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Citation	Plant and Cell Physiology, 51(8), 1341-1349 <a href="https://doi.org/10.1093/pcp/pcq094">https://doi.org/10.1093/pcp/pcq094</a>
Issue Date	2010-08
Doc URL	<a href="https://hdl.handle.net/2115/46761">https://hdl.handle.net/2115/46761</a>
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Plant and Cell Physiology following peer review. The definitive publisher-authenticated version Plant Cell Physiol (2010) 51(8): 1341-1349 is available online at: <a href="http://pcp.oxfordjournals.org/content/51/8/1341">http://pcp.oxfordjournals.org/content/51/8/1341</a>
Type	journal article
File Information	PCP51-8_1341-1349.pdf



# **Isolation and identification of an anti-bolting compound, hexadecatrienoic acid monoglyceride, responsible for inhibition of bolting and maintenance of leaf rosette in radish plants**

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**Abbreviations:** ABC, anti-bolting compound; EtOAc, ethyl acetate

**Abstract:** Generally, the bolting (stem elongation from rosette plants) of winter annuals is believed to be induced by an increase in the levels of GA that occurs after a certain period of chilling (vernalization), and a deficiency of GA allows the plant to maintain a rosette style. Lack of direct evidence proving the above assumption in radish plants (*Raphanus sativus* L.) urged us to assume the presence of an anti-bolting compound; actively maintaining the rosette habit through inhibition of bolting. Anti-bolting activity was detected in an extract of rosette shoots of radish plants by an assay using seedlings cultured *in vitro*. The causal compound that strongly inhibited bolting was isolated and identified as  $\alpha$ -(6Z,9Z,12Z)-hexadecatrienoic acid monoglyceride (16:3 monoglyceride). This compound did not inhibit leaf production at the apical meristem indicating that it merely inhibits growth at the internode. The compound disappeared completely after vernalization and bolting occurred thereafter. The results suggest that the release from inhibition by 16:3 monoglyceride induces the initiation of bolting. The possible mechanism by which the compound exerts the activity is discussed.

**Key words:** Anti-bolting compound, Hexadecatrienoic acid monoglyceride, Inhibition of bolting, Radish, *Raphanus sativus* L, Sensitivity to GA.

## **Introduction**

Many winter annual crops, such as radish, carrot, beet and spinach, are cold-requiring long day plants. The plants develop a leaf rosette on a dwarf stem and accumulate assimilation products in a thick root during the short days (SD) of autumn. After a certain period of chilling in winter, the plants initiate stem growth in response to the long days (LD) of spring and finally form flowers on the elongated stem. Exposure to chilling for a certain period is known as vernalization and the stem growth is called bolting. The rosette style and the subsequent bolting appear to have been acquired by evolutionary processes, since the former kept the plant body close to the soil surface to avoid damage from strong wind and large daily fluctuations in temperature. Flowers appear high on the bolted stem in a position that can facilitate the attraction of remote pollinators. Usually, the seeds of these plants are sown in summer (summer cropping), but when the seeds are sown in spring (spring cropping), unseasonable bolting sometimes occurs by occasional chilling in spring, and as a result it severely reduces their value as agricultural products. In beet plants, for example, bolting markedly lowers the sucrose content in the storage root and in radish plants the storage root becomes unpalatable due to the hardening of the vascular bundles. Breeders have developed many late-bolting cultivars of radish and beet that are somewhat resistant to bolting in spring cropping. In our district, however, almost all the late-bolting cultivars of radish gradually bolt when the seeds are sown in May. Recently, an attempt has been made to breed late-bolting cultivars of radish by gene transfer (Curtis et al. 2002). The late-bolting cultivars are used only for spring cropping. The cultivars not resistant to bolt are called early-bolting cultivars and popular for summer cropping because of its roots of high quality.

In many winter annual plants, an application of GA under SD conditions causes bolting. These observations have led to the idea that rosette plants on SD are deficient in GAs, while vernalization and LD would raise the level of endogenous GAs and stimulate bolting (Chailakhyan 1968). The inhibitors of GA biosynthesis have been shown to inhibit bolting of radish plants (Suge and Rappaport 1968). However, several studies have failed to detect a clear increase in the levels of GA in plants exposed to vernalization and LD. Zeevart (1971) found that spinach plants on SD had approximately the same GA content as plants on LD. Nakayama et al. (1995) reported that vernalization of

radish plants did not induce an increase in the levels of active GAs (GA<sub>1</sub> and GA<sub>4</sub>), and exposure to LD for 5 days after vernalization induced a slight increase in the GA levels. An increase in the active GAs was barely observed after the commencement of bolting. Both authors assumed that the sensitivity to GA is enhanced by vernalization or LD condition. In *Eustoma* and *Arabidopsis* plants, a similar elevation of sensitivity to GA was reported after the vernalization of the plants (Oka *et al.* 2001). But the nature of the sensitivity is currently unknown.

Garner and Allard (1923) reported that the overwintered beet plants ceased bolting and immediately initiated the production of leaf rosettes when exposed to SD. The rapid cessation of stem growth can hardly be explained by a mere deficiency of GA. It is possible that some endogenous anti-GA compound, in other word, an anti-bolting compound, is formed under SD and inhibits stem growth, and the levels of the compound determine the sensitivity; the lower the level, the higher the sensitivity. Previously, we have hypothesized that jasmonic acid and related compounds act as anti-bolting compounds in beet plants, but failed to substantiate this hypothesis; JA can induce leaf rosettes but the levels of JA in the plants were too low to account for the rosette habit (Koda *et al.* 2001)

Here we report isolation and identification of an anti-bolting compound in radish plants that is responsible for the formation of the leaf rosette, using two different bioassays for anti-bolting activity.

