High-frequency Floral Bud Regeneration from Petal Segment Cultures of *Sinningia speciosa* Hiern

Pang Jiliang[1]*, Wang Lilin[1], Xiang Taihe[1], Zhong Dan[1], Yu Hong[2]
([1]*College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China*;
[2]*Institute of Biotechnology, Hangzhou Academy of Agricultural Science, Hangzhou 310024, China*)

Abstract The influence of gibberellin and cytokinin on the regeneration of floral bud from petal segments of *Sinningia speciosa* Hiern *in vitro* was studied here. Two types of regeneration were regeneration of floral buds only (designated B^F), and regeneration of both floral and vegetative buds (designated B^{F+V}). The capacity of floral bud regeneration was influenced by light, the size of the floral bud and exogenous gibberellin (GA_3) and cytokinin in the media. In MS basic medium containing 1.0 mg/L GA_3, the addition of 6-benzyladenine (BA, 0.5 mg/L) significantly increased the frequency of total (B^F+B^{F+V}) flower bud formation to 100%. The culture was in darkness, the frequency of B^F regeneration was up to 93.4%. The highest percentage of B^F was 86.7% formed in petal segments from 7 mm floral buds in diameter. Morphological changes in tissue structure of petal segment cultures were observed at several stages (0 to 30 d) under the light microscopy.

Key words *Sinningia speciosa* Hiern; petal segment culture; direct regeneration of floral buds; gibberellin; benzyladenine; darkness

In vitro direct regeneration of flower buds plays important roles not only on the application in biotechnological industry but also on the understanding of the mechanism of floral bud differentiation. *In vitro* direct regeneration of flower buds was successfully achieved in only over twenty of species in past long period[1-3]. In most of these species such as *Nicotiana tatbacum*[4], *Hyccinthus orientalis*[5], *Lycopersucun esculentum*[6], applications of auxin and cytokinin have been used *in vitro* to induce direct regeneration of floral buds with different combinations. Only in few species, such as *Torenia fournieri*[7], applications of gibberellin and cytokinin *in vitro* induced direct regeneration of floral buds. We have successfully induced the direct formation of male and female flowers from excised cotyledons of cucumber[8], and induced flowering of *in vitro* cultures from a hybrid of *Cymbidium goeringii* and *C.hybridium*[9].

Our recent studies show that GA_3 combinated with cytokinin promotes the direct regeneration of floral buds from cultured floral buds and sepal segments in *Sinningia speciosa* Hiern[10-11]. Sinningia is a day-neutral ornamental flowering plant with a short (3~4 months) vegetative phase. In sinningia genetic transformation is performed easily[12], possibly making it an ideal species for further study of the mechanism of synergistic promotion by GA and cytokinin on floral bud differentiation. To optimize the experimental system, we further investigated the optimal conditions for floral bud regeneration from petal segment cultures of sinningia under different light, floral bud size and the combinations of GA_3 and cytokinin.

1 Materials and Methods

1.1 Plant material

Floral buds with diameters of about 7 mm and young leaves were excised from flowering plants of

Received: August 9, 2011 Accepted: January 10, 2012
This work was supported by the National Natural Science Foundation of China (No.31071818)
*Corresponding author. Tel: 86-571-28865327, E-mail: pangrenshuiliang@yahoo.com.cn

S. speciosa Hiern. The excised floral buds and young leaves were sterilized in 0.1% (v/v) mercuric chloride for 8 min and then rinsed three times with sterile distilled water. This was followed by cutting the detached petal into four segments longitudinally and cutting the young leaf into 5 mm^2 segments.

1.2 Culture of explants in media supplemented with GA_3 and BA

To investigate the effects of combinations of GA_3 and BA on regeneration of floral buds, three independent experiments were carried out. Firstly, petal segments were cultured on modified MS medium (Murashige and Skoog, 1962) containing 1.0 mg/L GA_3 and a range of concentrations of BA (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) in the light or in darkness. Secondly, young leaf segments were cultured on modified MS medium containing 1.0 mg/L GA_3 and a range of concentrations of BA (0, 0.3 and 0.5 mg/L) in the light or in darkness. Thirdly, petal segments were cultured on modified MS medium with combinations of 0.3 mg/L BA and a range of concentrations of GA_3 (0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L) in the light.

