エンドウの春化とジベレリン

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Vernalization and Gibberellins in Pea

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Summary

Vernalization treatment to pea seeds (cv. Atsumi-kinusaya) at 5℃ for 20 days exerted flower promotion by 2.4 nodes. Endogenous gibberellins were found to increase in response to vernalization treatment although no difference could be detected in the plant height between vernalized and nonvernalized plants. Gibberellin activities were also detected in the water which contained materials that had diffused out of the vernalizing seeds. Bioassay data suggested that detected gibberellins from vernalized seeds and shoots are gibberellins possessing no OH substitution on C-3 position of *ent*-gibberellane ring. Exogenously applied gibberellin A_3 and A_7, possessing OH-substitution at the position, increased stem length but rather acted in inhibitory manner to the flowering as indicated by increase of the first flower nodes.

Introduction

A flower promotion of several nodes can be observed after vernalization for 10 to 20 days in certain varieties of pea (5, 10). Gibberellins (GAs) usually induce or promote flowering in the plants that require long-day or low-temperature for flowering, but in peas GA with few exception always delays flower initiation by about 1 to 3 nodes (1, 2, 11). However, it must notice that presumably GA_3 might be the main component of GA complex exogenously applied in these early studies. In peas, effects of endogenous GA have been discussed in relation to light inhibition of stem growth (9, 13), tall and dwarf habit of growth (14, 23), seed development (7) and maturation (4), but no information is available in relation to vernalization. This experiment was done for studying on these points.

Materials and methods

Plant materials

"Atsumi-kinusaya", a dwarf variety known as one of the most sensitive varieties cultivated in Japan to vernalization (10), was used in all experiments. Seeds were imbibed in running water for 8 hr, allowed to germinate for 2 days at room temperature in petri dishes containing water, and then vernalized at 5±2℃ for 20 days. Non-vernalized (control) seeds were soaked at room temperature in dishes for 2 days just prior to the end of vernalization, thus providing germinating seeds of approximately the same size and appearance as the vernalized ones. Both lots of seeds were planted in plastic containers (28×23×11 cm) filled with the mixture of half vermiculite and half soil and grown in a growth cabinet under 16 hr photoperiod at 25±1℃. The irradiation during the light period was supplied by 10 of *Toshiba* FLR-60 HD fluorescent lamps for plant growth and 7 of *Toshiba* 40 watt incandescent lamps providing about 10,000 lx in total at the plant level.

As diffusate from vernalized pea seeds was known to contain flower promoting entity (6), this was obtained by the method described below. A 500 m*l* of dry seeds was sterilized by immersion in mercuric chloride solution for 30 min then washed completely in distilled water and soaked for 8 hr and then vernalized in 1,200 m*l* of distilled water at 5±2℃ for 20 days. Air supply was made using a small air pump during the chilling

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period. After 20 days, aqueous phase was separated from seeds and used as the diffusate.

Green pods with immature seeds were obtained from plants grown in field.

Extraction and fractionation

The germinating seeds and shoots (minus the roots) were covered with 70% acetone, ground via a blender and transferred to shaker for 8 hr. The homogenate was filtered through cheese cloth and filter paper. The filtrate was evaporated to the water phase under reduced pressure, adjusted to pH 2.0 with phosphoric acid and extracted 3 times with ethyl acetate. The ethyl acetate fraction was further extracted with phosphate buffer of pH 7.0. The buffer, adjusted to pH 2.0 with phosphoric acid, was extracted 4 times with ethyl acetate. After overnight dehydration of the combined fraction with sodium sulfate anhydride, the fraction was concentrated under reduced pressure.

Thin-layer chromatography

Each concentrated extract was applied in a small volume of acetone, as a 0.4 cm band on 20×20 cm thin-layer plates prepared with 0.6 mm thick silica gel. The solvent was permitted to run a distance of 10 cm on the plate. After drying, the chromatograms were divided into 10 equal zones (the first zone was further subdivided into 2 zones), and the silica gel was scraped into small beakers. About 3 m*l* of 50% acetone was added to each beakers, the eluate was taken up into a small tube (1.3×2.5 cm), and after evaporating each sample was redissolved in 100 μl of 50% acetone for bioassay. The developing system used was isopropyl ether-acetic acid (95 : 5, v/v).

