園学雑. (J. Japan. Soc. Hort. Sci.) 74 (1) : 60-62. 2005.

SHORT COMMUNICATION

Comparison of Selection Efficiency between the *bar* and *hpt* Genes in *Agrobacterium*-mediated Transformation of *Muscari armeniacum*

Sakae Suzuki[1]*, Masaru Nakano[2], Yosuke Koike[2], Kenji Ueda[3], Masayasu Inoue[3], Masahiro Nishihara[1] and Saburo Yamamura[1]

[1]*Iwate Biotechnology Research Center, Narita, Kitakami 024-0003*

[2]*Faculty of Agriculture, Niigata University, Ikarashi, Niigata 950-2181*

[3]*Faculty of Bioresource Sciences, Akita Prefectural University, Akita 010-0195*

Summary

Embryogenic calli of *Muscari armeniacum* cv. Blue Pearl were co-cultivated with *Agrobacterium tumefaciens* EHA101/pBH, which harbored a binary vector that carries the phosphinothricin acetyltransferase (*bar*) and hygromycin phosphotransferase (*hpt*) genes. The calli were then transferred onto the selection media containing either 4 mg·liter^{-1} bialaphos or 75 mg·liter^{-1} hygromycin. Four to five weeks after transfer to the selection media, both bialaphos-resistant (Bia^r) and hygromycin-resistance (Hyg^r) cell clusters were produced. Over 90% of callus lines selected on a bialaphos-containing medium were verified to be transgenic by PCR analysis; this selection efficiency is comparable to that based on the *hpt* gene. Leaf segments of plantlets regenerated from the transgenic Bia^r callus lines showed resistance to 4 mg·liter^{-1} of bialaphos, indicating that the *bar* gene is useful not only as a selectable marker but also as a producer of herbicide-resistant transgenic plants of *M. armeniacum*.

Key Words: *Agrobacterium tumefaciens*, *bar* gene, herbicide-resistant transgenic plants, *hpt* gene, *Muscari armeniacum*.

Introduction

The phosphinothricin acetyltransferase (*bar*) gene, which confers resistance to bialaphos and phosphinothricin, has been used for the successful production of transgenic herbicide-resistance plants in many crop species (Rathore et al., 1993). This gene has also been used in combination with bialaphos or phosphinothricin for selecting transformants in genetic transformation systems of several monocot species such as, *Oryza sativa* (Rathore et al., 1993), *Hordeum vulgare* (Wan and Lemaux, 1994), *Gladiolus* sp. (Kamo et al., 1995) and *Phalaenopsis* sp. (Anzai et al., 1996). However, few comparative studies on the effectiveness of the *bar* gene as a selectable marker have been reported so. Recently, we developed an *Agrobacterium*-mediated genetic transformation system in the Liliaceous monocot *Muscari armeniacum* by using the hygromycin phosphotransferase (*hpt*) gene as a selectable marker (Suzuki and Nakano, 2002). In this study, we compared the selection efficiency between the *bar* and *hpt* genes in the transformation system of *M. armeniacum* and produced herbicide-resistant transgenic plants of this species.

Received; March 16, 2004. Accepted; June 11, 2004.

* Corresponding author.

* Present address: Tokyo University of Agriculture and Technology, Fuchu 183-8509

Materials and Methods

Leaf-derived embryogenic calli of *M. armeniacum* cv. Blue Pearl (Suzuki and Nakano, 2001) were used for inoculation and co-cultivation with *Agrobacterium tumefaciens* strain EHA101/pBH. The T-DNA region of the binary vector pBH contains the *bar* and *hpt* genes, both under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. *Agrobacterium* inoculation, co-cultivation and selection of putative transgenic calli were performed according to our previous report (Suzuki and Nakano, 2002), in which putative transformants were selected by using 75 mg·liter^{-1} hygromycin (hygromycin B; Wako). In this study, 4 mg·liter^{-1} bialaphos (Meiji Seika Ltd.) was also used as a selective agent in addition to 75 mg·liter^{-1} hygromycin.

PCR analysis for detecting the *bar* and *hpt* genes was performed according to Anzai et al. (1996) and Suzuki and Nakano (2002), respectively. Southern analysis using the *bar* gene as a probe was carried out according to Suzuki and Nakano (2002). For Northern blot analysis, about 5 μg of total RNA was separated by agarose gel electrophoresis and blotted to Hybond-N$^+$ nylon membrane (Amersham). Labeling of the *bar* gene, hybridization, washing and detection were performed according to the instruction manual of DIG DNA Labeling Kit (Roche) and DIG Luminescent Detection Kit (Roche). For testing bialaphos resistance in trans-

genic plants, leaves were cut into segments of 20-30 mm in length, placed on half-strength MS medium (Murashige and Skoog, 1962) containing 4 mg・liter^{-1} bialaphos, 15 g・liter^{-1} sucrose and 2 g・liter^{-1} gellan gum, and incubated at 25℃ under continuous illumination with white fluorescent light (30 μmol・m^{-2}・s^{-1}).