1.3 Culture of petal segments from floral buds of different diameters

To investigate the effect of floral bud size on regeneration, petal segments from floral buds of a range of diameters (5, 7, 9, 12, 16, 30 mm) (Fig.1A) were cultured on modified MS medium with combinations of 1.0 mg/L GA_3 and 0.3 mg/L BA. In each experiment described above, GA_3 was always added to the medium after filter sterilization. Each treatment consisted of 60 explants and the experiment was repeated twice. The explants were cultured in a growth room at 25 °C, and examined using a Zeiss Stemi 2000-C Stereomicroscope equipped with a Canon PowerShot G_5 camera after 30, 40, 50, 60 d of culture.

1.4 Histological analysis

After 0, 8, 15, 20, 24, 30 d of culture, petal segments were fixed in FAA (formaldehyde/acetic acid/alcohol) for 48 h. Conventional paraffin embedding and sectioning was used for the preparation of slides. Sections of 8 μm thick were stained in HEIDENHAIN'S haematoxylin[13] and viewed with a microscope (Zeiss AXIO Imager A1), and micrographs were obtained using a Zeiss AxioCam MRc5 camera.

2 Results

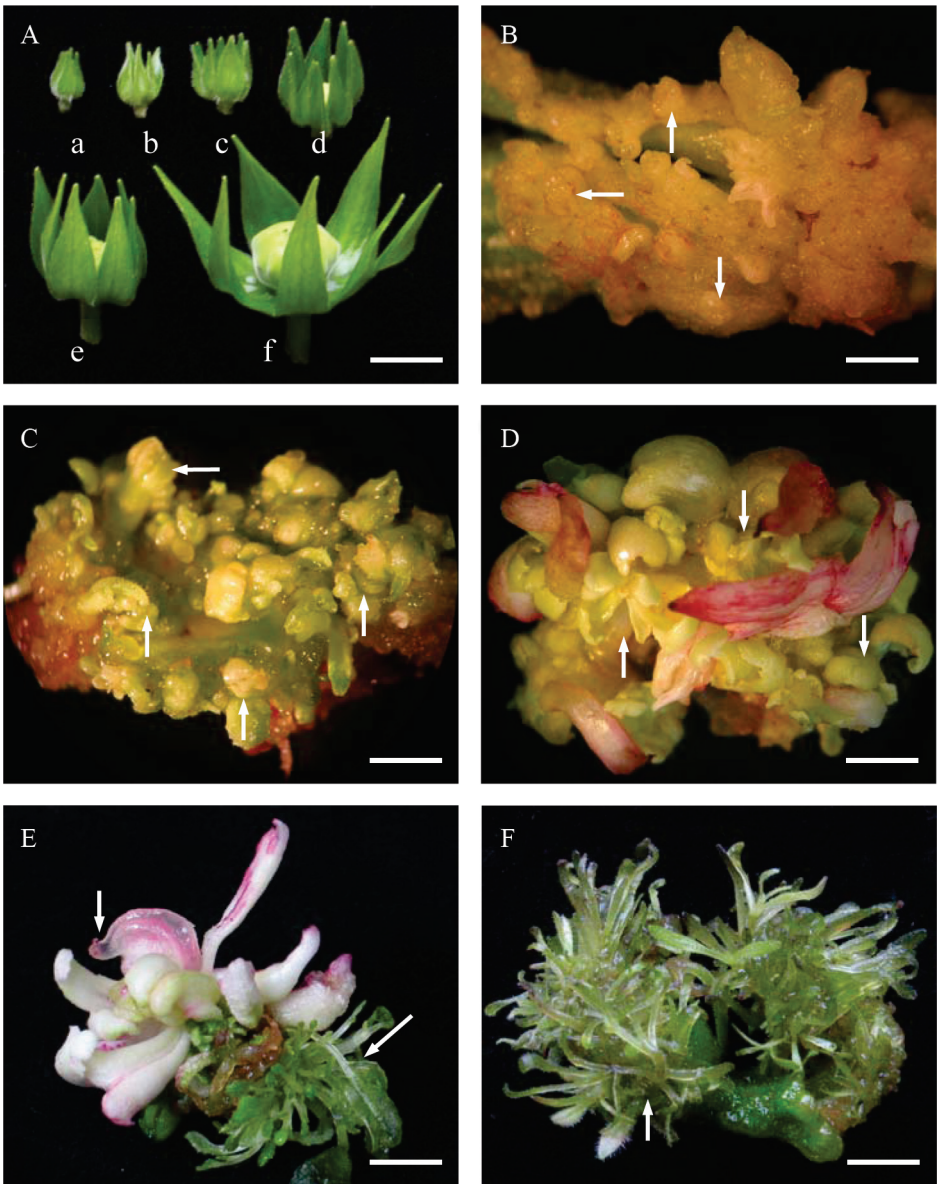
2.1 External morphological and internal structure changes in direct *in vitro* floral bud regeneration from petal segments

