

Effect of Absciscic Acid on Shoot Regeneration from Rice (*Oryza sativa* L.) Callus

Xin-Hui XING, Min HUANG, Naohiro SHIRAGAMI and Hajime UNNO*

*Department of Bioengineering, Faculty of Bioscience and Biotechnology,
Tokyo Institute of Technology,*

4259

Nagatsuta-cho, Midori-ku, Yokohama 227, Japan

(Received June 25, 1994)

(Accepted March 18, 1995)

The effect of abscisic acid (ABA) on shoot regeneration from rice callus was examined in a two-stage culture. ABA in regeneration and preculture media at the concentrations of 1, 10 and 100 mg/l inhibited the regeneration of precultured callus presumably by countering the effect of auxins and cytokinins in the media. However, ABA in 2, 4-dichlorophenoxyacetic acid (2, 4-D) free preculture medium at the concentrations of 1 and 10 mg/l enhanced the callus growth as observed in the medium containing 2, 4-D, and showed no inhibition on the shoot regeneration. These results show that ABA in the preculture stage can substitute 2, 4-D for the callus growth.

Introduction

In order to produce rice plantlets from calli a two-stage culture technique has been developed, wherein the calli are grown in a preculture medium and then transferred to a regeneration medium^{1,2)}. In the rice callus culture auxins and cytokinins are indispensable to callus growth and plantlet regeneration²⁻⁴⁾. Among auxins 2, 4-D is the most effective for the callus growth. However, for the practical scale production of clone rice plantlets, it is preferable to refrain from using 2, 4-D for public acceptance since it is a well-known herbicide which is harmful to human health. Therefore, a substitute for 2, 4-D needs to be sought.

ABA is considered an inhibitory phytohormone for plant growth by counteracting the effects of auxins and cytokinins⁵⁾. ABA is also known as an important hormone for seed germination and acquisition of resistance of plants to freezing, salt and water stress^{5,6)}. Somatic embryos treated by ABA showed desiccation tolerance⁷⁾. Callus treated with ABA was hardened and acquired freezing resistance⁸⁾. On the other hand, ABA added to the auxin free medium enhanced normal somatic embryo formation from cultured cells of caraway⁹⁾. Gene expression for an embryogenic cell protein, ECP31, in somatic embryos of carrot was improved in the presence of ABA in the auxin free medium¹⁰⁾. However, little is known about the role of ABA in the callus culture.

In the present research, in order to make clear whether ABA can be a substitute for 2, 4-D in rice callus culture, the effects of ABA on the rice callus growth and shoot regeneration processes were examined.

Materials and Methods

Callus was induced from rice (*Oryza sativa* L.) seed on a solid N6 medium¹¹⁾ containing 4% sucrose,

* To whom correspondence should be addressed.

12 mM proline, 100 mg/l casamino acid, 5 mg/l MES (2-(*N*-morpholino)ethanesulfonic acid), 4 mg/l 2, 4-D and 0.4% gelrite (pH = 5.8). Induced callus was inoculated in a 500 ml baffled flask containing 100 ml liquid N6 medium¹¹ with inoculum size of 5 g-fresh weight (F. W.)/l. The liquid medium contained 3% sorbitol, 1% sucrose and 4 mg/l 2, 4-D with the same components as the above solid N6 medium. The subculture was performed at 27°C on a reciprocal shaker (80 rpm) for 4 weeks changing the medium every week.

The calli obtained after 4 weeks subculture were inoculated into a 100 ml baffled flask containing 20 ml liquid N6 medium as a preculture medium (PM) which contained 4 mg/l 2, 4-D. In order to obtain a callus growth profile in PM, calli were precultured in 8 flasks containing the 20 ml medium for predetermined periods of 2, 3, 4, 5, 7, 9, 12 and 14 days for each flask. The effect of ABA in PM on the callus growth and shoot regeneration was examined with the additions of ABA to PM and 2, 4-D free PM (abbreviated as PM+ABA and PM-2, 4-D+ABA, respectively) at the concentrations of 1, 10 and/or 100 mg/l. As a control callus growth in 2, 4-D free PM (PM-2, 4-D) was also measured. The growth of callus was evaluated by measuring the F. W. after the preculture.

The precultured calli were placed on the petri plates containing solid MS regeneration medium¹¹ (RM) with 3% sucrose, 3% sorbitol, 2000 mg/l cosamino acid, 5 mg/l MES, 2 mg/l naphthaleneacetic acid (NAA), 1 mg/l kinetin (KIN) and 0.4% gelrite (pH=5.8) for shoot regeneration. In order to examine the effect of ABA in RM on shoot regeneration from the calli, RM was prepared with the addition of ABA (RM+ABA) at the concentrations of 1, 10 and 100 mg/l.