Results and Discussion

We have previously reported an *Agrobacterium*-mediated transformation system in *M. armeniacum*, in which transgenic calli were efficiently selected by the *hpt* gene in combination with 75 mg・liter^{-1} hygromycin (Suzuki and Nakano, 2002). In this study, embryogenic calli of *M. armeniacum*, which had been co-cultivated with an *A. tumefaciens* strain harboring both *bar* and *hpt* genes, were cultured on selection media that contain either 4 mg・liter^{-1} bialaphos or 75 mg・liter^{-1} hygromycin to examine the effectiveness of the *bar* gene as a selectable marker. Our previous study showed that 4 mg・liter^{-1} bialaphos was sufficient to inhibit the growth of the non-co-cultivated embryogenic calli of *M. armeniacum* (Suzuki and Nakano, 2003). When co-cultivated calli were transferred after 4-5 weeks of culture onto the selection media containing bialaphos or hygromycin, bialaphos-resistant (Bia^r) or hygromycin-resistant (Hyg^r) cell clusters, which were white to light-yellow in color, started to develop among unaltered, dead calli. An average of 41.6 Bia^r and 38.5 Hyg^r callus lines per 5 g fresh weight of co-cultivated calli were obtained 7 weeks after transfer (Table 1). The resistant callus lines were selected and subjected to PCR analysis for the detection of the *bar* or *hpt* gene; 92.3% of Bia^r callus lines and 91.7% of Hyg^r callus lines were verified to be transgenic (Table 1). No significant differences (1% level with *t* test) in the number of selected callus lines and the selection efficiency were observed between the *bar* and *hpt* genes, indicating that either gene is equally efficient for selection of transformants of *M. armeniacum*. Although the effect of selective agents, such as antibiotics and herbicides, has been examined prior to the development of transformation systems in many plant species (Dekeyser et al., 1989), only a few studies have been reported on the effect of selectable marker genes on the selection efficiency of transformants (Dekeyser et al., 1989; Hauptmann et al., 1988). In the present study, we showed the effectiveness of the *bar* gene in combination with bialaphos as a selectable marker in the genetic transformation of *M. armeniacum* by comparing the transformation efficiency between the *bar* and *hpt* genes.

Some of the Bia^r embryogenic callus lines, which had been verified to be transgenic by PCR analysis, were transferred onto a medium that lacks plant growth regulators (PGRs) but contains 3 mg・liter^{-1} bialaphos and 500 mg・liter^{-1} cefotaxime. Our previous study indicated that this level of bialaphos was sufficient to inhibit the formation of somatic embryo from non-co-cultivated embryogenic calli of *M. armeniacum* cv. Blue Pearl (Suzuki and Nakano, 2003). A couple of weeks after transfer, almost all of the callus lines developed numerous somatic embryos. When these elongated embryos were transferred onto a medium without both PGRs and antibiotics, over 80% of them developed into plantlets after 5-6 weeks. The efficiency of the formation of somatic embryos and their conversion into plantlets in the selection system using the *bar* gene in combination with bialaphos, was comparable with those in the previously reported selection system using the *hpt* gene in combination with hygromycin (Suzuki and Nakano, 2003). The *bar* gene was detected by PCR analysis in the regenerated plantlets. The Southern blot analysis of several plantlets positive for PCR analysis also indicated the presence of the *bar* gene in the genome of all plantlets analyzed. The copy number of the *bar* gene varied from 1 to 3. To examine the bar gene expression, 4 independent transgenic plants positive for Southern blot analysis and the control non-transgenic plantlets were subjected to Northern blot analysis. The *bar* gene was expressed in leaves of all the transgenic plantlets with different levels, but no signal was detected in the control. Leaf segments of transgenic plantlets positive for Northern blot analysis were then tested for bialaphos resistance. In the control plantlets, all the leaf segments that were placed on a medium containing 4 mg・liter^{-1} bialaphos turned white to yellow within 2 weeks and subsequently died with a week. In contrast, all the leaf segments of 4 independent transgenic plants remained green and healthy even after

Table 1. Comparison of the selection efficiency of transgenic calli between the *bar* and *hpt* genes in genetic transformation of *Muscari armeniacum* cv. Blue Pearl by *Agrobacterium tumefaciens* strain EHA101/pBH.[z]

Selectable gene (selective agent)	Number of callus lines selected for Bia^r or Hyg^r (A)[y]	Number of transgenic callus lines confirmed by PCR (B)[x]	Selection efficiency (B/A) (%)
Bar (bialaphos)	41.6 ± 2.9	38.4 ± 3.3	92.3
Hpt (hygromycin)	38.5 ± 3.1	35.3 ± 2.8	91.7

[z] Values represent the mean ± SE of 5 independent co-cultivation experiments, each using 5 g fresh weight of embryogenic calli.
[y] Data were recorded 7 weeks after transfer of co-cultivated calli to selection media containing 4 mg・liter^{-1} bialaphos or 75 mg・liter^{-1} hygromycin.
[x] PCR analysis for the *bar* or *hpt* gene.

