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Molecular breeding of herbicide resistant transgenic plants with bromoxynil specific nitrilase gene

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Abstract : Bromoxynil is an antidicot herbicide widely used on cereal crops and has a short half life in the soil. A *bxn* gene, encoding a specific nitrilase that converts bromoxynil to its primary metabolite 3,5-dibromo-4-hydroxybenzoic acid, was inserted in plant binary vector pGA482, and then introduced into tobacco and lettuce plants *via Agrobacterium* mediated leaf-disc transformation method. Transgenic plants with the *bxn* gene were selected by kanamycin and regenerated to whole plants. The regenerated transgenic plants were determined level of expression of *bxn* gene by Northern blot analysis. Leaf-disc analysis and pot-assay confirmed that the transgenic tobacco and lettuce plants were resistant to high doses of bromoxynil (Received June 10, 1994; accepted August 22, 1994).

Introduction

At present, two approaches have been developed for the engineering of herbicide resistance in plants: the modification of a plant enzyme or other sensitive biochemical target of herbicidal action or the overproduction of the unmodified target protein; the introduction of an enzyme or enzyme system to degrade and detoxify the herbicide in the plant prior to its action.[1-6] Resistance obtained by first mechanism has been developed for the herbicide glyphosate,[7-9] atrazine, sulphonylureas[10] and phosphinothricin. A streptomyces gene encoding a phosphinothricin acetyl transferase was transferred to plants and it resulted in phosphinothricin-resistant plants, establishing detoxification. Two advantages of a detoxification- degradation mechanism, as opposed to altering a biochemical target, are that specialized compartment of the detoxifying activity is not required and that greater herbicide resistance can be achieved with lower levels of detoxifying enzymes. Disadvantages include potential toxicity of one or more metabolites of herbicides and the possibility that detoxifying activities might react with endogeneous plant compounds to impair plant function. The herbicide bromoxynil (3,5-dibromo-4-hydroxy benzonitrile) is a photosynthetic (photosystem II) inhibitor in plants. Although the actual biochemical target localized in chloroplast is not well defined, there is evidence that bromoxynil acts by binding to a component of the quinone-binding protein complex of PSII, inhibiting electron transfer. It has been suggested that a low affinity binding site within this complex exists in 32 kD polypeptide. Bromoxynil has a very short half-life in the environment, as microbial populations and tolerant plant species can convert the cyano moiety of bromoxynil to the corresponding amino acid derivatives. A natural soil isolate, *Klebsiella ozaenae*, has been identified that transforms bromoxynil to 3,5-dibromo-4-hydroxybenzoic acid, releasing ammonia.[11] This reaction is carried out via a bromoxynil-specific nit-

Key words : *bxn*, bromoxynil, herbicide resistant transgenic plant
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rilase. A gene, *bxn*, encoding a specific nitrilase that converts bromoxynil to its primary metabolite 3,5-dibromo-4-hydroxybenzoic acid, was cloned from natural soil bacterium, *Klebsiella ozaenae*. The *bxn* gene was linked to plant promoters such as RUBP carboxylase small subunit and CaMV 35S promoters and these chimeric genes were introduced into tobacco and tomato plants. High levels of bromoxynil resistance were displayed (up to 10 fold of field level) by expressing the bromoxynil-specific nitrilase in photosynthetic plant tissues.[12]

In this study, the *bxn* gene of *Klebsiella ozaenae* was introduced to tobacco and lettuce plants and its herbicidal resistance was determined.

Materials and Methods

Construction of plant binary vector

pRPA-BL-429 vector containing *bxn* gene was obtained from *Rhone-Poulene Agro, Biologie Moleculaire et Cellulaire Vegetae*. The *bxn* gene was transferred to pGA482, that was a generous gift from prof. An, G., plant binary vector containing border sequences and virulence genes.[13,14]

Bacterial Culture and Plant tissue culture

E. coli strain was grown in Luria-Bertani (LB) medium. The nutrient medium (YEP) for *Agrobacterium* contains 10 g peptone, 10 g of yeast extract and 5 g of sodium chloride per liter. Plasmid bearing *Agrobacterium tumefaciens* was grown in YEP medium supplemented with 20 μg of kanamycin per m*l*. For the tissue culture of tobacco, Murashige and Skoog (MS) media and modified MS media were used. To develop the shoot and root, the concentration of sucrose and plant growth regulators were modified. The antibiotics such as carbenicillin and kanamycin were added to the medium at the final concentrations of 500 mg/*l* and 200 mg/*l* for the selection of transformants.

