The Effects of Different Types of Cytokinin on Shoot Formation and Plant Regeneration from Leaf Explants of Four Cultivars of Sagagiku Group in Chrysanthemum, *Dendranthema grandiflorum* (Ramat.) Kitamura

Masato TSURO, Yoshiaki SHIZUKAWA and Koji INABA

Kyoto Prefectural Institute of Agricultural Biotechnology, Kyoto 619-0244, Japan

(Received February 12, 2002)

Four cultivars of Sagagiku group in chrysanthemum were treated with different types of cytokinin to form adventitious shoots from leaf explants. The maximum adventitious shoot formation rate, 82.2%, was observed in the medium with 2.5×10^{-5} M of thidiazuron (TDZ), an urea-type cytokinin, in 'Ogura-nishiki,' followed by 'Sagano-aki' (56.7%) in the medium with 2.5×10^{-6} M of *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU), an urea-type cytokinin, 'Sagano-yuki' (12.2%) in the medium with 2.5×10^{-6} M of 6-benzylamino purine (BA), a purine-type cytokinin, and 'Sagano-haru' (1.3%) in the medium with 5.0×10^{-6} M of TDZ. Vigorous roots and significantly higher root formation rates were observed in the medium without 3-indolebutyric acid. In 'Sagano-haru,' however, no roots were differentiated and died during root induction culture. These results suggested that there were large differences in adventitious shoot formation ability among these four cultivars of Sagagiku group, and that TDZ and CPPU were effective for shoot formation in some cultivars of chrysanthemum.

Keywords: purine-type cytokinin, regeneration, Sagagiku chrysanthemum, urea-type cytokinin

INTRODUCTION

Chrysanthemum, *Dendranthema grandiflorum* (Ramat.) Kitamura, is now one of the most economically important cut- and potted-flower plants as well as rose and carnation. Traditionally, new cultivars have been released by means of conventional breeding programs in endemic chrysanthemum group Sagagiku.

Tissue culture technique is one of the most useful procedures to exploit a new trait via somaclonal mutation. This procedure has been applied to many crops and ornamental plants (Oosawa, 1994). In addition, gene transfer into plants, by *Agrobacterium*-mediation, requires regeneration through tissue culture. Plant regeneration and genetic transformation were reported in some cultivars of chrysanthemum. Fukai et al. (1987) and Shibata et al. (1992) compared plant regeneration rates in leaf and stem explants of 137 and 163 cultivars of chrysanthemum, respectively. Tanaka et al. (2000) established a method of plant regeneration from somatic embryo in cultivar 'Aboukyu.' In addition, several cultivars were successful in genetic transformation via *Agrobacterium*-mediation (Ledger et al., 1991; Lowe et al.,

Corresponding author: Masato Tsuru, fax: +81-774-93-3528, e-mail: tsuro@kab.seika.kyoto.jp

1993; Renou et al., 1993; De Jong et al., 1993, 1994; Urban et al., 1994; Fukai et al., 1995; Takatsu et al., 1998). However, in the previous experiments of chrysanthemum, 6-benzylamino purine (BA), a purine-type cytokinin, was mostly used for shoot induction and large differences in shoot regeneration capacity was reported among cultivars. Therefore, an efficient shoot formation system seems requisite for genetic transformation in each cultivar of chrysanthemum, independently. We have been searching an efficient procedure of plant regeneration, in order to establish a genetic transformation system.

In this paper, we describe effects of two types of cytokinin for shoot formation from leaf explant and plant regeneration in four cultivars of endemic chrysanthemum group Sagagiku.

MATERIALS AND METHODS

Plant materials. In Sagagiku group, 'Sagano-haru,' 'Ogura-nishiki,' 'Sagano-aki' and 'Sagano-yuki,' kindly supplied by Kyoto Flower Center (Soraku, Kyoto, Japan), were maintained in vitro on 1/2 Murashige and Skoog (1962) (MS) medium (MS macro nutrients in half strength) containing 0.3% gelrite and 3.0% sucrose (pH 5.8) in a plastic box (6×6×10 cm), at 25°C, under 16 h photoperiod illuminated by cool white fluorescent light (3 000 lx).