## **Results**

### **Varietal difference in sensitivity to GA and the effect of vernalization on sensitivity**

To initiate our studies, the sensitivity to GA was confirmed by two experiments; varietal differences in sensitivity to GA and the effect of vernalization on the sensitivity. Varietal differences were examined by application of GA<sub>3</sub> solutions to plants of the early- and late bolting cultivars grown in the field. Without application of GA<sub>3</sub>, the early-bolting cultivar began to bolt 6 weeks after sowing. On the other hand, the late-bolting cultivar did not bolt throughout the experimental period (Fig. 1A). Application of GA<sub>3</sub> induced bolting 5 weeks after sowing in both cultivars. However,

the stem growth of the early-bolting cultivar in response to GA<sub>3</sub> was much larger than that of the late-bolting cultivar (Fig. 1B), the length of the stem of the early-bolting cultivar reaching nearly 1 m after 7 weeks, while that of the late-bolting cultivar obtaining a length of only 5 cm (Fig. 1C).

The effect of vernalization on the sensitivity was studied using the early-bolting cultivar cultured *in vitro*. Vernalization enhanced the growth response of radish seedlings to GA<sub>3</sub> and the growth-promoting effect of GA<sub>3</sub> at a concentration of 10<sup>-5</sup> M was almost doubled (Fig. 2). These results demonstrate that the sensitivity to GA exists in radish plants with greater sensitivity in early-bolting cultivars than in late-bolting cultivars and that the sensitivity is enhanced by vernalization. The sensitivity might be explained by the level of an anti-bolting compound (an anti-GA), where the level is higher in late-bolting cultivars and lower in early-bolting cultivars, and decreases with vernalization.

### **Detection of anti-bolting activity**

To elucidate the presence of an anti-bolting compound in the rosette radish plants, an efficient bioassay method for the detection of anti-bolting activity was necessary with the requirement that all control plants must bolt. Initially, we developed a method utilizing radish seedlings cultured *in vitro*. But since the seedlings require vernalization at 4 °C for more than 4 weeks to bolt, the method was used occasionally to confirm the activity. For the sake of convenience, we also used seedlings of Qing geng cai (*Brassica rapa* var. *chinensis*, a kind of Chinese cabbage) cultured *in vitro*, which does not require vernalization to bolt. Since the late-bolting cultivars were assumed to be rich in anti-bolting compound, the amount was expected to be much higher in summer cropping when the plants never experience chilling temperatures. The plants of the late-bolting cultivar grown for 1 month in summer were used as materials for extraction of anti-bolting compound. The solvent fractions obtained from shoots and roots were subjected to an assay for anti-bolting activity utilizing seedlings of Qing geng cai. In roots, no activity was found in any fractions. But in shoots, a slight activity was found in the ethyl acetate (EtOAc) fraction. To confirm the activity, the EtOAc fraction was partially purified with a silica gel column (160 ml bed volume). The fraction was

dissolved in 50 ml 5% EtOAc in toluene and passed through the column and the column was washed with 450 ml 5% EtOAc in toluene. The compounds adsorbed onto the column were eluted sequentially with 10%, 20%, 40% and 80% EtOAc in toluene (500ml each) and finally with 200 ml MeOH. After complete drying, the fractions were subjected to the assay with seedlings of Qing geng cai. The inhibition of bolting (inhibition of internode growth) must be distinguished from the mere inhibition of growth. Therefore, in addition to the rate of bolting, the number of visible leaves that had developed during the experimental period was counted to assess the effects on vitality of the shoot apical meristem. Strong anti-bolting activity (81%) was found in the 40% EtOAc fraction (Fig. 3). Average numbers of the leaves of control seedlings and that of the seedlings treated with this fraction were almost identical, the former being  $5.0 \pm 0.8$  ( $\pm$ SD, n=14) and the latter being  $4.8 \pm 0.4$  ( $\pm$ SD, n=14). This result indicates that the anti-bolting activity detected here is attributable to the inhibition of growth at the internode and not to the inhibition of total vitality of the shoot apical meristem. JA-Me, used as a standard, showed strong anti-bolting activity in this assay system, the activity at  $0$ ,  $10^{-6}$ ,  $10^{-5}$  and  $10^{-4}$  M being 5%, 24%, 67% and 100%, respectively. The 40% EtOAc fraction also exhibited strong anti-bolting activity with radish seedlings (Fig. 4). Thus the presence of an anti-bolting activity in radish rosette shoots was confirmed.

### **Changes in the activity during the growth of early- and late-bolting cultivars**

If the compound that is responsible for the anti-bolting activity detected here is involved in the maintenance of rosette habit, the activity should show some relational change to the bolting of the plants. To examine the time course changes of the anti-bolting activity during the growth of radish plants, seeds of the early- and late-bolting cultivars were sown in the field middle in June. After 4 weeks, shoots were harvested once a week and the 40% EtOAc fraction were extracted. The bolting of early-bolting cultivar began 5 weeks after sowing and became frequent between 6 and 7 weeks. The anti-bolting activity, high in the 4-week sample, decreased sharply with time and became undetectable after 7 weeks and then increased (Fig. 5). By contrast, the anti-bolting activity in late-bolting cultivar was more or less constant and the plants did not bolt throughout the experimental period. These results suggest that the decrease in anti-bolting activity is a

prerequisite for the commencement of bolting.

