yield was 120 mg of a pure oily compound. ^{13}C NMR (CD_3OD, 125 MHz); 177.70 (s), 132.73 (d), 131.09 (d), 129.23 (d), 129.20 (d), 128.87 (d), 128.25 (d), 34.97 (t), 30.69 (t), 30.31 (t), 30.23 (t),

30.21 (t), 28.16 (t), 26.53 (t), 26.42 (t), 26.10 (t), 21.50 (t), 14.66 (q) ppm. ^{1}H NMR (CD_3OD, 500 MHz); 5.40-5.26 (m, 6H), 2.82-2.79 (m, 4H), 2.27 (t, J = 7.5 Hz, 2H), 2.12-2.04 (m, 4H), 1.63-1.56 (m, 2H), 1.41-1.30 (m, 8H), 0.97 (t, J = 7.5 Hz, 3H) ppm. HRESIMS (positive); *m/z* found 279.2328 $[M+H]^+$, calcd for $C_{18}H_{31}O_2$: 279.2324, found 301.2152 $[M+Na]^+$, calcd for $C_{18}H_{30}NaO_2$: 301.2143.

Time course change in the level of linolenic acid. The levels of linolenic acid were measured by HPLC using the same procedures for isolation of senescence-promoting compound (Fig. 5) and the authenticity of the compound at each peak was confirmed by EI mass spectrometry. The molecular ion peak at *m/z* 278 and the fragmentation pattern were identical to that of linolenic acid.

Disclosures

The authors have no conflict of interest to declare.

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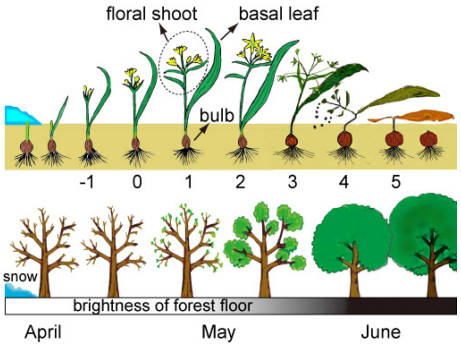


Fig. 1. Diagrams showing the changes in appearance of *Gagea lutea* during its rapid life cycle (top) and the changes in brightness of the forest floor, their original habitat, with closure of tee canopy (bottom). An adult plant consists of a floral shoot with a few small leaves, a basal leaf and a bulb with roots. Numbers in the top figure indicate weeks after flowering.

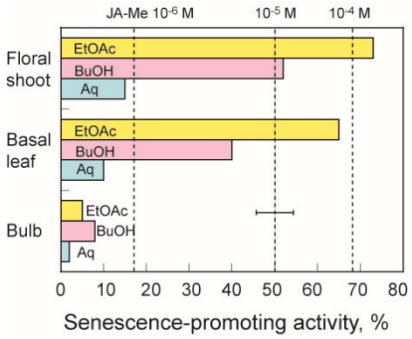


Fig. 2. Senescence-promoting activities of solvent fractions obtained from *Gagea* plants one week after flowering. Each extract equivalent to 5g FW was added to 5ml assay medium. Broken lines represent the activity of JA-Me at concentrations of 10^{-6}, 10^{-5} and 10^{-4}M, which was used as a standard senescence-promoting compound. The error bar on the line of activity of JA-Me at a concentration of 10^{-5} means ±SD (n=5).

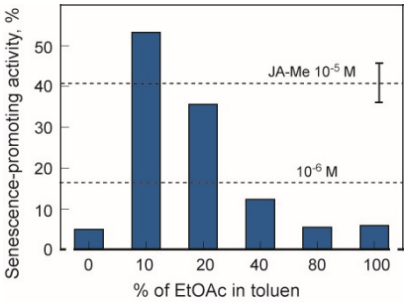


Fig. 3. Senescence-promoting activities of fractions separated by silica gel column chromatography. EtOAc fraction obtained from the basal leaves was separated by a silica gel column. Each extract equivalent to 10g FW was added to 5ml assay medium. Broken lines represent the senescence-promoting activities of JA-Me at concentrations of 10^{-6} and 10^{-5} M. The error bar on the activity of JA-Me at a concentration of 10^{-5} means ±SD (n=5).