Adventitious shoot induction. The fully developed leaves of each cultivar were detached and cut to 3×3 mm segments, then they were placed on 30 ml of MS solid medium supplemented with 2.5×10^{-6} , 5.0×10^{-6} and 2.5×10^{-5} M of BA, a purine-type cytokinin, and thidiazuron (TDZ), an urea-type cytokinin, and 2.5×10^{-7} , 5.0×10^{-7} and 2.5×10^{-6} M of *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU), an urea-type cytokinin, in plastic dishes (9 cm in diameter). For shoot induction, the explants were cultured at 25°C under 16 h photoperiod. After 2 months of culture, adventitious shoots formed on the callus were counted, and number of shoots per callus (number of shoots/number of shoot-formed calli) and shoot formation rates (number of shoots-formed calli/number of explants inoculated) were calculated.

Root formation and acclimatization. Shoots grown over 5 mm in length were separated from the calli and transferred to the 1/2 MS solid medium supplemented with or without 1.0×10^{-6} M of 3-indolebutyric acid (IBA). The shoots were cultured for root induction in the same conditions as shoot induction. After 1 month of culture, number of shoots forming roots was counted and root formation rates (number of shoots forming roots/number of shoots cultured) were determined.

For acclimatization, regenerated plantlets over 2 cm in height were transplanted to vermiculite medium moistened with 1/2 000 Hyponex® (N-P-K, 5-10-5% (w/w)) solution in top sealed plastic boxes (6×6×14 cm), and cultured at 25°C for 1 week. After complementation of acclimatization, the plantlets were transferred to soil in pots and grown at 15-25°C in the greenhouse of Kyoto Prefectural Institute of Agricultural Biotechnology in January, 2001.

RESULTS AND DISCUSSION

Leaf explants showed swelling at the initiation of culture. Then calli were formed at the cut surface of explants in all media within 2 to 3 weeks. After 3 weeks of culture, shoot primordia appeared on the surface of calli, from which one to four adventitious shoots developed. Effects of cytokinins on shoot formation were different among four cultivars. Adventitious shoot formation rates after 2 months of culture are shown in Table 1. In 'Ogura-nishiki,' significantly highest shoot formation rate, 82.2%, was obtained in the medium with 2.5×10^{-5} M of TDZ. Also in 'Sagano-aki' and 'Sagano-yuki,' highest shoot formation rates, 56.7 and 12.2%, respectively, were found in the medium with 2.5×10^{-6} M of CPPU and 2.5×10^{-6} M of BA. Adventitious shoots (2.6-3.5 shoots) per callus differentiated more in

Table 1 Effects of type and concentration of cytokinins on the number of shoots per callus and adventitious shoot formation rates in cultivars of Sagagiku group.

Cultivar	Cytokinin (M)		Number of explants cultured	Number of shoots with calli	Number of shoots per callus ^z	Adventitious shoot formation rates (%) ^z
'Sagano-haru'	BA	2.5×10^{-6}	62	0	0	0
		5.0×10^{-6}	90	0	0	0
		2.5×10^{-5}	90	0	0	0
	TDZ	2.5×10^{-6}	54	0	0	0
		5.0×10^{-6}	75	1	1.0 a	1.3 a
		2.5×10^{-5}	90	0	0	0
	CPPU	2.5×10^{-7}	90	0	0	0
		5.0×10^{-7}	90	0	0	0
		2.5×10^{-6}	90	0	0	0
'Ogura-nishiki'	BA	2.5×10^{-6}	90	9	1.7 a	10.0 abc
		5.0×10^{-6}	90	0	0	0
		2.5×10^{-5}	89	0	0	0
	TDZ	2.5×10^{-6}	89	63	3.5 c	70.8 g
		5.0×10^{-6}	90	62	2.7 b	68.2 g
		2.5×10^{-5}	90	74	2.6 b	82.2 h
	CPPU	2.5×10^{-7}	90	15	1.2 a	16.7 cd
		5.0×10^{-7}	81	18	2.3 b	22.2 d
		2.5×10^{-6}	71	2	1.0 a	2.8 ab
'Sagano-aki'	BA	2.5×10^{-6}	90	19	1.3 a	21.1 d
		5.0×10^{-6}	81	4	1.5 a	4.9 ab
		2.5×10^{-5}	90	8	1.6 a	8.9 abc
	TDZ	2.5×10^{-6}	90	43	1.6 a	47.8 ef
		5.0×10^{-6}	81	35	1.6 a	43.2 e
		2.5×10^{-5}	81	35	1.5 a	43.2 e
	CPPU	2.5×10^{-7}	90	4	1.0 a	4.4 ab
		5.0×10^{-7}	90	11	1.3 a	12.2 bcd
		2.5×10^{-6}	90	51	1.5 a	56.7 f
'Sagano-yuki'	BA	2.5×10^{-6}	90	11	1.3 a	12.2 bcd
		5.0×10^{-6}	90	2	1.0 a	2.2 a
		2.5×10^{-5}	90	0	0	0
	TDZ	2.5×10^{-6}	90	7	1.4 a	7.8 abc
		5.0×10^{-6}	80	5	1.0 a	6.3 ab
		2.5×10^{-5}	90	1	1.0 a	1.1 a
	CPPU	2.5×10^{-7}	81	7	1.7 a	8.6 abc
		5.0×10^{-7}	90	1	1.0 a	1.1 a
		2.5×10^{-6}	90	2	1.5 a	2.2 a