### **Isolation and identification of the anti-bolting compound**

An attempt at isolation of the active compound from the shoots of the late-bolting cultivar was made utilizing the assay for anti-bolting activity with seedlings of Qing geng cai. The 40% EtOAc fraction from the silica gel column was dissolved with 35 ml 70% MeOH and loaded onto a Sep-Pak Vac 35 cc C<sub>18</sub> cartridge (Waters). The cartridge was washed with 70 ml 70% MeOH and compounds adsorbed on the cartridge were eluted successively with 70 ml 90% MeOH and 100% MeOH. The activity was found in the 90% MeOH fraction. The active fraction was subjected to HPLC with a semi-preparative column, monitored at 210nm, with 90% MeOH that contained 0.1% acetic acid as the solvent. The activity corresponded to an absorbance peak at 13.9 min and the compound generated the peak was further purified by analytical HPLC with 60% acetonitrile that contained 0.1% acetic acid as the solvent. The retention time of the active compound was 17.5 min. The isolation procedure is summarized in Figure 6, with a 50 mg yield of the active compound. The spectrum by EI mass spectrometry revealed that the compound had a molecular ion peak at  $m/z$  324. From the <sup>1</sup>H and <sup>13</sup>C NMR spectral data this compound was identified as  $\alpha$ -(6Z,9Z,12Z)-hexadecatrienoic acid monoglyceride (Fig. 7). Hereafter the compound was designated as anti-bolting compound (ABC). The anti-bolting activity of this compound when assayed with seedlings of Qing geng cai at concentrations of 0, 0.1, 0.3 and 1.0 mM was 0%, 37%, 46% and 67%, respectively. The compound also showed anti-bolting activity with radish seedlings (Fig. 8); the activity at 0 and 1.0 mM being 0 and 62%, respectively. In this experiment, some of the control shoots of radish bore floral buds. The compound also induced some morphological changes in the first leaves that were in contact with the medium; rooting from petiole and loss of serration of the leaves (date not presented). Because of the shortage of the compound, we could not examine the effects of the compound above 1 mM.

### **Changes in the amount of ABC with the growth of the plants and with vernalization**

Changes in the amount of ABC during the growth of the radish plants were examined in the late-bolting cultivar sown in May (spring cropping). The compound was extracted by the procedure shown in Figure 6, and measured by analytical HPLC. Recovery of ABC calculated from the standard addition method was  $73.7 \pm 2.5\%$  ( $\pm$ SD, n=3). No corrections were made in the values obtained. The amount was very high in shoots harvested 5 weeks after sowing ( $0.43 \text{ m mol kg fr wt}^{-1}$ ). Thereafter the amount decreased sharply to one-hundredth the peak value at 8 weeks after sowing (Fig. 9). Bolting of the plants was evident at 10 weeks. In a different experiment, the shoots were divided into leaves and dwarf stems, and the levels of ABC were compared. A large amount of the compound ( $0.55 \text{ m mol kg fr wt}^{-1}$ ) was found in the leaves but no appreciable amount was detected in the dwarf stems (data not presented).

The effect of vernalization on the amount of ABC was examined using plants of early-bolting cultivars raised in the field. During vernalization of the plants at  $4^\circ\text{C}$  for 9 weeks, the old leaves had fallen by senescence and new leaves continued to emerge slowly. The new leaves began to grow rapidly just after transfer to the growth chamber at  $23^\circ\text{C}$ . Bolting of the plants became visible 1 week after transfer and the shoot grew rapidly thereafter. ABC was present in the shoots ( $65 \text{ }\mu\text{mol kg fr wt}^{-1}$ ) before vernalization (Fig. 10). After vernalization, it disappeared almost completely and remained undetectable until 1 week after the transfer to the growth chamber. ABC re-appeared 2 weeks after transfer with the growth of the bolted shoot.

## Discussion

In the present study, we used radish seedlings cultured *in vitro* as an experimental model system to detect anti-bolting activity. Vernalized radish seedlings grown *in vitro* bolted with floral buds (Fig. 8), just like field-grown plants (Fig. 9). The similar characteristics of bolting and floral bud formation indicates the validity of using seedlings as a model system for bolting studies.

The anti-bolting compound (ABC) of radish plants was identified as  $\alpha$ -6Z,9Z,12Z-hexadecatrienoic acid monoglyceride (16:3 monoglyceride). A sharp decrease in the amount of ABC was found before the commencement of bolting (Fig. 9) and after vernalization (Fig. 10). In both cases, the plants began to bolt after the

decrease, indicating that the release from inhibition by ABC can trigger the initiation of bolting. The results suggest that ABC is responsible for the maintenance of leaf rosettes of radish plants. In winter canola plants, an increase in the level of active GAs ( $GA_1$  and  $GA_3$ ) was found after vernalization (Zanewich and Rood 1995), but such an increase could not be detected in the levels of active GAs ( $GA_1$  and  $GA_4$ ) in radish plants (Nakayama et al. 1995). It is probable that the bolting in radish plants can be induced solely by a decrease in the levels of ABC instead of an increase in GA level. In other words, the bolting may be initiated by releasing the brakes by ABC, not by stepping on the accelerator by GA. A small amount of constitutive GA seems to be sufficient to initiate bolting of the stem. At present, we cannot explain why ABC, after decreasing before bolting, re-appeared near the completion of bolting (Figs. 5 and 10). Furthermore, effects of day-length on the amount should be examined.

In *Arabidopsis*, a winter annual, the bolting and subsequent floral bud formation are thought to be controlled by the same mechanism. In this context, various genes have been found to be involved in both phenomena (Piñeiro et al. 2003, Kim et al. 2005). However, the mechanism of bolting and that of floral bud formation need not be common in all winter annuals. The order of the commencement of bolting and floral bud formation is species-specific. For example, beet plants begin to form floral buds after the completion of bolting, but radish plants have already formed floral buds when the plants begin to bolt (Fig 9). ABC did not inhibit leaf production at the apical meristem and exclusively inhibited growth at the internode, thereby inhibiting only bolting. It is possible that ABC also inhibits floral bud formation.