Direct *Agrobacterium tumefaciens* transformation

Transformation was performed as described by freeze-thaw method.[15] *Agrobacterium* strain, LBA 4404, containing helper Ti plasmid was grown in 5 m*l* of YEP medium overnight at 28℃. 2 m*l* of overnight culture was inoculated into 50 m*l* YEP medium in 250 m*l* flask, and cultured with vigorous shaking, 250 rpm, at 28℃ until the mass of culture was reached to an A600 of 0.5~1.0. The culture was chilled on ice and centrifuged at 3000 g for 5 mins at 4℃. After the centrifugation, the supernatant was removed and the cells were resuspended in 1 m*l* of 20 mM $CaCl_2$ solution. The $CaCl_2$-shocked cells were combined with 1 μg plasmid DNA in prechilled tubes. The mixture was frozen in liquid nitrogen and then thawed in 37℃ water bath for 5 mins, diluted to fragment. The larger fragment in size 10.2 kb was eluted and ligated with 2.35 kb small fragment from pRPA-BL-429 digestion. This ligant was transferred in *E. coli* HB101 and selected the plasmid containing proper size by antibiotics kanamycin and tetrcycline. The selected plasmid pGB10 contains CaMV 35S promoter, *nos* terminator and *bxn* gene.

Introduction of pGB10 into *Agrobacterium tumefaciens*

Plasmid pGB10 was introduced into *Agrobacterium tumefaciens* LBA4404 containing helper Ti plasmid. The transformation of *Agrobacteriun tumefaciens* was performed according to the freeze-thaw method.[15] *Agrobacterium* transformants were screened by the resistance to kanamycin (20 μg/m*l*) on the YEP agar plate. The plasmid was isolated from the *Agrobacterium* transformants by alkaline lysis method.[15] The gel electrophoresis of the isolated plasmids showed that the correct construction is in the selected plasmid (data not shown). Both the transformed and the untransformed *Agrobacterium tumefaciens* showed a large plasmid band which is the helper Ti- plasmid.

Plant transformation and its regeneration

Tobacco (*Nicotiana tabaccum* Xanthi) and lettuce (*Lactuca sativa*) leaf discs were transformed by cocultivating with *Agrobacterium tumefaciens* contai-

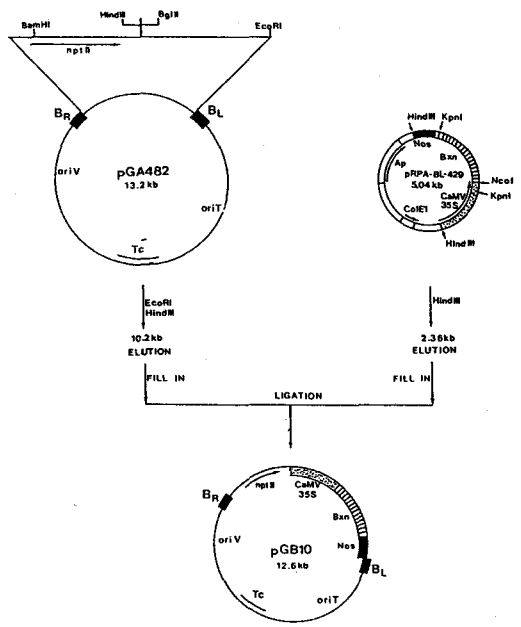

Fig. 1. Construction of the plant binary vector, pGB 10, containing bromoxynil specific nitrilase gene, *bxn*.