^z Different letters within a column represent significant difference at 5% level, by Fisher's PLSD test.

'Ogura-nishiki' with TDZ than in the other cultivars. These results suggested that the media for most effective in shoot induction in these three cultivars were different. BA, a purine-type cytokinin, has been applied for shoot induction in many cultivars of chrysanthemum (Kaul et al., 1990; Lu et al., 1990; Urban et al., 1994; Takatsu et al., 1998). Some cultivars, however, had not shown any feature of differentiation (Fukai et al., 1987; Shibata et al., 1992). Considering their and our results, some cultivars may depend on TDZ and CPPU, urea-type cytokinins, for effective shoot formation.

On the other hand, in 'Sagano-haru,' only one out of 75 explants formed shoot, 1.3% at the highest shoot formation rate, in the medium with 5.0×10^{-6} M of TDZ (Table 1). Fukai et al. (1995), Lu et al. (1990) and Shibata et al. (1992) demonstrated a clear polarity for shoot differentiation in stem segments in several cultivars of chrysanthemum. Those results suggest

Fig. 1 Root formation rates in cultivars of Sagagiku group after 1 month of culture.

□, without IBA ; ▨, with IBA. * and ** indicate significant difference within cultivar at 1% and 5% levels, respectively, by χ^2 -analysis.

a merit of using stem segment as an alternative explant for shoot formation in 'Sagano-haru.'

The developed shoots (> 5 mm) were excised from leaf explants and transferred to the root induction medium. Roots began to form after 1 to 1.5 weeks of culture in the medium without IBA. The shoots cultured in the medium with IBA, however, began to form roots after 3 weeks of culture. The root formation rates after 1 month of culture are shown in Fig. 1. The medium without IBA showed higher rates than that with IBA, in all the cultivars. In addition, the shoots formed roots in the medium with IBA grew very slowly. These results suggested that shoots in each cultivar had enough endogenous auxins and did not need any exogenous plant growth regulators for root induction. On the other hand, no root formation was observed in 'Sagano-haru' in any medium tested.

The regenerated plantlets over 2 cm in height were easily acclimatized within 1 week, and they continued to grow vigorously in the greenhouse.

As mentioned above, large variation in ability of shoot and root formation was observed among four cultivars of Sagagiku chrysanthemum group. Takatsu et al. (1998), Fukai et al. (1987) and Shibata et al. (1992) concluded that there were large differences in adventitious shoot regeneration capacity among chrysanthemum varieties. Our observation agrees with their results. On the other hand, TDZ or CPPU, urea-type cytokinin, was suggested to be alternative agents for efficient shoot induction in some cultivars of chrysanthemum which failed to form shoots in the medium with BA, purine-type cytokinin. In this study, differentiation of shoots at high frequency was achieved in 'Ogura-nishiki,' so that, experiments are in progress to insert a useful gene into this cultivar, using this regeneration system.

We thank Dr. T. Namiki of Kyoto Prefectural Institute of Agricultural Biotechnology (KAB) for his critical reading of this manuscript. We also thank Mrs. J. Yamagata and Miss M. Matsumoto of KAB for their technical assistance.

REFERENCES

- De Jong, J., Mertens, M. M. J., Rademaker, W. 1994. Stable expression of GUS reporter gene in chrysanthemum depends on binary plasmid T-DNA. *Plant Cell Rep.* **14**: 59-64.
- De Jong, J., Rademaker, W., Van Wordragen, M. F. 1993. Restoring adventitious shoot formation on chrysanthemum leaf explants following cocultivation with *Agrobacterium tumefaciens*. *Plant Cell, Tissue Organ Cult.* **32**: 263-270.
- Fukai, S., Chen, Z.-Y., Oe, M. 1987. Cultivar differences in adventitious shoot formation from leaf segments of chrysanthemum (*Dendranthema grandiflorum* (RAMAT. KITAMURA)). (Japanese text

