J. Plant Biol. 38(4) : 365~371 (1995)

Construction of Binary Vectors for the Rice Transformation Using a Rice Actin Promoter and Replication Origin of pTi12 Isolated from *Agrobacterium tumefaciens* KU12

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Binary vectors, pBI-ActR1, pBI-ActF1 and pBSH-ActR1, were constructed using pGA642, the replication origin of pTi12 and the rice actin promoter. The sizes of pBI-ActR1, pBI-ActF1 and pBSH-ActR1 were 12.9 kb, 13.2 kb and 11.95 kb, respectively. These vectors containing a rice actin promoter followed by a GUS structural gene could induce strongly the expression of GUS gene in transformed rice cells. Rice explants from 3-4 day old seedlings after germination were cocultured with *A. tumefaceins* harboring pBI-ActR1, pBI-ActF1 or pBSH-ActR1, and then GUS expression in the explants was assayed. Transformation of rice explants by these binary vectors was tissue-specific, such that the meristematic regions of shoot apex, root and hypocotyl were transformed by these binary vectors.

Keywords : binary vector, plant transformation, *A. tumefaciens*, rice

Agrobacterium tumefaciens can introduce foreign genes into plant genomes (Bean, 1984; Lurquin, 1987). Plant transformation by *A. tumefaciens* has several advantages over direct gene transfer methods. *A. tumefaciens* is capable of infecting intact plant cells and can efficiently transfer large fragments of DNA without substantial DNA rearrangement (Krens *et al.*, 1985). While the transformation of many dicotyledonous plants using *A. tumefaciens* has became routine, it is difficult for monocotyledonous plants, especially cereal crops, to be transformed by *A. tumefaciens*. One of the problems in transforming monocots using *A. tumefaciens* is finding of an efficient promoter to express foreign genes in the transgenic crop plants (McElroy *et al.*, 1990).

Since rice is a staple crop all over the world, it is very important to develop an efficient method for transformation of rice. Recently, it was reported that rice could be transformed by *A. tumefaciens* (Raineri *et al.*, 1990; Chan *et al.*, 1992; Li *et al.*, 1992). These authors used binary vectors which has CaMV 35S promoter and RK2 replication origin. However, those binary vectors had several disadvantages. CaMV 35S promoter not only has low activity in transformed rice cells (Peterhans *et al.*, 1990), but also is not active in all cell types (Terada and Shimamoto, 1990). Besides, the binary vectors constructed using RK2 origin is unstable in *A. tumefaciens* and has a low copy number in *E. coli*.

It has been reported that the rice actin promoter is 20 fold stronger than the CaMV 35S promoter in transgenic rice protoplasts (McElroy *et al.*, 1990) and is active in all tissues (Zhang *et al.*, 1991). Lee *et al.* (1994) reported that pYWXP having replication origins of pTi12 and *ColE*1 is very stable in *A. tumefaciens* and has a high copy number in *E. coli*. Therefore, in order to overcome the instability and low copy of the binary vectors containing RK2 origin, we constructed a new binary vector using a strong constitutive promoter of rice actin gene from the plasmid pActI-F (McElroy *et al.*, 1990) and the replication origins of pTi12 and *ColE*1 (Cha *et al.*, 1983; Lee, 1993) in this study. At the same time we found

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that Japonica type rice cultivars could be transformed by *A. tumefaciens* harboring the new binary vector, pBSM-ActR1.

MATERIALS AND METHODS

Bacterial strains and plasmids

We chose *A. tumefaciens* stranin A281 that contains an agropine type Ti plasmid, pTiBo542. The *A. tumefaciens* strain A281 (pTiBo542) induced larger and earlier appearing tumors on a wider range of plant than other strains (Jin *et al.*, 1987; Komari, 1990) and could infect rice (Raineri *et al.*, 1990; Chan *et al.*, 1992). The *E. coli* strain JM109 was used as a host cell and was grown in LB medium at 37℃. The *A. tumefaciens* strain was grown in MG/L medium (Chilton *et al.*, 1974) or in AB minimal medium (Chilton *et al.*, 1974) at 28℃.