When the assay with seedlings of Qing geng cai was employed, it took 2 weeks to obtain results from one step of the isolation procedure. During the course of isolation, the anti-bolting activity based on a unit fresh weight of plant material gradually decreased. The amount of ABC determined by analytical HPLC also gradually decreased with the duration of the storage period at  $-20\text{ }^{\circ}\text{C}$ . A faster-eluting peak of an unknown compound appeared concomitantly on the chromatogram obtained by HPLC after 1-month in storage. These results indicate that the compound is not so stable and gradually degraded to an inactive compound. Therefore, to examine changes in the levels of ABC shown in Figure 9 and 10, the isolation was carried out as soon as we obtained the samples and isolation and quantitation were completed within 3 days.

The maximum level of ABC in shoots of the late-bolting cultivar subjected to spring cropping was  $430 \mu \text{ mol kg fr wt}^{-1}$ , and that of the early-bolting cultivars subjected to summer cropping was  $60 \mu \text{ mol kg fr wt}^{-1}$  (Figs 9 and 10). Although we have not made a precise comparison of the levels of ABC in the early- and late-bolting cultivars, it is likely that the amount is higher in the late-bolting cultivar. As an indicator for late-bolting characteristics, the levels of ABC could be used in breeding for selection of late-bolting cultivars.

Free fatty acids are almost insoluble in water, and the solubility of ABC may be quite low, even after conjugation to glycerol. However, an increase in anti-bolting activity correlated with an increase in the concentration of ABC in the medium and considerable activity was found at 1.0 mM. This result indicates that the compound was incorporated into the tissue in a concentration-dependent manner. But it is likely that the rate of incorporation is low and the level in the seedling treated with 1 mM is much lower than 1 mM. The highest level of ABC in the shoot of field grown plants (0.4 mM) seems to be enough to induce complete inhibition of bolting.

The mechanism by which ABC exerts anti-bolting activity remains to be elucidated. Hexadecatrienoic acid (16:3) as well as linolenic acid (18:3) are important constituents of plastid membranes and they can affect cold tolerance (Khodakovskaya 2006) and defense responses (Yaeno et al. 2004). These free fatty acids released from chloroplast membrane are known to be precursors of JA synthesis (Weber et al. 1997). JA-Me has various growth-inhibiting activities (Koda et al. 1992), and in the present study it was shown to have strong anti-bolting activity (Fig.3). During the course of the isolation of ABC, guided by an assay for anti-bolting activity, we could not detect JA or JA-Me in any fractions that had anti-bolting activity. The detection limit of these compounds by HPLC was ca. 20 n mol. Therefore, ABC does not exert its activity after conversion to JA or JA-Me. The molecular weight (324) and fragmentation pattern of the isolated compound on EI-mass spectrum somewhat resembles that of hydroperoxy-linolenic acid methyl ester, a reactive oxygen species (ROS). We initially assumed that this compound had anti-bolting activity. Ohta et al. (1990) reported that 9-hydroperoxy-linolenic acid has antifungal activity. When hydrogen peroxide was applied to seedlings of Qing geng cai, it strongly inhibited the bolting at low concentrations with no apparent damage to the plants (unpublished data). It is known

that free fatty acids, including 18:3, have antibacterial activities (Andrew et al. 2008) and one of the suggestions for the mechanism by which they exert their activity is that they initiate production of ROS (Wang and Johnson 1992). Our preliminary experiment showed that both 16:3 and 18:3 have considerable anti-bolting activity (unpublished data). In *Arabidopsis*, free 18:3 is capable of activating plasma membrane-bound NADPH oxidase that generates ROS, but 16:3 does not (Yaeno et al. 2004). In any event, we have to compare the effects of these fatty acids on the levels of ROS in radish plants to substantiate the involvement of ROS in the maintenance of the leaf rosette. On the other hand, Yanagida et al. (2004) reported that glutathione is capable of inducing bolting of *Eustoma grandiflorum*. It is highly likely that the redox state determines the fate of the stems; in an oxidative state the plants maintain a leaf rosette on dwarf stems, and in a reductive state the stems begin to bolt.

Nowadays, various genes are known to be involved in bolting and flowering (Jung and Müller 2009). We have to elucidate target gene of ABC. ABC was found only in the leaves and not found in the stems. Linolenic acid (18:3) is known to be a precursor of various biologically active compounds such as  $\alpha$ -ketol linolenic acid (Suzuki et al. 2003). It is also possible that some unstable metabolite of 16:3 translocates from leaves to stems to exert its activity.

## **Materials and Methods**

### **Plant materials**

Early- (Taibyousoubutori, Takii Seed Co. Ltd.) and late-bolting cultivars (Haruhajime, Tohoku Seed Co. Ltd.) of radish (*Raphanus sativus* L.) were used. Seed of these cultivars were sown at the experimental farm of Hokkaido University at various times from May to August. Since radish plant inevitably forms floral buds before commencement of bolting, the criterion for bolting was visible floral buds at the apical region.

### **Varietal difference in sensitivity to GA**

Seeds of the early- and late-bolting cultivars were sown late in May. Twenty ml of 1 mM GA<sub>3</sub> solution that contained 1% Tween-20 was sprayed twice on each plant 3 and 4 weeks after sowing. The length of the stem of the sprayed plants was measured every week until 8 weeks after sowing.