The cut edges of explants swelled after 15 d of culture with many floral organ primordia appearing at the segment edges and on the surfaces on 30 d (Fig.1B). On 40 d small floral buds formed (Fig.1C). The floral buds were about 4 mm to be seen by the naked eye on 50 d (Fig.1D). After 60 d, the buds opened revealing many petals (Fig.1E). The regeneration of two types of floral buds from petal segment cultures was observed: floral buds (designated B^F) only (Fig.1D) and both floral buds and vegetative buds (designated B^{F+V}) (Fig.1E). Only vegetative buds were regenerated in the cultures of young leaf segments (Fig.1F).

The histological studies revealed the origins and early development process of the floral buds regenerated *in vitro* directly from petal segments. Sections of petal segments before culture showed the cells of the petal segment tissues to be arranged regularly with no cell division (Fig.2A). After 8 d of culture some highly meristematic centers of dividing cells appeared on the cut edges and under the epidermis (Fig.2B). After 15 d of culture, the meristematic centers became larger and formed meristematic protuberances (Fig.2C). By 20 d, floral organ primordia differentiated on the meristematic protuberances (Fig.2D). The floral organ primordia grew larger by 24 d (Fig.2E). Floral buds with petals and stamen primordia were observed on 30 d (Fig.2F).

2.2 Effect of combinations of GA_3, cytokinin and light on floral bud regeneration

To study the effects of GA_3, cytokinin and light on the efficiency of direct floral bud regeneration from petal segments, we used six different media in the light and three different media in the dark (Table 1). The frequencies of floral buds and vegetative buds regenerated directly from explants were calculated after