Bioassay

The bioassay was run according to Murakami's micro drop method (17). Two dwarf rice mutants, Tan-ginbozu and Waito-C, were used for the assay. Waito-C dwarf rice is highly responsive as well as Tan-ginbozu dwarf to those GAs having the OH radical at the C-3 position of *ent*-gibberellane ring but shows no or only weak activity to GAs that have no OH radical at the position (18). The length of the second leaf sheath was measured 3 days after the application. Test plants were grown at 32℃ under continuous light of about 4,000 lx. GA content was expressed as GA_3 equivalent calculated using a standard curve obtained from the response of the rice mutant, Tan-ginbozu, to GA_3.

Effect of GA_3 and GA_7 on the flowering of nonvernalized and vernalized plants

The effects of GA_3 and GA_7 exogenously applied on the flowering response of vernalized and nonvernalized pea plants were studied. Vernalized and nonvernalized seeds were sown in plastic containers as described above. Each treatment had 10 replications. Application of the GA was started 7 days after planting. GA was applied in a drop of 20 μl to the apex of the plant in each treatment.

Results

GAs in seeds

Distributions of GA activities in eluates separated from thin-layer chromatograms of extracts from vernalized or nonvernalized seeds, from green pods with immature seeds and from diffusate are illustrated in Fig. 1. As shown in the figure, vernalization to seeds had resulted in an increase of GA activities. In the Tan-ginbozu assay, single peak of activity was detected in the extract from nonvernalized seeds (Rf 0.3−0.5) but two peaks of activity were detected in the extract from vernalized seeds (Rf 0.05−0.1 and 0.2−0.6). However, the extracts did not respond to Waito-C dwarf at all. Distribution of GA activities detected in diffusate is also shown in Fig. 1. The highest peak of the activity was present at the position of Rf 0.5−0.6. Except this new peak, other pattern of distribution is almost similar to that of vernalized seeds. As shown in Fig. 1, two peaks of activity were also obtained in the extract from green pods with immature seeds. However, one peak with lower Rf value (0.05−0.1) responded to Waito-C dwarf but another peak with higher Rf value (0.3−0.4) did not respond to the same variety at all.