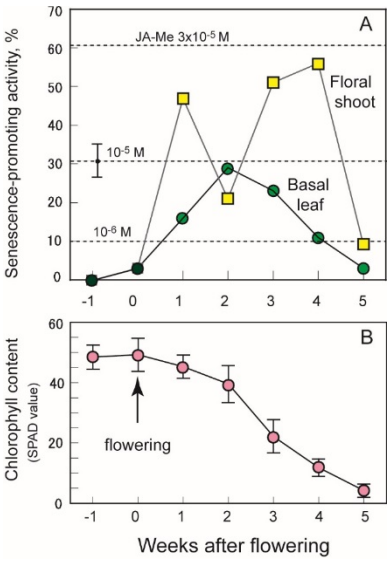


Fig. 4. Time course changes in the senescence-promoting activity in basal leaves and floral shoots during the growth of the plants (A). Fractions obtained by eluting a silica gel column with 10% EtOAc in toluene were assayed. Broken lines represent the activity of JA-Me at concentrations of 10^{-6}, 10^{-5} and $3x10^{-5}$ M. The error bar on the activity of JA-Me at a concentration of 10^{-5} means ±SD (n=5). To determine the progress of senescence in the plant, changes in the chlorophyll content in the leaves (B) were also measured (±SD, n=20).

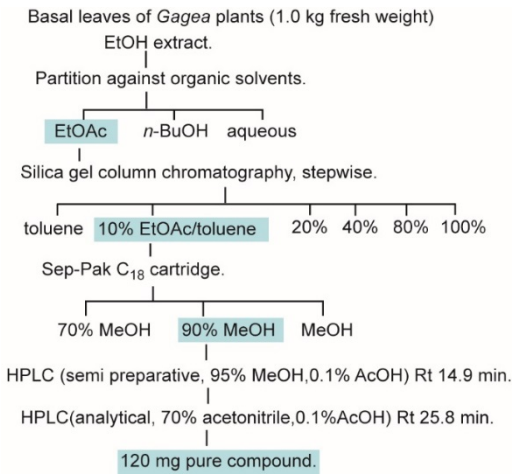


Fig. 5. Procedure for isolation of senescence-promoting compound from basal leaves.

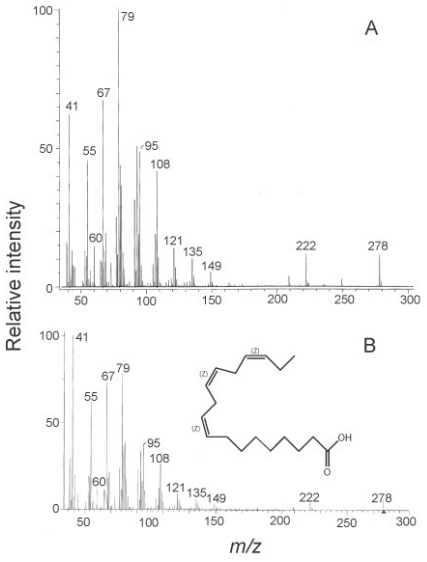


Fig. 6. EI mass spectrum of the isolated compound (A) and that of α-linolenic acid (B).

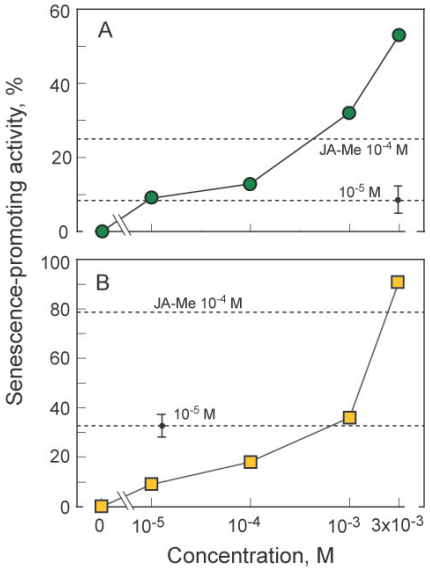


Fig. 7. Effects of linolenic acid on senescence of *Gagea* (A) and wheat (B) leaves. Broken lines represent the senescence-promoting activities of JA-Me at concentrations of 10^{-5} and 10^{-4} M. The error bars on the activities of JA-Me at a concentration of 10^{-5} M mean ±SD (n=5).