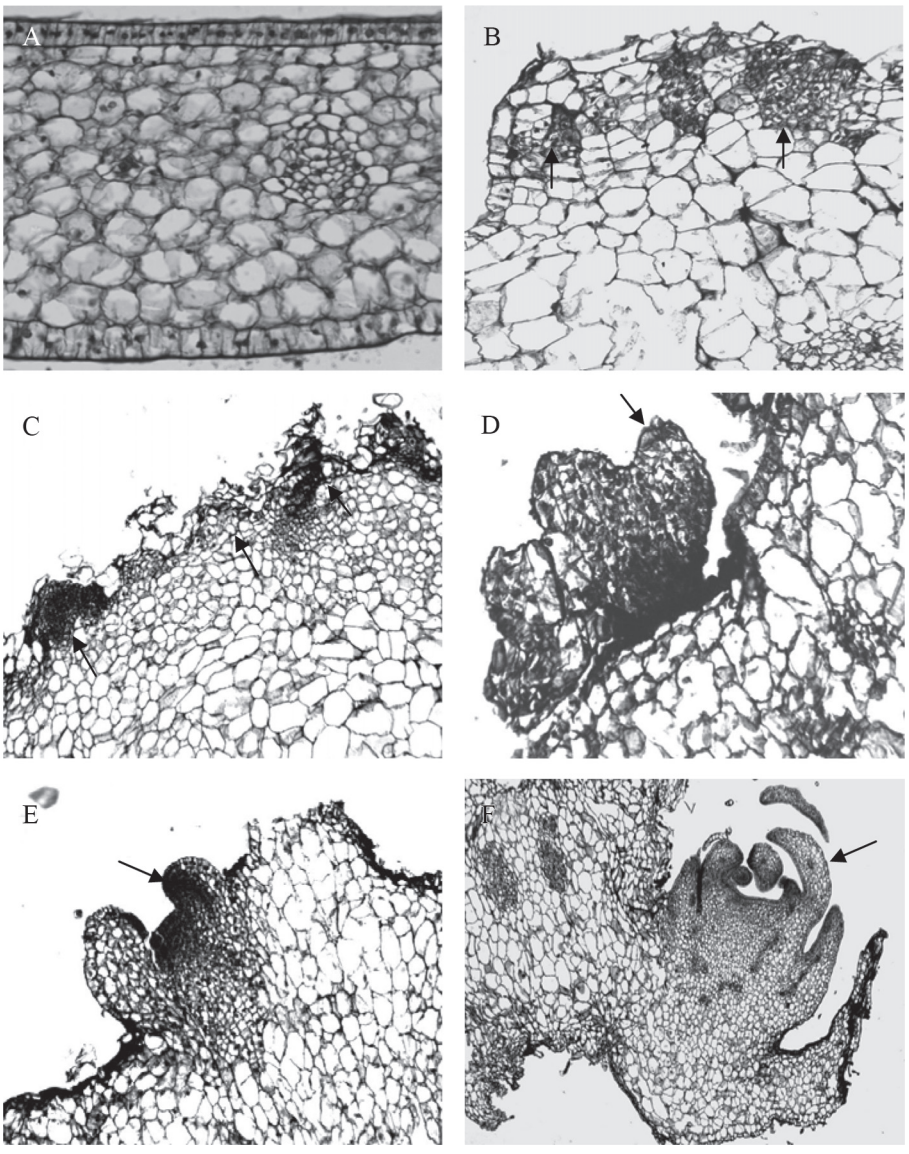


A: floral buds from a to f are 5, 7, 9, 12, 16, 30 mm in diameter respectively; B: many floral organ primordia (arrow) formed on the edges and surface of petal segments cultured for 30 d; Bar=0.5 mm; C: many floral buds (arrow) formed on the surfaces of petal segments cultured for 40 d; Bar=1.0 mm; D: larger floral buds (arrow) could be seen with the naked eye after culture for 50 d; Bar=2.5 mm; E: both floral buds and vegetative buds (arrow) regenerated from petal segments cultured for 60 d, and the floral bud opened; Bar=3.5 mm; F: only vegetative buds (arrow) regenerated from young leaf segments cultured for 60 d; Bar=3.5 mm.

Fig.1 Direct regeneration of floral buds from petal segment cultures in *Sinningia speciosa* Hiern

60 d of culture. The results showed that no flower buds were developed in the absence of BA in the light and in the dark (Table 1). In the light, with 1.0 mg/L GA_3 in the medium, the frequency of B^F increased gradually with increasing concentrations of BA from 0 to 0.3 mg/L. The highest frequency of B^F was 86.6%, but the frequency decreased with BA over 0.3 mg/L in the media. The frequency of (B^F+B^{F+V}) (total flower buds) increased from 0 to 100% with increasing concentrations of BA from 0 to 0.5 mg/L. In the dark, when the medium was supplemented with 0.3 mg/L BA in combination with 1.0 mg/L GA_3, the frequency of B^F was up to 93.4%.