GAs in shoots

Distributions of GA activities in eluates

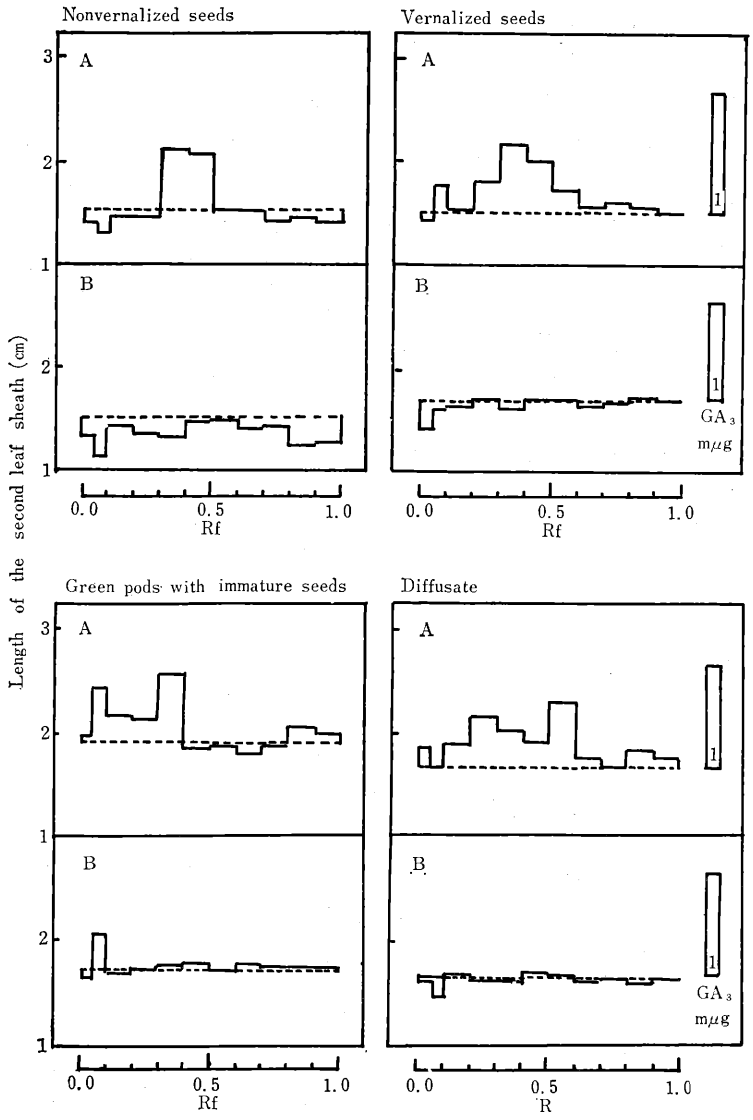

Fig. 1. Histograms indicating GA activities of extracts from nonvernalized or vernalized seeds, green pods with immature seeds and diffusats. Extracts were obtained from 125g (nonvernalized or vernalized seeds) and 30g (green pods with immature seeds) materials in fresh weight. Diffusate was obtained by the method described in the text. A and B indicate bioassay using Tan-ginbozu and Waito-C as test plants, respectively.

obtained from shoot extracts from vernalized or nonvernalized plants are shown in Fig. 2. Changes in total activities are shown in Fig. 3, as well as the plant height at the time of extraction. Highest peak is always detected at Rf 0.3−0.4 in both of vernalized and nonvernalized plants. Another peak with lower Rf value (0.1−0.2) was also detected in the extracts from vernalized plants but presence of this peak was obscure in nonveralized ones. As shown in Fig. 3, no difference was found in plant height between vernalized and nonvernalized plants although total GA content was increased by vernaliza-