### **Effect of vernalization on sensitivity to GA of radish seedling cultured *in vitro***

The seeds of the early-bolting cultivar were surface sterilized with a 1% solution of sodium hypochlorite for 40 min and rinsed thoroughly with sterile water. Twelve seeds were sown in a 100-ml conical flask that contained 20 ml Murashige-Skoog medium with half strength of inorganic salts (1/2 MS medium). The flask were kept at 4 °C in the dark for 4 week to vernalize the seeds and then transferred to 25 °C under a photoperiod of 16 h of light and 8 h of darkness. The seedlings were grown for 4 days. Illumination (ca. 2.0 mW cm<sup>-2</sup>) was provided by white fluorescent lamps. Non-vernalized control seedlings were obtained without vernalization. Since the root tip is one of the sites for GA biosynthesis and seems to obstruct sensitive detection of the effect of exogenous GA, roots were removed from the seedlings at the lower part of the hypocotyl and the remaining shoots (ca. 2 cm) were planted into the medium supplemented with GA<sub>3</sub> at concentrations of 0, 10<sup>-6</sup> and 10<sup>-5</sup> M. The shoots were grown for 2 weeks as above. Two flasks with 7 shoots each were prepared.

### **Assay for anti-bolting activity**

The vernalized radish seedlings were obtained as above and roots and the lower part of the hypocotyls were removed from the seedlings to facilitate incorporation of the compound to be tested. Seven shoots were planted in a conical flask that contained 20 ml assay medium (1/2 MS medium supplemented with an extract or JA-Me). JA-Me was used as a standard compound that had the ability to inhibit bolting (anti-bolting activity). Three flasks were prepared for each fraction. The cultures were kept for 2 weeks under the same condition as above and bolting was observed. The criterion of bolting was a visible internode between the cotyledon and the first leaf. Anti-bolting activity was calculated as 100(the number of shoots not bolted)/(the number of total

shoots). For the sake of convenience, we also used seedlings of Qing geng cai (*Brassica rapa* var. *chinensis*, a kind of Chinese cabbage) cultured *in vitro*, which does not require vernalization to bolt. Since practical difficulties in experimental space did not permit sufficient replications of each assay, experiments were repeated at least 3 times for 3 years, and the results were found to be similar in all cases.

### Isolation procedure of ABC

To obtain plant materials for isolation of anti-bolting compound, the seeds of the late-bolting cultivar were sown in the field early in August and shoots and roots (each 2 kg fr wt) were harvested 1 month later. Plant materials were homogenized with a sufficient amount of ethanol to give a final 70% ethanolic extract, kept at 4°C overnight and then filtered. The filtrate was concentrated and the resultant aqueous residue was partitioned 3 times against ethyl acetate (EtOAc) and *n*-BuOH. After each isolation step, the fractions were evaporated to dryness, sealed with N<sub>2</sub> gas and kept at -20 °C until use. The solvent fraction and the remaining aqueous fraction were added to test solution at a concentration of 0.5g or 1.0 g fresh weight equivalent ml<sup>-1</sup> medium and their anti-bolting activities were examined. Further purification of ABC in the EtOAc fraction was carried out by column chromatography on a silica gel column (Wakogel C-300; solvent, toluene/ethyl acetate). The active fraction was purified successively by a Sep-Pak C<sub>18</sub> cartridge (Vac 35 cc, Waters) and by a semi-preparative HPLC column (column, Prep Nova-Pak HR C<sub>18</sub>, 25×100 mm, Waters; solvent, 90% MeOH that contained 0.1% acetic acid; flow rate, 4ml min<sup>-1</sup>). The elution profile was monitored by absorbance at 210 nm. Final purification was carried out by analytical HPLC (Radial Pak Cartridge Nova-Pak C<sub>18</sub>, 4.6×250 mm; solvent, 60% acetonitrile that contained 0.1% acetic acid; flow rate, 1ml min<sup>-1</sup>). Finally, a 50 mg pure, oily compound was obtained. <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) δ 5.40-5.26 (6H, m), 4.13 (1H, dd, *J* = 5.0, 10.0 Hz), 4.06 (1H, dd, *J* = 5.0, 10.0 Hz), 3.81 (1H, quint, *J* = 5.0 Hz), 3.55 (1H, dd, *J* = 5.0, 10.0 Hz), 3.53 (1H, dd, *J* = 5.0, 10.0 Hz), 2.80 (4H, m), 2.35 (2H, t, *J* = 5.0 Hz), 2.11-2.05 (4H, m), 1.62 (2H, quint, *J* = 7.5 Hz), 1.42-1.31 (4H, m), 0.97 (3H, t, *J* = 7.5 Hz); <sup>13</sup>C NMR (CD<sub>3</sub>OD, 125 MHz) δ 175.4 (c), 132.7 (d), 130.9 (d), 129.24 (d), 129.17 (d), 129.0 (d), 128.2 (d), 71.1 (t), 66.5 (d), 64.1 (t), 34.0 (t), 30.4 (t), 29.8 (t), 28.0 (t), 26.5 (t), 26.4 (t), 25.9 (t), 21.5 (t), 14.6 (q);

EIMS  $m/z$  324 [M]<sup>+</sup>; HREIMS  $m/z$  324.2313 (calcd for C<sub>19</sub>H<sub>32</sub>O<sub>4</sub>, 324.2301).

### **Effect of vernalization on the amount of ABC**

The seeds of the early-bolting cultivar were sown in the field early in August and 24 uniform small plants (320g±28 g fr wt, ±SD) were harvested 1 month later. The plants were surface sterilized with a 0.2% solution of sodium hypochlorite for 20 min and rinsed thoroughly. The plants were wrapped in wet paper towels and kept at 4 °C for 9 weeks. The plants were planted in a plastic pot that contained vermiculite moistened with Hyponex solution (x1,000) and transferred to a growth chamber (23 °C, a photoperiod of 16 h of light and 8 h of darkness), and grown for 2 weeks. Illumination (ca. 6.0 mW cm<sup>-2</sup>) was provided by sunlight mercury-vapor lamps.