To study the effect of GA_3, cytokinin and light on



A: the cells of petal segment tissues before culture arranged regularly with no dividing cells visible, 400×; B: small meristematic centers of dividing cells at the cut edges and the lower epidermis of petal segment cultured for 8 d, 200×; C: after 15 d of culture, the meristematic centers grew bigger and formed meristematic protuberances, 200×; D: by 20 d of *in vitro* culture, floral organ primordia had differentiated on the meristematic protuberances, 200×; E: the floral organ primordia became larger after 24 d of culture, 100×; F: floral bud with petal, stamen primordia on a petal segment cultured for 30 d, 50×.

Fig.2 Sections of *in vitro* petal segments in *Sinningia speciosa* Hiern

organ regeneration from young leaf segments, we used three different media in the light and in the dark, respectively (Table 2). The results showed that no flower buds were regenerated in these conditions, indicating that the internal physiological condition of the explants is very important for the regeneration of floral buds.

As shown in Table 1, the combination of 1.0 mg/L GA_3 with 0.3 mg/L BA resulted in the highest frequency of pure floral bud regeneration (B^F). To further optimize the combination of BA and GA_3, explants were cultured on the medium containing 0.3 mg/L BA with different concentrations of GA_3 (0~4.0 mg/L) in the light. After 60 d of culture, no floral bud formation was observed on explants grown on the medium containing 0.3 mg/L BA without GA_3. The addition of 0.5~4.0 mg/L GA_3 in the medium containing 0.3 mg/L BA clearly promoted floral bud regeneration (Table 3). The highest frequency of B^F was 83% at 1.0 mg/L GA_3.

Table 1 Effects of combinations of 1.0 mg/L GA_3 with different concentrations of BA on different organs regenerated directly from cultures of petal segments in the light and in the dark

Illumination	Growth regulator		Frequencies of different organs regenerated directly(%)				
	GA_3(mg/L)	BA(mg/L)	B^F	B^{F+V}	B^V	B^F+B^{F+V}	B^F/B^T
Light	1.0	0	0	0	0	0	0
	1.0	0.1	20.0±3.2a	0	0	20.0±3.2a	100 d
	1.0	0.2	70.8±3.5b	0	0	70.8±3.5b	100 d
	1.0	0.3	86.6±4.1c	0	0	86.6±4.1c	100 d
	1.0	0.4	76.7±2.7b	6.7±1.6ab	0	83.4±2.1c	92±3.2bc
	1.0	0.5	68.1±5.2b	31.9±4.5c	0	100d	68.1±5.2a
Dark	1.0	0	0	0	0	0	0
	1.0	0.3	93.4±6.6d	1.7±1.7a	0	95.1±4.2d	98.2±1.8cd
	1.0	0.5	86±5.8c	10.7±2.5b	3.3±3.3a	96.7±3.3d	86.0±5.6b

Explants were evaluated after 60 d of culture. B^F: frequency of flower buds; B^{F+V}: frequency of floral and vegetative buds; B^V: frequency of only vegetative buds; B^T: induction frequency (percentage of petal segments developing organs of the total petal segments plated). Each value represents the mean±standard error of two replications, each with 60 explants. Means within a column followed by the same letter are not significantly different using Duncan's multiple range test ($P \geqslant 0.05$).

Table 2 Effects of combinations of 1.0 mg/L GA_3 with different concentrations of BA on different organs regenerated directly from cultures of young leaf segments in the light and the dark

Illumination	Growth regulator		Frequencies of different organs regenerated directly(%)				
	GA_3(mg/L)	BA(mg/L)	B^F	B^{F+V}	B^V	B^F+B^{F+V}	B^F/B^T
Light	1.0	0	0	0	0	0	0
	1.0	0.3	0	0	90.5±3.0a	0	0
	1.0	0.5	0	0	91.7±2.5a	0	0
Dark	1.0	0	0	0	0	0	0
	1.0	0.3	0	0	100b	0	0
	1.0	0.5	0	0	100b	0	0

The note is the same as Table 1.

