Light and Scanning Electron Microscopic Analysis of Benzyl Adenine Induced Multiple Shoot Regeneration in Kenaf (*Hibiscus cannabinus* L.)

Samanthi P. Herath^{*1)}, Takayuki Suzuki²⁾ and Kazumi Hattori¹⁾

¹⁾ Laboratory of Plant Genetics and Breeding, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan

²⁾ Araco Corporation, Toyota, Aichi 470-0395, Japan

Morphological changes in shoot apices of kenaf treated with benzyl adenine (BA) were compared with those of untreated control plants using light and scanning electron microscopy (SEM). Seven days after the start of BA treatment (day 7), meristematic regions appeared adjacent to the axillary buds, and on the abaxial surface of the primary leaf primordia. Some of these meristematic regions were later developed into shoots. Further, the cells in the primary shoot apical meristem were reprogrammed to induce several meristematic loci. At day 28 in culture, supernumerary vegetative shoot buds were observed in and near the axillary buds. In the control plants neither axillary nor adventitious buds developed. These results suggest that the treatment with BA reprogrammed the developmental fate of a large number of cells in the shoot apex of kenaf. Further, it reconfirmed the ability of BA to overcome the apical dominance of shoots.

Key Words: benzyl adenine, cytokinin, histology, kenaf, scanning electron microscopy.

Introduction

Kenaf is a fiber crop belonging to family Malvaceae. Traditionally it is used as a raw material for rope and textiles etc. Recently, it is being used in many industries such as the paper pulp manufacturing and automobile industries. However, commercial cultivation of kenaf is hindered with competition from weeds at the seedling stage (Srivatanakul *et al.* 2001). Herbicide-resistant kenaf cultivars would reduce seedling weed competition and make kenaf cultivation more profitable. Several studies were made to develop an *in vitro* regeneration method, which would facilitate the subsequent development of genetically transformed plants of kenaf (Zapata *et al.* 1999, Srivatanakul *et al.* 2000, Herath *et al.* 2004). Multiple shoots were induced in a young shoot of kenaf by treatment with benzyladenine (BA) (Herath *et al.* 2004). However, genetic transformation of kenaf through the *Agrobacterium*-mediated method has not been successful so far. Srivatanakul *et al.* (2001) reported the transformation parameters that enhance the T-DNA expression in kenaf, but no stable transformants were obtained. We succeeded in achieving stable genetic transformation although the transformation efficiency was low (manuscript under preparation). This was achieved mainly by wounding the explant with a sterile needle before *Agrobacterium* inoculation at the location for axillary shoot development. None of the unwounded explants were transformed. This method resulted

in production of low number of stable transformants while producing a very high percentage of chimeras. Knowledge on the exact origin of adventitious shoots would be beneficial since it facilitates the wounding of the explant at locations suitable for adventitious shoot development. This would be important for increasing the transformation efficiency as well as reducing the generation of chimeras. Among the cytokinins, BA is the most frequently used and the most successful plant growth regulator used for micro propagation (Thomas and Blakesley 1987). Treatment with BA is known to induce multiple shoots in many plant species (Flick *et al.* 1983). According to Buising *et al.* (1994), a single exposure to a low concentration of BA causes cells that would normally remain quiescent to divide repeatedly and form numerous vegetative buds. In addition to shoot induction, cytokinins are reported to have a stimulatory or inhibitory effect on different developmental processes such as root growth and branching, apical dominance in the shoot, chloroplast development and leaf senescence (Mok and Mok 2001). According to Cheng *et al.* (1980), treatment with BA caused diversion of normal shoot apex development to form multiple shoot development in *Glycine max*. So far, early events of multiple shoot formation have not been characterized in kenaf. Knowledge on the precise origin of the induced shoots would be helpful in developing a method of apex based genetic transformation through many adventitious shoot development. In this study, by using light and scanning electron microscopic methods we investigated the morphological changes in the shoot tips of kenaf cultured in BA. We show that treatment with BA causes the development of axillary shoots and numerous adventitious shoots in

Communicated by T. Kondo

Received July 20, 2004. Accepted October 13, 2004.

*Corresponding author (e-mail: samanthiherath@hotmail.com)

kenaf. Further, we show that the primary shoot apical meristem of the BA-treated shoot tip is reprogrammed to induce several meristematic loci.