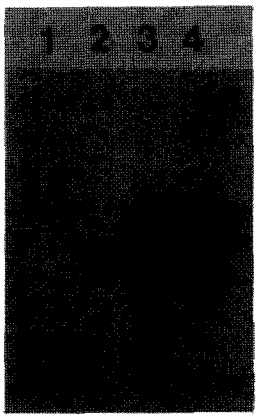

Fig. 2. Northern hybridization analysis for transcript of *bxn* gene in the transformed tobbaco plants (R0). Probe was synthesized from purified *Kpn*I restriction fragment of the plasmid pRPA-BL-429 specific to the *bxn* coding region.
Lane 1: Non-transformed tobacco plant.
Lane 2: Transformed tobacco plant XB102.
Lane 3: Transformed tobacco plant XB110.
Lane 4: Transformed tobacco plant XB111.

ning the plasmid pGB10 and helper Ti plasmid. In he cocultivation of leaf discs and *Agrobacterium*, the bacteria were efficiently killed by carbenicillin treatment, but *Agrobacterium* cells were coaggregated and attached to tobacco leaf discs. During the coculltivation, the culture was carried out in gloomy dark. Transformed tobacco and lettuce were developed from the periphery of the leaf sections on a shoot-inducing MS agar plate containing 200 μg /m*l* kanamycin and 500 μg/m*l* carbenicillin for 4 weeks. Within this period, shoots were developed from kanamycin-resistant green ieaf-disc. The shoots having more than two small leaves were removed from leaf-disc and then transferred root inducing media for 4 weeks. Plants regenerated from the transformed tissues showed resistance to kanamycin and they were subjected to further analysis for herbicide bromoxynil resistance.

Northern hybridization analysis

In order to find transcription efficiency of the *bxn* gene in transgenic tobacco and lettuce, Northern hybridization analysis were performed. For these hybridization experiments, the probe was prepared from the pRPA-BL-429 digested with *Kpn*I and the 1 kb fragment containing only the *bxn* gene was used as the probe. Samples were prepared from the wholely regenerated plants leaves. The results are shown in Fig. 2. Lane 1 is the nontransformed tobacco and lane 2, 3, 4, are the transgenic tobacco plants containing *bxn* gene. Lane 1 showed no signal and lane 2 showed a weak signal but lane 3 and 4 showed very strong signals. Theses results showed that the transformed plants normally transcribed the *bxn* gene from *Klebsiella ozaenae* and the transcriptional efficiency was different from the plant to plant, but in the most two fold with YEP broth and allowed to grow at 28℃ for 2~4 hrs. The tubes were centrifuged for 30 seconds in microfuge, discarded the supernatant and the cells were resuspended in 0.1 m*l* of YEP medium containing the appropriate antibiotics and incubated at 28℃.

Plant transformation and its regeneration

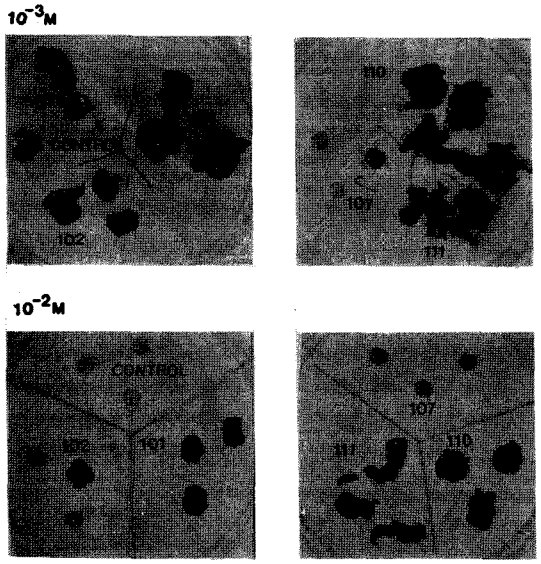

Fig. 3. Leaf-disc analysis of the transgenic tobacco plants (101, 102, 107, 110 and 111). Leaf-disc obtained from transformed plants, placed in the concentrations of bromoxynil indicated. Photographs were 3 weeks after initiation of the experiment.

Transformants of *Nicotiana tabaccum* Xanthi and *Lactuca sativa* L. were obtained by co-cultivating leaf disc with *A. tumefaciens* LBA4404.[16,17] The transformed shoots were selected on selection plates (full strength MS, appropriate hormones, 500 μg/ml carbenicillin and 200 μg/ml kanamycin). After the shoot formation, shoots were transferred to rooting media. Shoots that were rooted in the presence of kanamycin were transferred to fresh rooting media for further analysis.