Table 3 Effects of combinations of 0.3 mg/L BA with different concentrations of GA_3 on different organs regenerated directly from the cultures of petal segments

Growth regulator		Frequencies of different organs regenerated directly(%)				
GA_3(mg/L)	BA(mg/L)	B^F	B^{F+V}	B^V	B^F+B^{F+V}	B^F/B^T
0	0.3	0	0	5.6±3.4a	0	0
0.5	0.3	53±3.6a	13.3±2.7b	3.3±1.7a	66.3±3.2b	76.1±5.2a
1.0	0.3	83±3.2c	0	0	83±3.2c	100b
2.0	0.3	72.2±4.5b	5.6±1.4a	0	77.8±3.0c	92.8±5.7b
4.0	0.3	50±2.3a	0	0	50±2.3a	100b

The note is the same as Table 1.

2.3 Effect of floral bud size on the direct regeneration of floral buds

Petal segments from floral buds of different diameters (5~30 mm) were cultured on media supplemented with combinations of 1.0 mg/L GA_3 and 0.3 mg/L BA for 60 d. It was significantly different in the frequencies of direct floral bud regeneration from petal segments of floral buds of different diameters (Table 4). The frequency of B^F regenerated from petal segments from 7 mm flower buds was highest (86.7%), followed by petal segments from 5 mm buds (80%) and petal segments from 9 mm flower buds (75%) (Table 4). The frequency of B^F decreased with increasing floral bud diameter from 9 to 30 mm.

Table 4 Effects of floral bud size on direct regeneration of floral buds from the cultures of petal segments

Diameter of floral buds(mm)	Frequencies of different organs regenerated directly(%)				
	B^F	B^{F+V}	B^V	B^F+B^{F+V}	B^F/B^T
5	80±4.3bc	0	0	80±4.3bc	100b
7	86.7±3.8c	0	0	86.7±3.8c	100b
9	75±2.7b	0	0	75±2.7b	100b
12	72.2±4.5b	0	0	72.2±4.5b	100b
16	37.5±2.3a	0	0	37.5±2.3a	100b
30	36.7±1.8a	3.3±1.3a	0	40±1.6a	91.8±4.5a

The note is the same as Table 1.

3 Discussion

The genes of *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CO1/AGAMOUS-LIKE20* (*SOC1/AGL20*) and *LEAFY* (*LFY*) are the flowering integrators that play the critical role in the transition from vegetative to reproductive development. GA promotes the expression of *LFY*, *SOC1 and FT*[14-16]. The combination of GA and BA enhances *SaSOC1* expression in the SAM of *Sinapis alba*[17]. Cytokinin activates *TSF*, *FT* homologue[18]. Floral buds regenerated directly from sepals of 35S::*CFL* (cucumber *LEAFY* homologue) Gloxinia *in vitro*[12]. So GA and BA may promote synergistically the direct regenerations of floral buds by promoting *FT*, *SOC1* and *LFY* expression.

Some long-day (*Beta vulgaris*) and short-day (*Pharbitis nil*) plants flower in darkness[19-20]. *Arabidopsis* could flower in complete darkness on liquid mineral media[21] and solid medium supplemented with sucrose[22]. Here floral bud regeneration from petal segments in darkness was also observed (Table 1). Moreover the regeneration frequency of B^F from petal segments in darkness could reach 93.4% and much higher than in the light. *Arabidopsis* lacking phytochrome B, results in higher gibberellin biosynthesis and sensitivity[23], dark treatments of the upper shoots of rose significantly increased their cytokinin levels[24], possibly indicated the culture of petal segments in darkness increases their gibberellin biosynthesis, sensitivity and endogenous cytokinin levels, thus promoted floral bud formation.