Materials and Methods

Plant materials

Seeds of the kenaf cultivars Tainung 2 (T2), Everglades 41 (E41) and SF 459 were kindly provided by the Oji Paper Company Ltd, Japan, and the seeds of the Thimpi 3 (Th3) from the Araco Corporation, Japan. Surface sterilization and germination of seed, and the tissue culture conditions, unless otherwise stated, were carried out according to the earlier described method (Herath *et al.* 2004). Shoot tips together with the cotyledonary nodes were isolated from 7-day-old seedlings and cultured on the medium containing MS (Murashige and Skoog 1962) basal salts and vitamins, 3% (w/v) sucrose, BA (2.2, 4.4, 8.8 or 22 μM) and solidified with 0.8% (w/v) agar (INA AGAR BA-30). The medium was adjusted to pH 5.8. The same medium but without BA added was used as the control. Cultures were maintained at $28 \pm 1^\circ\text{C}$ under continuous light ($25 \mu\text{mol}^{-2}\text{s}^{-1}$), renewing the culture medium every two weeks. After 8 weeks of culture, the number of shoots regenerated was recorded. Judging from the results of an independent experiment samples were isolated from only T2 cultured on 8.8 μM BA and control medium at 7, 14, 21 and 28 days after incubation of the shoot tips for the light microscopic and SEM analysis.

Light microscopy

Twenty explants each cultured on the control, and BA (8.8 μM) medium were sampled at each age. Samples were fixed in FAA (formalin, acetic acid, 70% ethanol at 5:5:90 ratio), dehydrated in a graded ethanol series (30, 50, 70, 90, 95 and 100% v/v), embedded in paraffin, cut into 10 μm ribbons, floated on distilled water and placed on slides laced with 70% albumin. Slides were dewaxed in xylene and rehydrated in a graded ethanol series. Sections were stained with Toluidine Blue (0.05% w/v; 40 seconds) and again dehydrated in a graded ethanol series, cleared in xylene and mounted under a cover slip with resin and observed under a light microscope.

Scanning electron microscopy

Samples were fixed in FAA, dehydrated in a graded series of acetone (30, 50, 70, 80, 90, 95 and 99% v/v) at 30-minute interval and stored overnight in 100% acetone. The samples were dried to critical point with carbon dioxide for 50 minutes using a Hitachi Critical Point Dryer HCP-1 and then coated with platinum palladium for 180 s using a Hitachi E-1030 Ion Sputter. Coated samples were mounted on metal blocks and observed under a Hitachi S-2300 scanning electron microscope.

Results

Effect of BA on multiple shoot regeneration in kenaf

BA induced multiple shoot regeneration in kenaf shoot apices and no shoots developed in the absence of BA at 8 weeks after the start of culture (Table 1). The number of shoots produced increased with the concentration of BA up to 8.8 μM . BA at higher than 8.8 μM did not increase the number of shoot buds although the response varied with the cultivar. Cultivar T2 produced the highest number of shoot buds at 8.8 μM BA. Hence, samples isolated from this cultivar were used for the analysis with light and scanning electron microscopies (Fig. 1).

Light microscopy

In the sections of 7-day-old seedlings of T2 (prior to culture), two cotyledonary petiole residues and the first leaf primordium on the primary apical meristem was observed (Fig. 1A). No axillary bud meristems were present. The effect of BA was evident at day 7 of culture (Fig. 1B). In BA-treated seedlings, the shoot tip was enlarged and deeply stained meristematic regions were observed adjacent to axillary bud meristem (Fig. 1C) and also on the basal abaxial side of the leaf primordium (Fig. 1D). On these regions, cell division was observed in the epidermal cells of meristematic nodules (Fig. 1D). In the control shoot tips, the development of the first axillary bud meristem was observed at day 7 (Fig. 1E), and that of the second axillary shoot meristem at day 14 (Fig. 1F). In BA-treated explants multiple shoots developed from the primary shoot apical meristem at day 14 (Fig. 1G). In BA-treated apices at day 21 the primary shoot and the axillary shoots developed simultaneously, developing three shoots in total (Fig. 1H). Further, in the region between the axillary shoot and the cotyledon another adventitious shoot meristem appeared (Fig. 1I). In the control the primary shoot elongated and the leaves grew vigorously while none of the axillary shoots developed. At day 28 in the BA medium, further development of the reprogrammed primary shoot apex programmed to develop multiple shoots and