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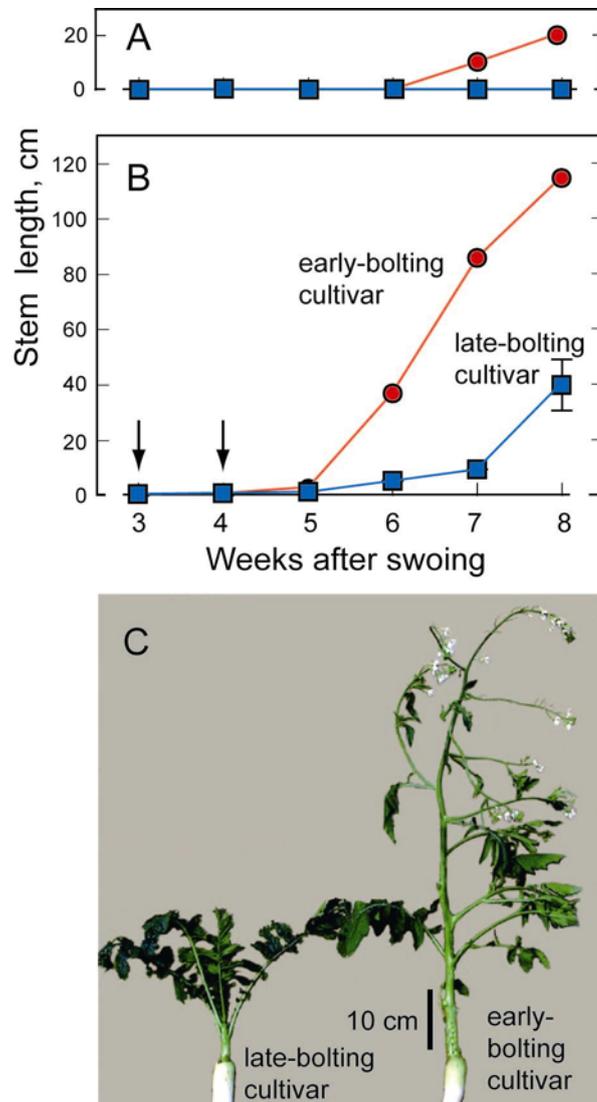


Fig. 1. Effects of GA<sub>3</sub> on stem growth of early- and late-bolting cultivars of radish. Changes in the stem lengths of early- and late-bolting cultivars were compared without application of GA<sub>3</sub> (A), with two applications of GA<sub>3</sub> (1 mM, 20 ml each) (B), and the typical appearance of the GA<sub>3</sub>-treated plants 7 weeks after sowing (C). Seeds were sown in June. Arrows indicate the time of GA application. Each value in A and B represents the mean  $\pm$  SE (n = 15).

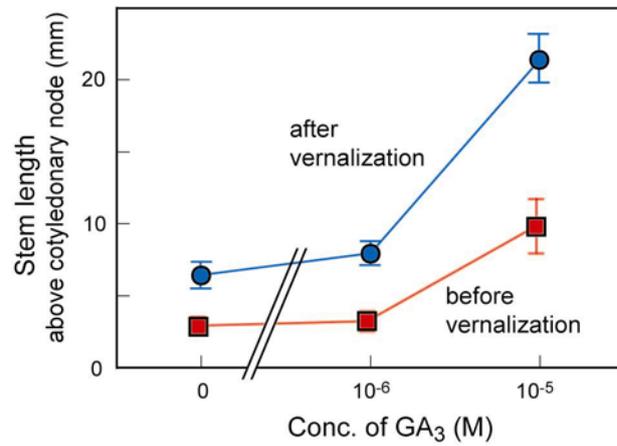


Fig. 2. Effects of vernalization and GA<sub>3</sub> on the stem growth of early-bolting cultivar of radish cultured *in vitro*. Vernalization was carried out at 4 °C for 4 weeks. Each value represents the mean  $\pm$  SE (n = 14).

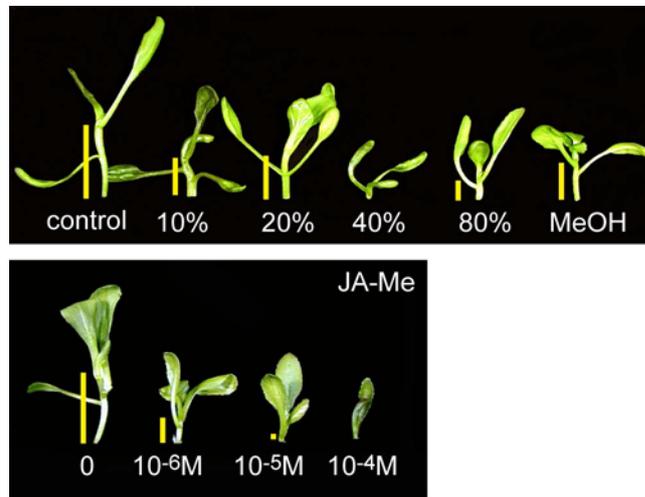


Fig. 3. Effects of fractions of radish leaf extracts separated by silica gel column chromatography and JA-Me on bolting of seedlings of Qing geng cai (*Brassica rapa* var. *chinensis*). Each fraction, equivalent of 20 g fr wt was incorporated into 20 ml of assay medium. For the purpose of comparison, the cotyledons and shoot under the cotyledonary node were excised. Yellow bars to the left of the shoots indicate the length of the stem above the cotyledonary node. The anti-bolting activities of the control (basal medium) and the 40% EtOAc fraction were  $1.9 \pm 2.6\%$  ( $\pm$ SD, n=5) and 81%, respectively.