Shoot regeneration from the calli was carried out using 4 petri plates containing solid MS medium with 16 calli each plate, and the regeneration process was evaluated by using regeneration frequency which was defined by the number ratio of shoot-regenerated calli to the total calli. Standard deviation of the regeneration frequency was calculated from the results obtained in the 4 petri plates.

Results and Discussion

Fig. 1 shows a typical growth profile of the calli precultured in PM. The calli grew exponentially and reached the stationary phase after about 9 days. As shown in **Fig. 1** the data were scarcely scattered even though the calli precultured in different flasks, which had been inoculated at the same time, were sampled at the respective preculture period for measuring F. W. **Table 1** shows the comparison of F. W. of the calli precultured for 8 days in PM, PM-2, 4-D+ABA and PM-2, 4-D. The F. W. of the calli precultured in PM as plotted in **Fig. 3** shows good coincidence with the growth profile. The F. W. s of the calli cultured with ABA addition of 1 and 10 mg/l were the same as that in PM, while they were more than three times of F. W. in PM-2, 4-D. This result showed a strong dependency of the callus growth on both 2, 4-D and ABA. The calli precultured for 8 days in PM were in the later exponential growth phase (**Fig. 1**). The result shown in **Table 1** implies that ABA in 2, 4-D free PM promoted the callus growth just as 2, 4-D in PM did.

Fig. 2 shows the effect of ABA added to the RM on the shoot regeneration process of rice callus precultured in PM for one week. The shoot regeneration from the calli in RM was faster and its frequency was higher (above 0.9) than those in RM+ABA. In RM with 100 mg/l ABA the shoot regeneration was scarcely observed and the calli were hardened without browning. These results show that the shoot regeneration from the calli was inhibited by ABA in the regeneration medium. It is well known that the growth or elongation of plants induced by auxins/cytokinins is inhibited by ABA^{5,6}. ABA was also reported to counter the effect of cytokinin on organ formation of plant cells¹². These facts suggest that ABA inhibited the shoot regeneration of the callus by counteract-

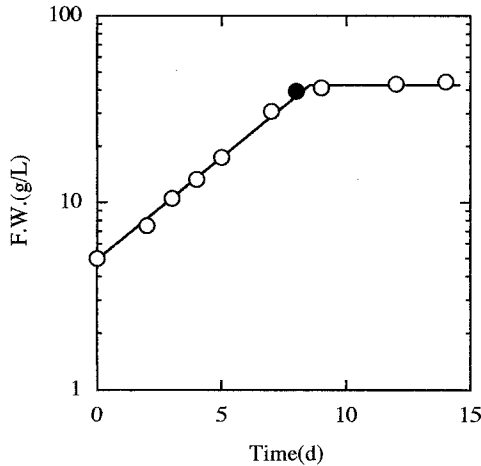


Fig. 1 Growth profile of rice callus precultured in preculture medium containing 2, 4-D.
○: F. W. of the calli precultured in the flasks with different culture period,
●: F. W. of the calli precultured in PM as listed in **Table 1**.

Table 1. Comparison of callus fresh weights cultured in PM, PM-2, 4-D+ABA and PM-2, 4-D for 8 days.

Medium	Callus concentration
	(g-F. W./l)
PM	39.6
PM-2, 4-D+1 mg/l ABA	38.8
PM-2, 4-D+10 mg/l ABA	37.6
PM-2, 4-D	12.5

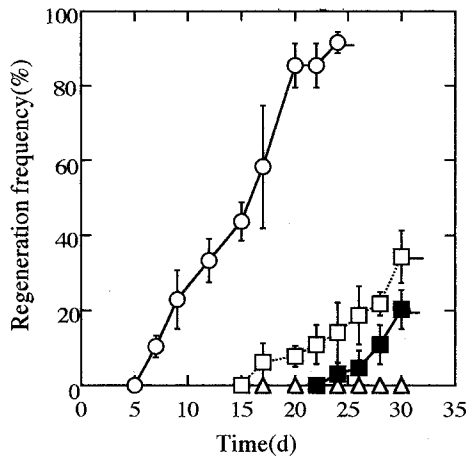


Fig. 2 Effect of ABA in regeneration medium on the shoot regeneration process of rice callus precultured for 1 week.
○: RM, □: RM+ABA (1 mg/l), ■: RM+ABA (10 mg/l), △: RM+ABA (100 mg/l).
Bars: standard deviations ($n=4$).

ing the actions of auxin(NAA)and cytokinin(KIN)in the regeneration medium. In the following experiments the calli precultured in the preculture medium containing ABA were used for shoot regeneration in RM.