Table 1. Effect of BA concentration on the number of axillary and adventitious shoot buds regenerated from shoot apex of four kenaf cultivars

BA (μM)	Number of shoot buds generated ¹⁾			
	T2	E41	Th3	SF459
0.0	0.00	0.00	0.00	0.00
2.2	3.62 ^{ab}	3.72 ^{bc}	3.55 ^{bcd}	3.03 ^{ab}
4.4	3.56 ^{ab}	4.00 ^{bcd}	5.80 ^e	4.44 ^{bc}
8.8	11.00 ^d	4.18 ^{cd}	9.40 ^f	4.62 ^{bcd}
17.6	4.22 ^{bc}	4.10 ^{bcd}	4.16 ^{cd}	4.02 ^b

¹⁾ Each value is the mean of 3 trials, each with 30 replicates, collected 8 weeks after inoculation.

Means within a column followed by different letters are significantly different from each other at 5% level by Duncan's Multiple Range Test (DMRT).

the development of axillary shoots were observed (Fig. 1K) and adventitious shoots developed from the basal parts of the explant (Fig. 1L and Fig. 1M). In the control cultures at day 28 normal growth with vigorous leaf growth was observed, but development of axillary shoots was not (Fig. 1J).

Scanning electron microscopy

Differences between the control and BA-treated plants were observed at day 7. The control apices developed normally but the apices treated with BA enlarged dramatically and showed reduced growth of trichomes (Fig. 2B). At day 14, no further differences were observed between the control and the

Fig. 1. Light microscopy of the shoot apical meristem of kenaf (cultivar T2) treated with 8.8 μ M BA and control as seen in longitudinal sections. **A.** 7-day-old seedling prior to culture (day 0). **B.** Shoot tip cultured on BA for 7 days (day 7); the boxed region is magnified in C, showing the emergence of two meristematic regions; meristematic nodule pointed by the arrow is magnified in D. **E.** Shoot tip from the control after a 7 day culture (day 7), arrow points to the first axillary bud meristem. **F.** Control shoot tip at day 14, Thin arrow points to the first axillary bud meristem and the thick arrow the second axillary bud meristem). **G.** Shoot tip isolated from BA medium at day 14; arrows point to two meristematic loci on the primary shoot tip.

BA-treated cultures except for the enlargement of the BA-treated apices. In the BA culture, at day 21, nodular structures appeared adjacent to the axillary buds (Fig. 2C) and on the basal regions of the explant (Fig. 2D). In the control cultures at day 21, prolific growth of leaves and trichomes was observed (data not shown). At day 28 in BA-treated cultures, development of nodular structures into adventitious shoot buds was observed (Fig. 2E). The control cultures showed normal elongation of the shoot and leaf development (Fig. 2F).

Discussion

In the presence of BA, several axillary and adventitious shoots developed in kenaf but such shoots were not observed in the untreated control. This result confirms the results of our previous study on multiple shoot regeneration in kenaf (Herath *et al.* 2004). This indicates that in normal kenaf plants apical dominance prevents the axillary shoot development but the apical dominance is eliminated by the treatment

Fig. 1. (continued)

H. Shoot tip from BA medium at day 21; boxed region is magnified in I to show the emergence of an adventitious bud meristem from the side of the axillary shoot **J.** Shoot tip from the control at day 28. **K.** Shoot tip cultured in BA at day 28. **L, M.** Emergence of adventitious buds (L) and further development of meristematic regions from the basal part of the explant (M) at day 28 on BA medium. Bar in all figures = 50 μ m. ax-axillary shoot, cp- cotyledonary petioles, lf- leaf, ps-primary shoot meristem.

Fig. 2. Scanning electron micrographs of control and BA treated shoot apices of kenaf. **A.** 7-day-old seedling (day 0) prior to culture $\times 40$. **B.** Shoot apex cultured on BA for 7 days $\times 50$. **C, D.** Shoot apex cultured on BA for 21 days $\times 40$; arrows point to the emerging meristematic nodules. **E.** Shoot apex cultured on BA for 28 days $\times 40$, arrows point to adventitious shoots developed on the basal region of the explant. **F.** Control shoot apex cultured for 28 days $\times 40$. Bar = 100 μm in all figures. ax- axillary bud, br- basal region of the explant, cp- cotyledonary petioles, lf- leaf, ps- primary shoot.