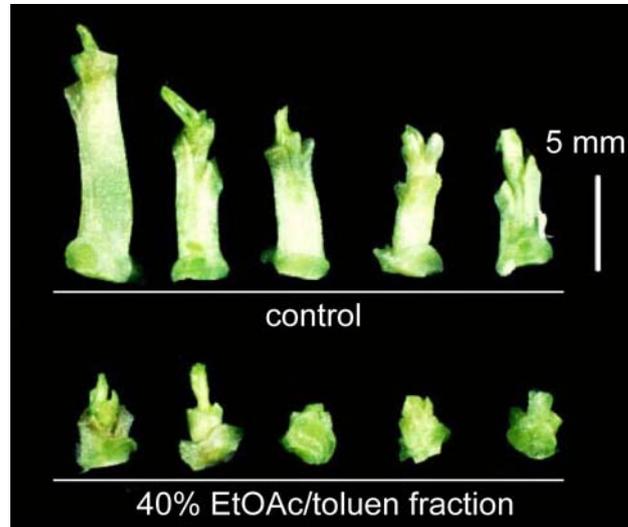


Fig. 4. Effect of the 40% EtOAc fraction obtained by silica gel column chromatography on bolting of radish seedlings. The fraction, equivalent to 20 g fr wt, was incorporated into 20 ml of assay medium. The seedlings were cultured on the medium for 2 weeks. For the purpose of comparison, the cotyledons and shoot under the cotyledonary node and all the leaves were removed from the seedlings. The anti-bolting activities of the control and 40% EtOAc fractions were 0% and 86%, respectively.

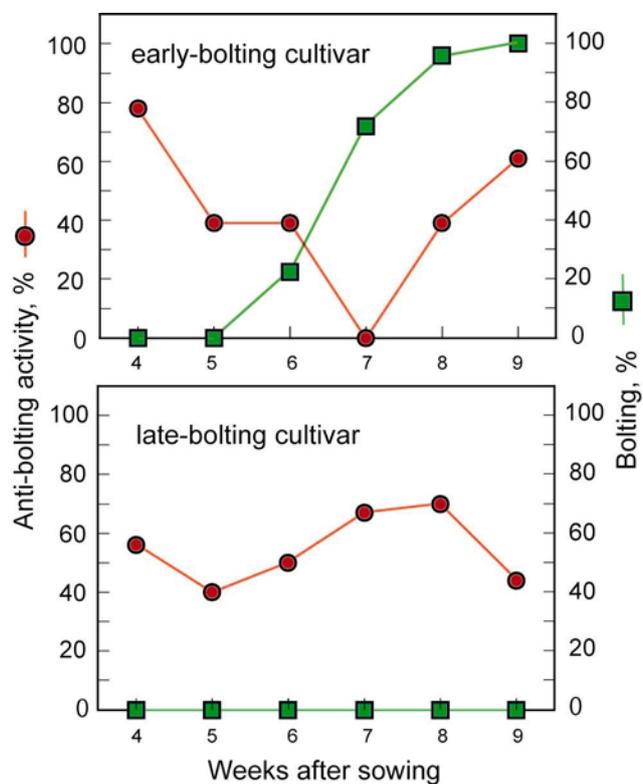


Fig. 5. Changes in the anti-bolting activity in shoots during the growth of the early- and late-bolting cultivars. The seeds of the cultivars were sown in the field early in June. The 40% EtOAc fractions extracted from the shoot samples were subjected to assay with seedlings of Qing geng cai.

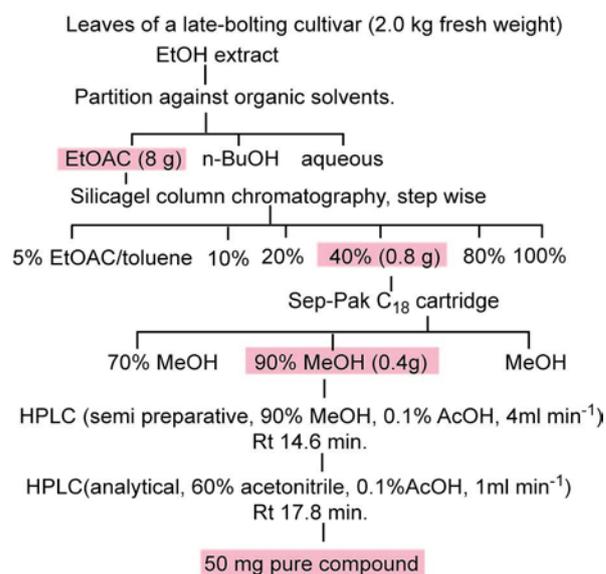


Fig. 6. Isolation procedure for ABC from radish leaves.

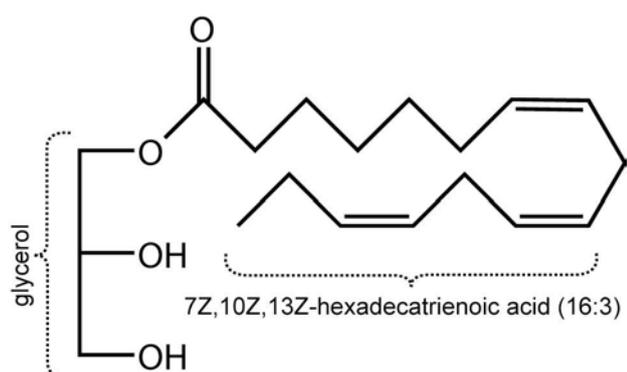


Fig. 7. Chemical structure of ABC,  $\alpha$ -7Z,10Z,13Z-hexadecatrienoic acid monoglyceride.