Northern blotting

The acid guanidium-phenol-chloroform method[18] was used to isolate RNA from tissues. The isolated RNA was dissolved in 50 μl formamide and stored at −20℃.[19] The nick-translation reaction was used to introduce radioactive nucleoside phosphates into unlabelled DNA for the purpose of making a probe. Amersham's nick translation kit was used in this study. Unlabelled DNA to be used in nick-translation was prepared by digestion of the pGB10 plasmid with *Kpn*I. The 10 μg RNA for Northern analysis was seperated in formaldehyde denaturing gel, transferred to nylon membrane by capillary transfer and hybridized to the ^{32}P-labeled *Kpn*I fragment.[20]

Leaf disc analysis for bioassay

To measure the resistance to bromoxynil of the transformant containing *bxn* gene, MS shooting media containing bromoxynil was prepared. Bromoxynil was dissolved in tetrahydrofuran at the concentration of 1M. It was serially diluted and added to the MS shooting media. Leaf discs were placed on the media and their viability was checked after 3 weeks.

Pot analysis

The transformants were transferred to pots and hardened for 1~2 weeks. When they reached about 10 cm in size, they were sprayed at dose of 50 mg~100 mg/m^2 of bromoxynil and their viability was checked after 5~10 days.

Results and Discussion

Construction of plant binary vector

The scheme for the construction of the vector is shown in Fig. 1. pRPA-BL-429 was digested with *Hind*III, and small fragment (2.35 kb) containing *bxn* gene was eluted and then filled in by Klenow fragment. pGA482 was digested with *Eco*RI and *Hind*III and then filled in by Klenow of them their transcriptional levels were very high. In lettuce (Fig. 4), lane 1 is of the non-transformed lettuc and lane 2, 3, 4 are the transgenic lettuce plants containg *bxn* gene. Land 1 and 4 did not show any signal, but lane 2 and 3 showed any signal, but lane 2 and 3 showed a signal.

Leaf disc analysis of bromoxynil resistance in transgenic tobacco and lettuce

To analysis the bromoxynil resistance of transgenic plants *in vitro*, leaf discs were placed on shoot-inducing MS agar plates containing appropriate concentrations of bromoxynil. Since bromoxynil was dissolved in tetrahydrofuran, leaf discs were also

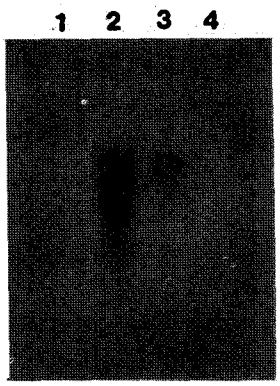

Fig. 4. Northern hybridization analysis for transcript of *bxn* gene in the transformed lettuce plants (R0).
Lane 1: Non-transformed lettuce plant.
Lane 2: Transformed lettuce plant SB102.
Lane 3: Transformed lettuce plant SB110.
Lane 4: Transformed lettuce plant SB111.

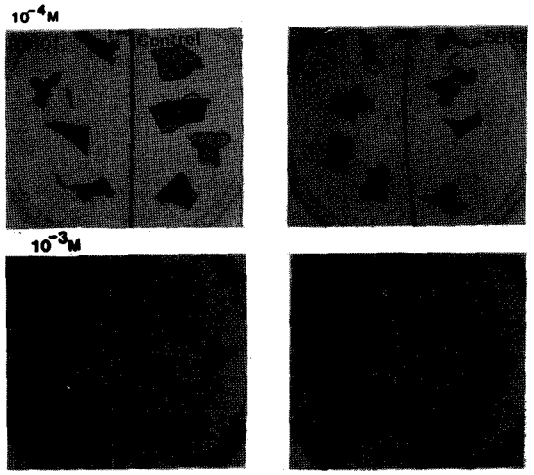

Fig. 5. Leaf-disc analysis of the transgenic lettuce plants (SB101, SB102 and SB103). Leaf-disc obtained from transformed plants, placed in the concentrations of bromoxynil indicated. Photographs were 3 weeks after initiation of the experiment.