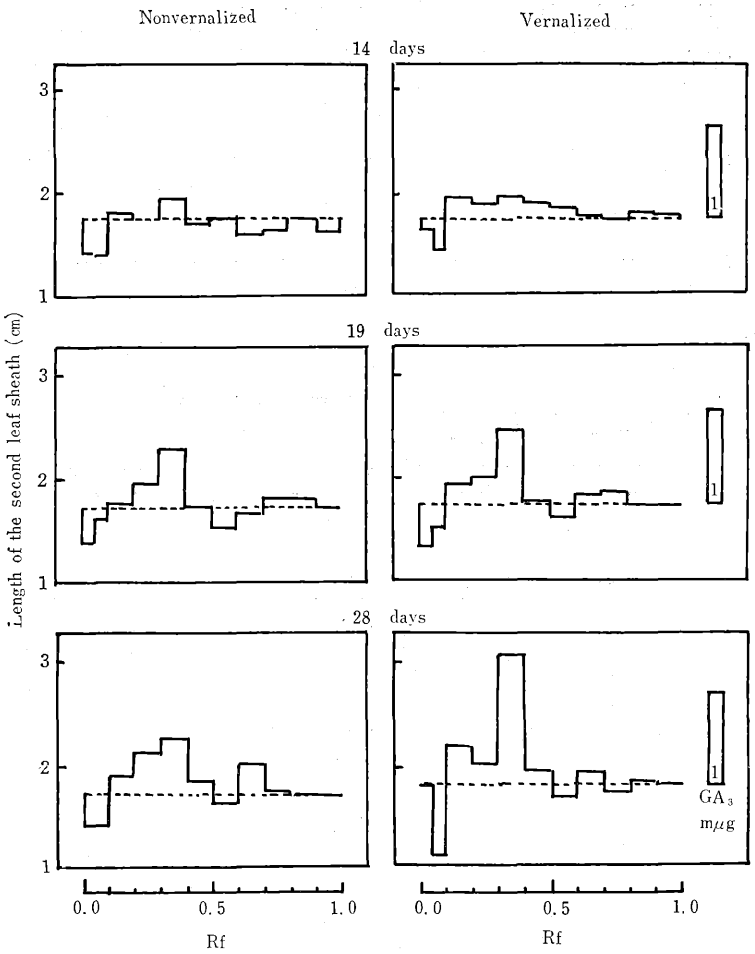

Fig. 2. Histograms showing GA activity in extracts of plants grown from nonvernalized or vernalized seeds harvested in different days from the end of vernalization treatment. Extracts were obtained from 43g (14 days), 68g (19 days) and 94g (28 days) materials in fresh weight. Tan-ginbozu was used as the assay plants.

tion; GAs much more 4-fold were detected in vernalized plants comparing with nonvernalized ones in 28 days after the end of the vernalization. Shoot extracts did not respond to Waito-C dwarf at all notwithstanding the vernalization treatment.

Effects of GA_3 and GA_7 on the flowering of vernalized and nonvernalized plants

Application of GA_3 or GA_7 to vernalized and nonvernalized plants caused stimulation of stem elongation and delay of flowering as shown in Table 1. Promotion of flower formation of 2.4 nodes was obtained after vernalization treatment for 20 days in this variety. However application of GA_3 or GA_7, especially in higher dosages, increased stem length but inhibited flowering as indicated in the number of first flower nodes in both vernalized and nonvernalized plants.

Discussion

Kende *et al.* (13) reported that dwarf pea shoots contain two major fractions with GA-like activity. The chromatographic and biological properties of one of them resemble those of GA_1, while the chromatographic

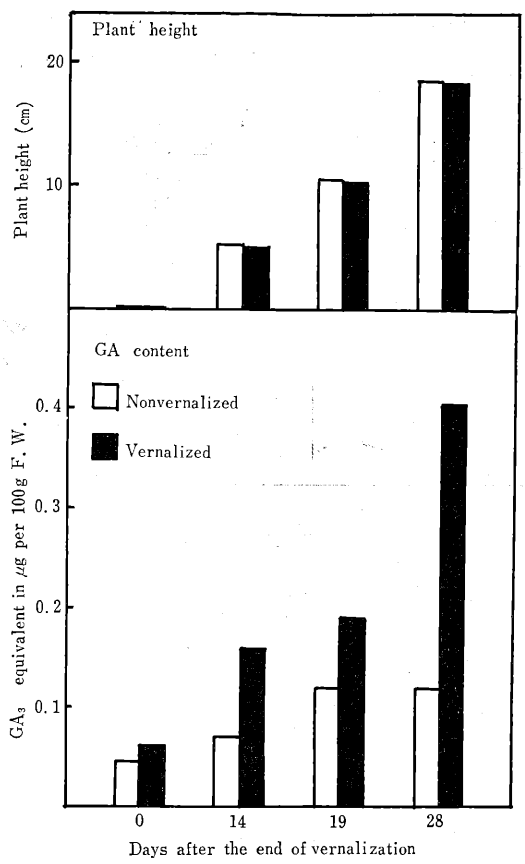

Fig. 3. Changes of plant height and GA content in plants grown from nonvernalized or vernalized seeds.

and biological properties of the other are similar to those of GA_5. Jones (8) also reported that both GA_1- and GA_5-like substances were obtained from pea tissue by extraction. However, by diffusion only the GA_1-like GA was found. There was no quantitative difference in the level of extractable or diffusible GA obtained from light- and dark-grown peas. For explaining this discrepancy between diffusible and extractable GA, Jones (8) suggested that GA_5 will serve as a precursor of GA_1. Metabolism of radioactive GA_5 in dwarf pea shoots was studied by Musgrave and Kende (22) in order to determine whether it is active *per se* or through conversion to GA_1-like compound. The biological activity of the metabolite did not account for the response to applied GA_5. GA_5 is therefore assumed to be biologically active *per se*.