- with English abstract). Bull. Osaka Agric. Res. Cent. **24**: 55-58.
- Fukai, S., De Jong, J., Rademaker, W. 1995. Efficient genetic transformation of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura) using stem segments. Breed. Sci. **45**: 179-184.
- Kaul, V., Miller, R. M., Hutchinson, J. F., Richards, D. 1990. Shoot regeneration from stem and leaf explants of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). Plant Cell, Tissue Organ Cult. **21**: 21-30.
- Ledger, S. E., Deroles, S. C., Given, N. K. 1991. Regeneration and *Agrobacterium*-mediated transformation of chrysanthemum. Plant Cell Rep. **10**: 195-199.
- Lowe, J. M., Davey, M. R., Power, J. B., Blundy, K. S. 1993. A study of some factors affecting *Agrobacterium* transformation and plant regeneration of *Dendranthema grandiflora* Tzvelev (syn. *Chrysanthemum morifolium* Ramat.). Plant Cell, Tissue Organ Cult. **33**: 171-180.
- Lu, C., Nugent, G., Wardley, T. 1990. Efficient, direct plant regeneration from stem segments of chrysanthemum (*Chrysanthemum morifolium* Ramat. cv. Royal Purple). Plant Cell Rep. **8**: 733-736.
- Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.
- Oosawa, K. 1994. An Elementary Knowledge of Plant Biotechnology. (Japanese text). Noubun-Kyo, Tokyo, p 79-86.
- Renou, J. P., Brochard, P., Jalouzet, R. 1993. Recovery of transgenic chrysanthemum (*Dendranthema grandiflora* Tzvelev) after hygromycin selection. Plant Sci. **89**: 185-197.
- Shibata, M., Aida, R., Ikeda, H., Shimizu, A. 1992. Difference of adventitious shoot formation ability from stem segments in chrysanthemum cultivars and species. (Japanese text). Jpn. J. Breed. **42** (Suppl. 2): 556-557.
- Takatsu, Y., Tomotsune, H., Kasumi, M., Sakuma, F. 1998. Differences in adventitious shoot regeneration capacity among Japanese chrysanthemum [*Dendranthema grandiflorum* (Ramat.) Kitamura] cultivars and the improved protocol for *Agrobacterium*-mediated genetic transformation. (Japanese text with English abstract). J. Jpn. Soc. Hortic. Sci. **67**: 958-964.
- Tanaka, K., Kanno, Y., Kudo, S., Suzuki, M. 2000. Somatic embryogenesis and plant regeneration in chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura). Plant Cell Rep. **19**: 946-953.
- Urban, L. A., Sherman, J. M., Moyer, J. W., Daub, M. E. 1994. High frequency shoot regeneration and *Agrobacterium*-mediated transformation of chrysanthemum (*Dendranthema grandiflora*). Plant Sci. **98**: 69-79.

〈和文抄録〉

異なるタイプのサイトカイニンが嵯峨ギク4品種の葉片からの不定芽形成に及ぼす影響

津 呂 正 人・ 静 川 幸 明・ 稲 葉 幸 司

京都府農業資源研究センター

種々のタイプおよび濃度のサイトカイニンを添加した MS 固形培地で嵯峨ギク葉片から不定芽を誘導したところ、不定芽誘導に対する最適培地および形成率が品種間で大きく異なった。‘小倉錦’では、ウレア型サイトカイニンである TDZ を 2.5×10^{-5} M 添加した培地で 82.2% と最も高い不定芽形成率が得られ、続いて‘嵯峨の秋’でウレア型サイトカイニンである CPPU を 2.5×10^{-6} M 添加した培地で 56.7%、‘嵯峨の雪’でプリン型サイトカイニンである BA を 2.5×10^{-6} M 添加した培地で 12.2%、および‘嵯峨の春’で 5.0×10^{-6} M TDZ 添加培地での 1.3% の順であった。一方、‘嵯峨の春’を除くいずれの品種もホルモンフリーの培地で容易に発根できたが、‘嵯峨の春’で得られた不定芽からは発根せず、再分化植物体を得ることができなかった。以上の結果から、嵯峨ギク品種群内において葉片からの不定芽形成能に大きな品種間差異が存在することが明らかとなった。また、ギク品種ではこれまでに極めて報告の少ない TDZ あるいは CPPU 等のウレア型サイトカイニンが不定芽形成に有効であることが示唆された。