4 weeks on this medium. For both control and transgenic plantlets, leaf segments remained green for more than 4 weeks on a bialaphos-free medium. These results indicate that the regenerated transgenic plantlets for the *bar* gene are resistant to 4 mg・liter^{-1} bialaphos, which is lethal to the control plantlets.

In conclusion, the *bar* gene in combination with bialaphos is useful as a selectable maker in genetic transformation of *M. armeniacum*. Furthermore, this gene is also useful for the production of transgenic herbicide-resistant plants in *M. armeniacum*. Detailed characterization of transgenic plants obtained in this study with respect to herbicide-resistance as well as morphological alteration is now in progress.

Literature Cited

Anzai, H., Y. Ishii, M. Shichinohe, K. Katsumata, C. Nojiri, H. Morikawa and M. Tanaka. 1996. Transformation of phalaenopsis by particle bombardment. Plant Tiss. Cul. Lett. 13: 265-272.

Dekeyser, R., B. Claes, M. Marichal, M. Van Montagu and A. Caplan. 1989. Evaluation of selectable markers for rice transformation. Plant Physiol. 90: 217-223.

Hauptmann, R. M., V. Vasil, P. Ozias-Akins, Z. Tabaeizadeh, S. G. Rogers, R. T. Fraley, R. B. Horsch and I. K. Vasil. 1988. Evaluation of selectable markers for obtaining stable transformants in the Gramineae. Plant Physiol. 86: 602-606.

Kamo, K., A. Blowers, F. Smith, E. J .Van and R. Lawson. 1995. Stable transformation of *Gladiolus* using suspension cells and callus. J. Amer. Soc. Hort. Sci. 120: 347-352.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Rathore, K. S., V. K. Chowdhury and T. K. Hodges. 1993. Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. Plant Mol. Biol. 21: 871-884.

Suzuki, S. and M. Nakano. 2001. Organogenesis and somatic embryogenesis from callus cultures in *Muscari armeniacum* Leichtl. ex Bak. In Vitro Cell Dev. Biol. Plant. 37: 382-387.

Suzuki, S. and M. Nakano. 2002. *Agrobacterium*-mediated production of transgenic plants of *Muscari armeniacum* Leichtl. ex Bak. Plant Cell Rep. 20: 835-841.

Suzuki, S. and M. Nakano, 2003. Effect of antibiotics and bialaphos on the growth and development of embryogenic callus cultures of *Muscari armeniacum*. Biol. Plant. 47: 425-427.

Wan, Y. and P. G. Lemaux. 1994. Generation of large number of independently transformed fertile barley plants. Plant Physiol. 104: 37-48.

アグロバクテリウム法によるムスカリの形質転換における*bar*遺伝子と*hpt*遺伝子の選抜効率の比較

鈴木　栄[1]*・中野　優[2]・小池洋介[2]・上田健治[3]・井上正保[3]・西原昌宏[1]・山村三郎[1]

[1]岩手生物工学研究センター　024-0003　北上市成田

[2]新潟大学農学部　950-2181　新潟市五十嵐2の町

[3]秋田県立大学生物資源科学部　010-0195

秋田市下新城中野

摘　要

アグロバクテリウム法によるムスカリ(*Muscari armeniacum* cv. Blue pearl)の形質転換における選抜マーカー遺伝子*bar*の有効性を調査した．葉片由来のエンブリオジェニックカルスを*A. tumefaciens* EHA101 / pBH (ビアラホス耐性遺伝子*bar*およびハイグロマイシン耐性遺伝子*hpt*がT-DNA上にコードされている)との共存培養後，4 mg・liter^{-1}ビアラホスまたは75 mg・liter^{-1}ハイグロマイシンを含む選抜培地に移植した．選抜開始4~5週間後，ビアラホス耐性またはハイグロマイシン耐性カルスがそれぞれの選抜培地上で形成された．PCR分析の結果，*bar*遺伝子を用いた場合の形質転換効率は90%以上となり，この効率は*hpt*遺伝子を用いた場合の選抜効率と同等であった．また，ビアラホス耐性形質転換カルスから再生した植物体の葉片は4 mg・liter^{-1}ビアラホスに耐性を示した．これらの結果より，アグロバクテリウム法によるムスカリの形質転換において，*bar*遺伝子は有効な選抜マーカーとして利用できるだけでなく，除草剤耐性形質転換体の作出にも利用可能なことが明らかとなった．

*現在：東京農工大学，府中市．