On the other hand, Komoda *et al.* (15) identified the presence of GA_{20} in the extract of green pea pods with immature seeds. Addition to this, Murofushi *et al.* (21) and Frydman and MacMillan (3) detected GA_{29} in immature seeds of a cultivar of pea using gas chromatography and mass spectrometer. Frydman and MacMillan (3) concluded that the GA_1- and GA_5-like fraction from immature pea seeds were GA_{29} and GA_{20}, respectively. In addition to GA_{20} and GA_{29}, the existence of GA_9, GA_{17}, GA_{38} and GA_{44} has been identified in immature seeds of pea. However, GA_{20} and GA_{29} are the major GAs in terms of quantity, the other GAs remain at very low levels throughout development of the seeds (4).

Murakami (19) has made the comparison of GAs between immature seeds and pods in a pea cultivar. He reported that 3 different GAs, GA_{19}, GA_{20} and an unidentified GA, are present in immature seeds, whereas GA_{19} and GA_1/GA_3 are detected in green pods based on the chromatographic properties and the bioassay using different kinds of dwarf rice, Tan-ginbozu and Waito-C. Recently, GA_{19} was identified chemically in the developing pea seeds (7).

In the present experiment, the extracts obtained from germinating seeds, both of vernalized and nonvernalized ones, and from shoots did not respond to Waito-C dwarf at all. This implys that GAs having OH substitution at the C-3 position of *ent*-gibberellane ring may not present in the germinating seeds and shoots of Atsumi-Kinusaya pea in lorge quantity. The highest peak of the activity (Rf 0.3−0.4) detected in the extracts from these tissues seems to correspond with that of GA_5 but this activity might be due to GA_{20} rather than GA_5 since GA_{20}, dehydro derivative of GA_5, has been isolated from pea seeds. It has been known that GA_{20} shows similar chromatographic property as that of GA_5. Biological activity, observed in this experiment, supports the view that this activity might be due to GA_{20} since

Table 1. Effects of GAs on the flowering and stem elongation of vernalized and nonvernalized pea plants.

Vernalization	GAs applied (μg/plant)	Days to first flower	Plant height at the time of first flower (cm)	Number of first flower nodes
Nonvernalized	No GA	38.0±0.86	20.4±1.21	16.5±0.35
	GA_3 1	38.8±2.05	25.5±8.26	17.7±1.27
	GA_3 5	37.1±2.40	32.4±4.13*	17.3±0.76
	GA_3 10	35.4±1.82	43.5±6.15*	17.9±1.04*
	GA_7 1	37.3±1.65	26.2±4.27*	17.7±1.12
	GA_7 5	37.4±1.72	33.7±6.05*	18.0±1.09*
	GA_7 10	36.5±1.49	38.5±5.03*	17.9±1.04*
Vernalized	No GA	29.8±0.94	17.0±1.12	14.1±0.41
	GA_3 1	31.1±1.90	23.8±5.18*	15.1±0.09
	GA_3 5	30.6±1.54	28.2±4.51*	15.8±1.10*
	GA_3 10	30.3±0.42	39.3±8.39*	16.0±1.34*
	GA_7 1	30.8±2.67	20.9±4.04	15.0±1.18
	GA_7 5	29.9±1.52	28.9±8.10*	15.4±0.77*
	GA_7 10	30.1±1.53	38.3±3.50*	15.6±0.70*

* : Statistically significant at 5% level from control (no GA).

Murakami (20) reported GA_{20} and GA_5 show 90 and 30% activity to that of GA_3, respectively, in Tan-ginbozu dwarf, whereas GA_{20} and GA_5 show 1 and 5% activity to that of GA_3, respectively, in Waito-C dwarf. Another GA activity at low Rf value seems to also depend on the GA which possess no OH radical at the C-3 position of *ent*-gibberellane ring although final conclusion awaits chemical identification.