Fig. 3 shows the effect of ABA in PM containing 2, 4-D on shoot regeneration process from the callus. The calli were precultured in PM and PM containing ABA for one week. Compared with the calli precultured in PM, the shoot regeneration of the calli precultured in PM+ABA was

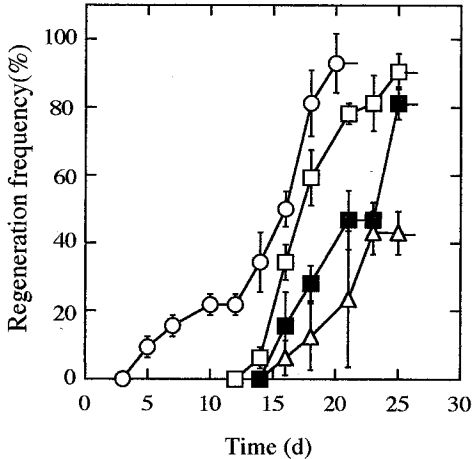


Fig. 3 Effect of ABA in preculture medium containing 2, 4-D on the shoot regeneration process of rice callus precultured for 1 week.
○: PM, □: PM+ABA (1 mg/l), ■: PM+ABA (10 mg/l), △: PM+ABA (100 mg/l).
Bars: standard deviations (n=4).

decreased and the degree of reduction was dependent on the ABA concentration. Inoue *et al.*²⁾ showed that ABA (10^{-4} M=26.4 mg/l) in their preculture medium containing 2, 4-D (10^{-5} M=2.2 mg/l) did not affect the shoot and plantlet formation of rice calli precultured for one month. The difference between the results obtained by Inoue *et al.* and the present experiment might be due to the different preculture periods, which were 30 and 7 days, respectively. The authors have found that intracellular 2, 4-D content of the calli precultured in PM for more than 5 days decreased to a very low level which did not affect the shoot regeneration of the callus¹³⁾. From these results, it was considered that the intracellular 2, 4-D and/or ABA in Inoue *et al.*'s experiment of 30 days' preculture was reduced to too low a level to suppress the shoot regeneration, while its repressive effect on the regeneration still remained larger in our experiment after 7 days' preculture.

Fig. 4 shows the shoot regeneration process of the callus precultured for 8 days in PM and PM-2, 4-D+ABA. The precultured calli with the additions of ABA of 1 and 10 mg/l regenerated shoots similarly to that in PM. The same tendency was observed for the calli of the stationary phase

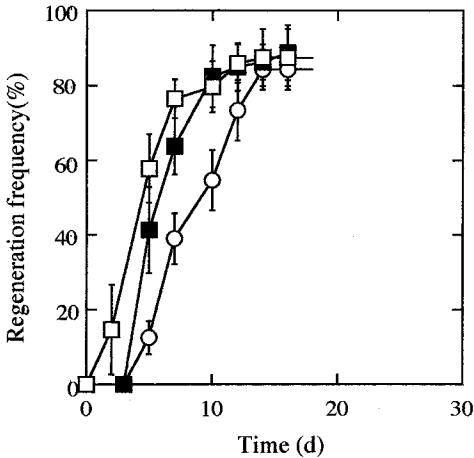


Fig. 4 Effect of ABA in 2, 4-D free preculture medium on the shoot regeneration process of rice callus precultured for 8 days.
○: PM, □: PM-2, 4-D+ABA (1 mg/l), ■: PM-2, 4-D+ABA (10 mg/l).
Bars: standard deviations (n=4).