with cytokinin. In the plants treated with BA, in addition, several meristematic regions developed from the adaxial side of the cotyledonary petiole residue, and from the abaxial side of the basal parts of the leaf primordium of the primary shoot apex. Furthermore, the present study showed that these regions developed by the cell division of the epidermal cells. This fact indicates that BA was able to re-determine the developmental fate of many cells in the explant, resulting in inducing shoots from undifferentiated cells. Similar observations were reported in *Vigna radiata* (Mendoza *et al.* 1993) and *Pisum sativum* (Jackson and Hobbs 1990). In these plants the shoots differentiated directly from epidermal and

sub-epidermal cells without callus formation. Our results showed that the treatment with BA reprogrammed the development of the shoot apex resulting in emergence of multiple meristematic loci. However, the maximum number of shoots observed per plant was 11 (Table 1). This indicates that some of the meristematic regions did not develop into shoots. According to our previous study, excision of the elongating shoots stimulated the growth of the dormant shoots showing that all of these shoots are able to develop into normal plants.

In conclusion, this study showed that exposure of shoot tips to BA induced the growth of both axillary and

adventitious shoots in kenaf. Also, it indicated the precise origin of these shoots. This knowledge would be important for improving the *Agrobacterium*-mediated genetic transformation method for kenaf, since it would facilitate the wounding of the explant at the locations suitable for axillary and adventitious shoot development and thereby reducing the production of chimeras. To our understanding this is the first detailed report on light and scanning electron microscopic analysis of BA-induced multiple shoot regeneration in kenaf.

Acknowledgement

The postgraduate scholarship from the Japanese Ministry of Education, Science, Sports and Culture (Monbusho) to S P Herath is gratefully acknowledged.

Literature Cited

- Buising, C.M., R.C. Shoemaker and R.M. Benbow (1994) Early events of multiple bud formation and shoot development in soybean embryonic axes treated with the cytokinin 6-benzylaminopurine. *Am. J. Bot.* 81: 1435–1448.
- Cheng, T.-Y., H. Saka and T.H. Voqui-Dinh (1980) Plant regeneration from soybean cotyledonary node segments in culture. *Plant Sci. Lett.* 19: 91–99.
- Flick, C.E., D.A. Evans and W.R. Sharp (1983) Organogenesis. In "Handbook of Plant Culture" Evans, D.A., W.R. Sharp, P.V. Ammirato and Y. Yamada (eds.), Macmillan, New York, NY. p. 13–81.
- Herath, S.P., T. Suzuki and K. Hattori (2004) Multiple shoot regeneration from young shoots of kenaf (*Hibiscus cannabinus* L.). *Plant Cell Tissue Organ Cult.* 77: 49–53.
- Jackson, J.A. and S.L.A. Hobbs (1990) Rapid multiple shoot production from cotyledonary node explants of pea (*Pisum sativum* L.). *In vitro Cell Dev. Biol.* 26: 835–838.
- Mendoza, A.B., K. Hattori, T. Nishimura and Y. Futsuhara (1993) Histological and scanning electron microscopic observations on plant regeneration in mung bean cotyledon (*Vigna radiata* L. Wilczek) cultured *in vitro*. *Plant Cell Tissue Organ Cult.* 32: 137–143.
- Mok, D.W. and M.C. Mok (2001) Cytokinin metabolism and action. *Annual Rev. Plant Physiol. Plant Mol. Biol.* 52: 89–118.
- Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497.
- Srivatanakul, M., S.H. Park, J.R. Sanders, M.G. Salas and R.H. Smith (2000) Multiple shoot regeneration of kenaf (*Hibiscus cannabinus* L.) from a shoot apex culture system. *Plant Cell Rep.* 19: 1165–1170.
- Srivatanakul, M., S.H. Park, M.G. Salas and R.H. Smith (2001) Transformation parameters enhancing T-DNA expression in kenaf (*Hibiscus cannabinus*) J. *Plant Physiol.* 158: 255–260.
- Thomas, T.H. and D. Blakesley (1987) Practical and potential uses of cytokinins in agriculture and horticulture. *Brit. Plant Growth Regul. Group Mono.* 14: 69–83.
- Zapata, C., M. Srivatanakul, S.H. Park, B.M. Lee, M.G. Salas and R.H. Smith (1999) Improvements in shoot apex regenerations of two fiber crops: Cotton and kenaf. *Plant Cell Tissue Organ Cult.* 56: 185–191.