In general, floral buds can only be regenerated directly from explants from reproductive organs. In our study, floral buds could not be regenerated directly from cultures of young leaf segments (Table 2). It is further confirmed that there are some unknown substances inhibiting floral differentiation in vegetative organs.

Cytokinin activity in young rose petals is higher than that in old ones[25]. In *Cosmos sulphureus Cav*, cytokinin activity is low at early stage in flower development and increases before full bloom, but decreases at full bloom[26]. The highest concentration of free GA_1/GA_3 was found in dormant flower buds of peach and diminished thereafter. The highest concentration of free GA_1/GA_3 did not increase immediately before flower bud break[27]. Our experiment (Table 4) showed that the B^F frequency of petal segments changed according to the size of floral buds, possibly mainly resulted from the differences in the activity and content of endogenous cytokinin and gibberellin in floral buds of different sizes.

References

1 Dickens CWS, Staden JV. The induction and evocation of flowering *in vitro*. S Afr J Bot 1988; 54(4): 325-44.

2 Chen YN. An enumeration of *in vtrio* flowering plants (preliminary catalog). Plant Physiol Comm 1990; (3): 71-6.

3 Chen YN. An enumeration of *in vtrio* flowering plants (sequel). Plant Physiol Comm 1995; 31(4): 318-20.

4 Tran Thanh Van K. Direct flower neoformation from superficial tissue of small explants of *Nicotiana tabacum* L. Planta 1973; 115(1): 87-92.

5 Lu WL, Bai SN, Zhang XS. Induction of continuous tepal differentiation from *in vitro* regenerated flower buds of *Hyacinthus orientalis*. Acta Bot Sin 1999; 41(9): 921-6.

6 Wu ZJ, Cheng ZH, Sun SW. Floral and fruit differentiated directly from callus of tomato cotyledon. Plant Physiol J 2011; 47: 991-3.

7 Tanimoto S, Harada H. Chemical factors controlling floral bud

formation of torenia stem segments cultured *in vitro* II. Effects of growth regulators. Plant Cell Physiol 1981; 22(3): 543-50.

8 Pang JL, Liang HM, Liu FY, Guo DC. Direct formation of male and female flowers from excised cotyledons of Cucumbers (*Cucumis sativus* L.). Chinese J Bot 1993; 5(2): 185-8.

9 Zheng LM, Pang JL. *In vitro* Flowering of cultures from a hybrid of *Cymbidium goeringii* and *C.hybridium*. J Plant Physiol Mol Biol 2006; 32(3): 320-4.

10 Pang JL, Wang LL, Hu JQ, Liang HM. Effect of gibberellin on direct regeneration of floral buds from young flower buds in *Sinningia speciosa* Hiern. Acta Biol Exp Sin 2004; 37(3): 241-6.

11 Pang JL, Wang LL, Hu JQ, Xiang TH, Liang HM. Synergistic promotion of gibberellin and cytokinin on direct regeneration of floral buds from *in vitro* cultures of sepal segments in *Sinningia speciosa* Hiern. *In vitro* Cell Dev Biol Plant 2006; 42(5): 450-4.

12 Zhang MZ, Ye D, Wang LL, Pang JL, Zhang YH, Zheng K, *et al.* Overexpression of the cucumber *LEAFY* homolog *CFL* and hormone treatments alter flower development in gloxinia (*Sinningia speciosa*). Plant Mol Biol 2008; 67(4): 419-27.

13 Heidenhain M. Neue Untersuchungen ober die Centralk6rper und ihre Beziehungen zum Kern-und Zellen-protoplasma. Arch Mikr Anat 1894; 3: 423-758.

14 Blázquez M, Green R, Nilsson O, Sussman M, Weigel D. Gibberellins promote flowering of *Arabidopsis* by activating the *LEAFY* promoter. Plant Cell 1998; 10(5): 791-800.

15 Moon J, Suh SS, Lee H, Choi KR, Hong CB, Paek NC, *et al.* The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis*. Plant J 2003; 35(5): 613-23.