It is interesting that the new peak of activity at Rf 0.5–0.6 was detected in the extract from diffusate, since Highkin (6) reported that flowering in at least one vernalizable variety of pea can be promoted by first allowing the seeds to imbibe in diffusate prepared from other pea seeds. But we can not draw conclusion simply from the data at hand whether this new peak of activity contributes flower promotion or not, since diffusate probably contains many metabolically important compounds other than GAs. As shown in the figure, extract from diffusate also did not respond to Waito-C dwarf at all. Kagawa *et al.* (12) identified cytidylic acid as the flower promoting entity in pea diffusate but their conclusion may also be tentative since flower promoting activity of cytidylic acid is small.

Total amounts of GA in shoots increased 4-fold in response to vernalization although no difference was detected in the plant height. External application of either GA_3 or GA_7 was very effective for increasing stem length but rather gave inhibitory effect on the flowering (Table 1). Actually, application of GA_3 or GA_7 to the vernalized plants has nullified the promotive effect of vernalization on the flowering. Bond and Moore (2) and Kagawa (11) have shown that exogenously applied GA devernalized or prevents vernalization in a variety of pea depending on whether it is applied after or before the cold treatment. They did not mention what kinds of GAs were used in their experiments. However it might be GA mixture that main component is GA_3.

Vernalization treatment to seeds had resulted in increase of endogenous GAs but gave no effect on stem length (Fig. 3). In other words, increased GA accompanying with vernalization treatment, did not contribute to the stem elongation. GA activity detected in the extracts from shoots might be due to the GAs that have no OH substitution at the C-3 position of *ent*-gibberellane ring and might not be due to GAs such as GA_1/GA_3 which have high activity in stem elongation. It was reported that it requires only one tenth or one hundredth of GA_3 to restore the flower inhibition induced by CCC, an inhibitor of GA biosynthesis, than to

restore of stem elongation in *Bryophyllum daigremontianum* (26) and *Pharbitis nil* (24), respectively.

Michiniviz and Lang (16) reported that the different GAs exhibited considerable difference in their activity with respect to flower induction and different plants exhibited in certain specific differences in their sensitivity to the various GAs. It is specially interesting to note here that GA_{20} applied to spinach plants grown in short day was considerably more active in promoting petiole growth than GA_3 (25).

In peas, it is premature to draw conclusion from the data at hand whether the increase in the level of endogenous GAs in vernalized plants is the cause or only consequence of the chilling effect but present results indicate that more detailed study must be necessary until when we find final conclusion on the role of GAs in the flowering of vernalizable plants.

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エンドウの春化とジベレリン

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摘　要

エンドウは品種によって低温春化処理に感応し，第一花着生節位が二，三節低下する．従来の研究によるとジベレリン（GA）処理は，春化，非春化植物共に開花を遅延させ第一花着生節位を高くし，その効果は矮性品種ほど大きい．これら初期の研究において用いられたGAは種類についてはっきり記載はないが，いずれも GA_3 を主成分とした混合物であったと推定される．

我が国で栽培される品種中，最も低温感応の高い品種の一つである渥美絹莢（矮性）を用いて低温春化とGAの関係を検討した．低温処理により第一花着生節位は，2.4節低下した．種子春化（5℃，20日間）により，種子内及びそれから生育した植物葉条内の内生GAが増加するが，開花後も草丈は非春化のものと同じである．エンドウ発芽種子及び葉条内の内生GAは，イネ苗生物検定において短銀坊主によく反応するが矮稲-Cには全く反応しない．このことは，この内生GAがジベレラン環C-3位に水酸基を欠くGAで茎伸長には活性の低いGAであることを強く示唆している．

一方，ジベレラン環C-3位に水酸基を有する GA_3 と GA_7（矮稲-Cにも反応する）を供試品種に与えると春化したものでも非春化のものでも，茎伸長を著しく促進すると共に，春化したものに与えたときは春化効果をキヤンセルし第一花着生節位を統計的に有意に高くした．

なお，通気した水中で種子春化して得られるいわゆる"diffusate"にもGA活性が現われ特に春化種子より得られるGA活性と異なったピークが一か所出現した．しかし，これらのGAもすべて矮稲-Cには反応しなかった．

未熟種子を含む若い莢から得たGAには，矮稲-Cに反応するGAが存在している．