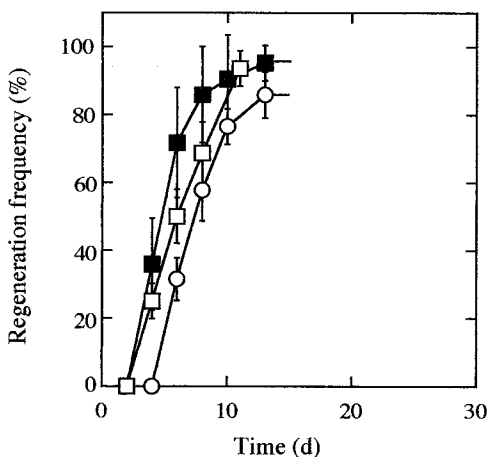


Fig. 5 Effect of ABA in 2, 4-D free preculture medium on the shoot regeneration process of rice callus precultured for 14 days.
 ○: PM, □: PM-2, 4-D+ABA (1 mg/l), ■: PM-2, 4-D+ABA (10 mg/l).
 Bars: standard deviations ($n=4$).

precultured for 14 days (**Fig. 5**). Although the variation of intracellular ABA of the calli during preculture has not been measured yet, from the results shown in **Fig. 2** and **3** it is suggested that a high intracellular ABA level would inhibit the shoot regeneration of the callus in RM. Based on the result shown in **Fig. 4**, it was considered that intracellular ABA of the calli precultured for 8 days in PM-2, 4-D+ABA was too low to inhibit shoot regeneration.

From the present research together with the results reported so far^{2,9,10}, it can be concluded that the utilization of ABA in the absence of auxins and cytokinins in the preculture medium enhances callus growth and shows no repressive effect on its shoot regeneration.

In most research of callus culture reported so far, 2, 4-D has been used as an indispensable hormone for callus growth. However, according to the present research, ABA, which is found widely in plants, could be an alternative to 2, 4-D. The calli used in this research were induced by 2, 4-D. Therefore, the next necessary step is to establish an appropriate callus induction method without using 2, 4-D. This research is now under way together with the analysis of intracellular ABA content in the preculture.

Acknowledgments

The authors thank Nursery Technology Inc. for kindly donating the rice seeds and advising us on medium preparation.

References

- 1) Hirosawa, T., 1992. In "Transplant Production Systems" (eds. by Kurata, K., T. Kozai), p. 195-212, Kluwer Academic Publishers.
- 2) Inoue, M., E. Maeda, 1981. Japan J. Crop Sci., **50**: 318-322.
- 3) Maeda, E., 1968. Proc. Crop Sci. Soc. Japan, **43**: 151-160.
- 4) Saka, H., E. Maeda, 1969. Proc. Crop Sci. Soc. Japan, **38**: 668-674.
- 5) Davies, W. J., J. Zhang, 1991. Annu. Rev. Plant Physiol. Plant Mol. Biol., **42**: 55-76.
- 6) Radin, J. W., 1991. In "Plant Physiology" (eds. by Taiz L., E. Zeiger), p. 482-487, The Benjamin/Cummings Publishing Company, Inc.
- 7) Iida, Y., K. Watanabe, H. Kamada, H. Harada, 1992. J. Plant Physiol., **40**: 356-360.

- 8) Chen, T. H., V. G. Lawrence, 1983. *Plant Physiol.*, **73**: 71-75.
 - 9) Ammirato, P. V., 1977. *Plant Physiol.*, **59**: 579-586.
 - 10) Kiyosue, T., K. Yamaguti, K. Shinozaki, K. Higashi, S. Satoh, H. Kamada, H. Harada, 1992. *Plant Mol. Biology*, **19**: 239-249.
 - 11) Matsuno, T., K. Ishizaki, M. E. Horn, 1990. *J. Breeding (in Japanese)*, **40**: 266-267.
 - 12) Yamaguti, T., T. Nakajima, 1974. In "Plant growth Substances", p. 1121-1127, Hirokawa publishing Co. Ltd. Tokyo.
 - 13) Mitsuoka, K., H. Honda, X. -H. Xing, H. Unno, 1994. *Appl. Microbiol. Biotechnol.*, **42**: 364-366.
-

《和文要約》

イネカルのシュート再生に及ぼすアブシジン酸の影響

邢 新会・黄 敏・白神直弘・海野 肇

東京工業大学生命理工学部生物工学科

イネカルの二段階培養におけるアブシジン酸(ABA)のカルスからのシュート再生への影響について調べた。カルの再生培地及び前培養培地に1, 10, 100 mg/lの濃度で添加したABAは培地中のオーキシニンやサイトカイニンとの拮抗作用によってカルのシュート再生を阻害した。しかし、2, 4-ジクロロフェノキシ酢酸(2, 4-D)無添加の前培養培地に1, 10 mg/lの濃度で添加したABAはカルの増殖に対して2, 4-Dと同程度の効果が認められ、その後のカルのシュート再生を阻害しなかった。これらの結果から、イネカルの前培養段階においてABAは2, 4-Dの代替として用いることが可能であることを明らかにした。