16 Hisamatsu T, Rod WK. The nature of floral signals in *Arabidopsis*.II.roles for *FLOWERING LOCUS T*(*FT*) and gibberellin. J Exp Bot 2008; 59(14): 3821-9.

17 Bonhomme F, Kurz B, Melzer S, Bernier G, Jacqard A. Cytokinin and gibberellin activate *SaMADS A*, a gene apparently involved in regulation of the floral transition in *Sinapis alba*. Plant J 2000; 24(1): 103-11.

18 D'Aloia M, Bonhomme D, Bouche F, Tamseddak K, Ormenese S, Torti S, *et al.* Cytokinin promotes flowering of Arabidopsis via transcriptional activation of the *FT* paralogue *TSF*. Plant J 2011; 65(6): 972-9.

19 Fife JM, Price C. Bolting and flowering of sugar beet in complete darkness. Plant Physiol 1953; 28(3): 475-80.

20 Takimoto A. Effect of sucrose on flower initiation of *Pharbitis nil* in aseptic culture. Plant Cell Physiol 1960; 1(4): 241-6.

21 Araki T, Komeda Y. Flowering in darkness in *Arabidopsis thaliana*. Plant J 1993; 4(5): 801-11.

22 Roldán M, Gómez-Mena C, Ruiz-García L, Salinas J, Martínez-Zapater JM. Sucrose availability on the aerial part promotes morphogenesis and flowering of *Arabidopsis* in the dark. Plant J 1999; 20(5): 581-90.

23 Reed JW, Foster KR, Morgan PW, Chory J. Phytochrome B affects responsiveness to gibberellins in *Arabidopsis*. Plant Physiol 1996; 112(1): 337-42.

24 van staden J, Zieslin N, Spiegelstein H, Halevy H. The effect of light on the cytokinin content of developing rose shoots. Ann Bot 1981; 47(1): 155-7.

25 Mayak S, Halevy AH. Cytokinin activity in rose petals and its relation to senescence. Plant Physiol 1970; 46(4): 497-9.

26 Saha S, Nagar PK, Sircar PK. Changes in cytokinin activity during flower development in *Cosmos sulphureus Cav*. Plant Growth Regul 1985; 3(1): 27-35.

27 Luna V, Lorenzo E, Reinoso H, Tordable MC, Abdala G, Pharis RP, *et al*. Dormancy in peach (*Prunus persica* L.) flower buds: I. Floral morphogenesis and endogenous gibberellins at the end of the dormancy period. Plant Physiol 1990; 93(1): 20-5.

大岩桐花瓣切块离体培养高频率花芽再生

庞基良[1]* 王利琳[1] 向太和[1] 钟 丹[1] 余 红[2]

([1]杭州师范大学生命与环境科学学院, 杭州 310036; [2]杭州市农业科学研究院生物技术研究所, 杭州 310024)

摘要 该文报道了大岩桐花瓣切块离体培养再生花现象，花瓣切块再生花有两种方式: 一种是仅再生花芽(命名为B^F); 另一种是既再生花芽也再生营养芽(命名为B^{F+V})。花芽再生的能力与光照、花芽大小及培养基中赤霉素和细胞分裂素浓度紧密相关。当培养基中含有1.0 mg/L GA_3时，BA的添加会显著增加总花芽(B^F+B^{F+V})的形成率，添加0.5 mg/L BA时，总花芽形成率达100%。在暗中培养时，B^F达93.4%。不同大小花芽的花瓣再生花的能力不同，7 mm直径花芽的B^F最高，达86.7%。同时，对花芽再生过程中花瓣切块的组织结构形态变化也进行了观察。

关键词 大岩桐; 花瓣切块; 直接花芽再生; 赤霉素; 细胞分裂素; 黑暗

收稿日期: 2011-08-09 接受日期: 2012-01-10

国家自然科学基金(No.31071818)资助项目

*通讯作者。Tel: 0571-28865327, E-mail: pangrenshuiliang@yahoo.com.cn