placed on the shoot-inducing MS media only containing tetrahydrofuran as control. After three weeks, leaf discs were checked. In 10^{-4} M concentration of bromoxynil, the control plants that had no *bxn* gene showed some growth inhibition, but transformants grew well (data not shown). In 10^{-3} M of bromoxynil, controls showed some necrosis but transformants grew well. In 10^{-2} M of bromoxynil, controls showed no growth and died. The transformants showed a little growth inhibition (Fig. 3), but still showed bromoxynil resistance. Leaf discs on MS media containing only tetrahydrofuran showed no growth inhibition (data not shown). In case of lettuce, control plants that had not *bxn* gene showed some growth inhibition and some necrosis in 10^{-4} M of bromoxynil, but transformed explant grew well. In 10^{-3} M of bromoxynil, the growth of control plants were completely inhibited, but transformed lettuce plants showed only little growth inhibition (Fig. 5). Both control and transgenic plants did not grow in 10^{-2} M of bromoxynil.

Pot assay of bromoxynil resistance in transgenic tobacco and lettuce plants

The transformants were transferred to pots and hardened for 1~2 weeks. When they reached about 10 cm in size, transgenic tobacco plants were sprayed at dose of 100 mg/m^2 of bromoxynil and their viability was checked after 10 days. The control tobacco plants were died, but transgenic tobacco plants grew well (Fig. 6). Transgenic lettuce plants were less resistant than transgenic tobacco plants. When transgenic lettuce plants were sprayed at dose of 50 mg/m^2 of bromoxynil and their viabilities were checked after 5 days, while the control lettuce plants died, the transgenic lettuce plants grew well (Fig. 7).

Fig. 6. Effect of a 100 mg/m^2 bromoxynil spray on transgenic tobacco plant with the *bxn* gene and non-transformed tobacco plant. Photograph was taken 10 days after initiation of the experiment.

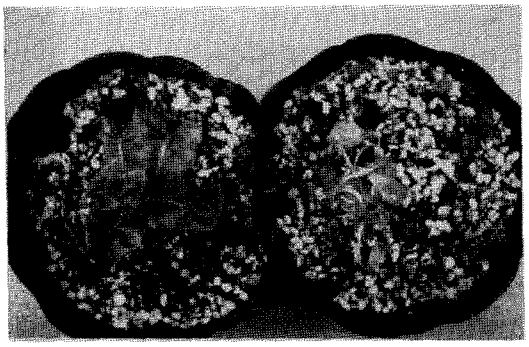

Fig. 7. Effect of a 50 mg/m² bromoxynil spray on transgenic lettuce plant with the *bxn* gene and non-transformed lettuce plant. Photograph was taken 5 days after initiation of the experiment.

These results show that the transgenic tobacco and lettuce plants with bromoxynil specific nitrilase gene, *bxn*, were resistant to high doses of bromoxynil and that the gene encoding the bromoxynil specific nitrilase can be used as a selective marker for rapid screening at whole plant level.

Further analysis are in progress to test the stable inheritance of the *bxn* gene in transformed plants and the tissue specific expression with diverse diverse promoters.

Acknowledgement

The *bxn* gene of *Klebsiella ozaenae* was a generous gift from *Rhone-Poulenc Agro, Biologie Moleculaire et Cellulaire Vegetae*. This research was supported by a grant from KOSEF II PMBBRC.

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Bromoxynil 특이성 nitrilase 유전자를 이용한 제초제 저항성 형질 전환 식물의 분자육종

민복기 · 박은성 · 박연홍 · 송재영 · 이세영(고려대학교 농화학과)

초록 : Bromoxymil은 쌍떡잎 특이적 제초제로써 폭 넓게 이용되고 있으며 반감기가 매우 짧다. Bromoxynil을 3,5-dibromo-4-hydroxybenzoic acid로 분해하는 nitrilase를 암호화한 *bxn* 유전자를 식물 벡터인 pGA482에 도입하고 *Agrobacterium*과의 동시배양을 통해 담배와 상추에 형질전환하였다. Kanamycin을 이용해 형질전환 식물체를 선별하고 완전한 식물체로 분화시켰다. Northern hybridization을 통해 *bxn* 유전자의 발현정도를 검정하고 liaf-disc와 pot assay를 통해 형질전환 식물체가 고농도의 bromoxynil에 저항성을 보임을 확인하였다.