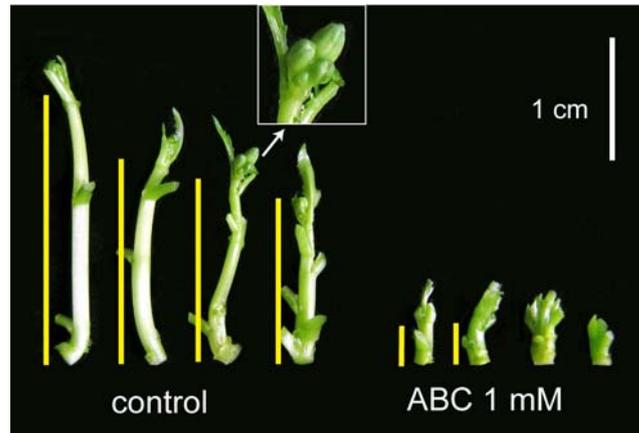


Fig. 8. Effect of ABC on bolting of radish seedlings at a concentration of 1 mM. The seedlings were cultured for 3 weeks. For the purpose of comparison, the cotyledons and shoot under the cotyledonary node and all the leaves were removed from the seedlings. Yellow bars to the left of the shoots indicate the length of the stem above cotyledonary node. Some of the shoots of the control seedlings bore floral buds (enlarged view, indicated by the arrow). The anti-bolting activities of control and 1 mM ABC were 0 and 62%, respectively.

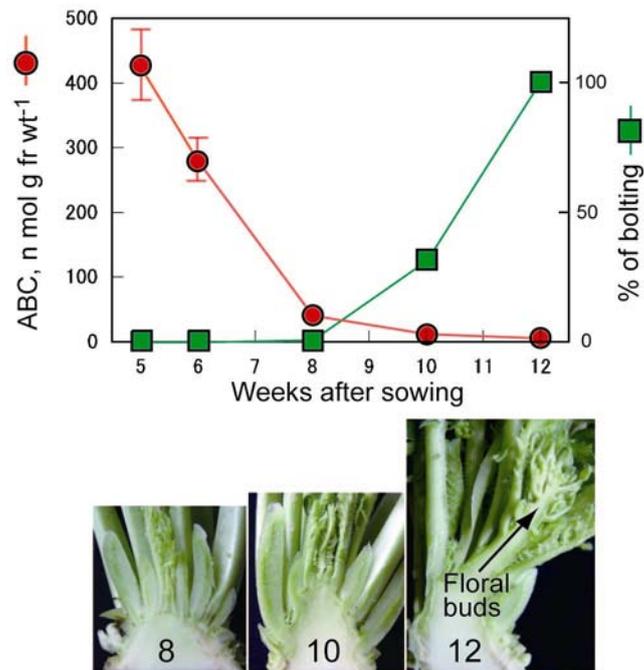


Fig. 9. Changes in the amount of ABC during the growth of the late-bolting cultivar. The seeds were sown in the field in the middle of May. Each value represents the mean  $\pm$  SD ( $n = 3$ ). Longitudinal sections of the shoot apices of the plants grown for 8, 10 and 12 weeks were also presented.

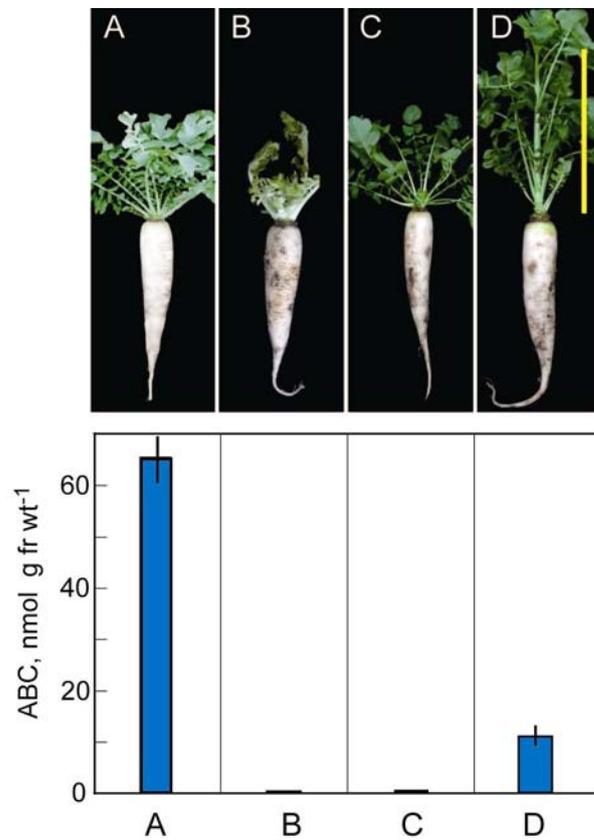


Fig. 10. Effect of vernalization on bolting of radish plants (top) and the amount of ABC in the shoot (bottom,  $\pm$ SD,  $n=3$ ). The whole plants of the early-bolting cultivar were vernalized at 4 °C for 9 weeks. A, before vernalization; B, just after vernalization; C, 1 week after transfer to a growth chamber (23 °C, 16h photoperiod); D, 2 weeks after transfer. A yellow bar means a stem (25 cm long) that had floral buds.