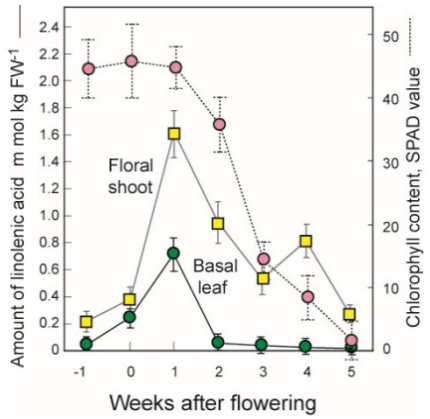
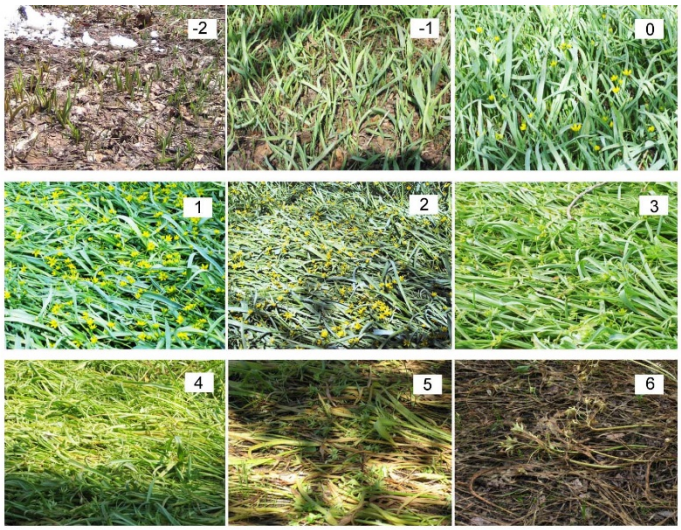
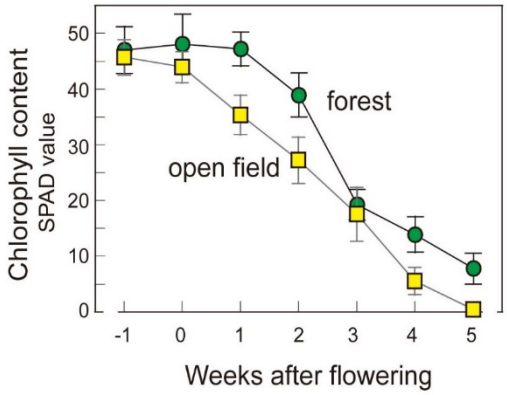


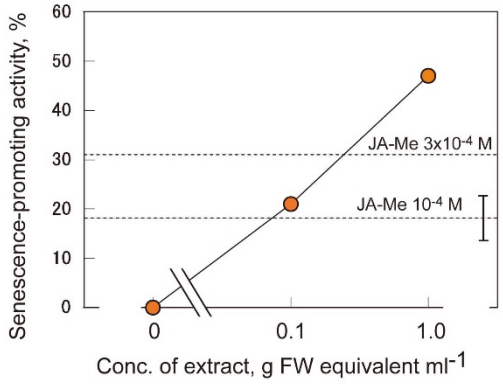
Fig. 8. Changes in the levels of linolenic acid in basal leaves and floral shoots during the growth of *Gagea* plants (±SD, n=3). To show the progress of senescence of the plants, changes in the chlorophyll content in the basal leaves are also presented (broken line, ±SD, n=20).



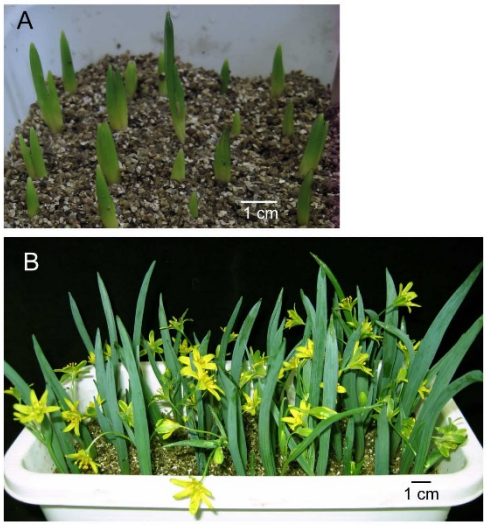
Supplementary Fig. S1. Changes in the typical appearance of the *Gagea* colony at the experimental site. Numbers indicate weeks after flowering.



Supplementary Fig. S2. Time course changes in the chlorophyll content in basal leaves grown in a forest and in an open field. The values were measured by SPAD meter (±SD, n=20).



Supplementary Fig. S3. Effect of 10%EtOAc in toluene fraction obtained from basal leaves on senescence of *Gagea* leaves. Broken lines represent senescence-promoting activities of JA-Me at 10^{-4} and 3 x 10^{-4}M. The error bar on the line of activity of JA-Me at a concentration of 10^{-4}M means ±SD (n=5).



Supplementary Fig. S4. Germination of *Gagea* bulbs at 4°C in a refrigerator (A), and flowering of the plants 2 weeks after transfer to a growth chamber (B) (15°C, 16 h photoperiod). The bulbs had been stored at 4°C for 4 months to break dormancy.