The plasmid, pAct-F (MeElroy *et al.*, 1990), containing rice actin promoter was kindly provided by Dr. Ray Wu and was used for constructing the binary vectors in this study. The plasmids, pBI121 (Jefferson *et al.*, 1987) and pGA642 (Gelvin *et al.*, 1991) were used to obtain T-DNA border regions and the nptII gene, respectively. New recombinant plasmid, pBSXP containing replication origins of pTi12 and *ColE1* from pYWXP was constructed in this study. pBA1, pBA2, pGB01, pGB02, pGB03, pBI-ActR1, pBI-ActF1 and pBSH-ActR1 were also constructed in this study (Figs. 1, 2).

Construction of pBI-ActR1 and pBI-ActF1

Plasmids pBI-ActR1 and pBI-ActF1 were constructed in the following way. A 1.0 kb *Eco*R1-SmaI fragment and a 6.4 kb *Kpn*I-*Xho*I fragment of pAct1-F were subcloned in pBluescriptSKII to make pBA1 and pBA2, respectively. A 1.0 kb *Hin*dIII-*Sma*I fragment containing rice actin promoter of pBA1 was inserted into *Hin*dIII-*Sma*I sites of plasmid pBI121 to make new plasmid pBI-ActR1. A 1.3 kb *Hin*dIII-*Sma*I fragment of the plasmid pBA2 was inserted into *Hin*dIII-*Sma*I sites of plasmid pBI121 to make another new plasmid pBI-ActF1. Overall construction scheme is shown in Fig. 1.

Construction of pBSH-ActR1

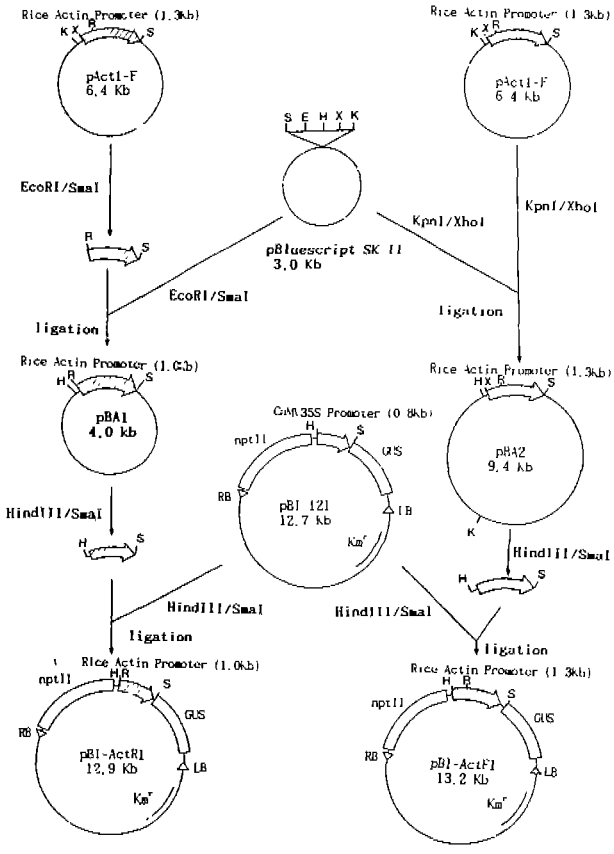

Fig. 1. Strategy for construction of pBI-ActR1 and pBI-ActF1. pBI121, pBI-ActR1 and pBI-ActF1 are shuttle vectors which can replicate in *E. coli* and *A. tumefaciens*. Each abbreviation is as followes: LB, left border; RB, right border; nptII, neomycin phosphotransferase; GUS, β-glucuronidase. Restriction sites denoted by H, *Hid*III; K, *Kpn*I; P, *Pst*I; R,*Eco*RI; S, *Sma*I; X, *Xho*I.

As shown in Fig. 2, we constructed pBSH-ActR1 using pGA642, pBI121, pBA1 and pBSXP. pGB01 was constructed by replacing *Pst*I-*Hin*dIII fragment of pGA642 with a 1.9 kb *Pst*I-*Hin*dIII fragment containing nptII from pBI121. pGB02 was constructed by replacing the *Hin*dIII-*Eco*RI fragment of pGB01 with 3.0 kb *Hin*dIII-*Eco*RI from pBI121. As a result, pGB02 had the nptII-CaMV 35S promoter-GUS cassette. To replace the CaMV 35S promoter of pGB 02 was replaced with the rice actin promoter by replacing the *Hin*dIII-*Sma*I fragment of pGB02 with a *Hin*dIII-*Sma*I fragment of pBA1. In order to replace the RK2 origin of pGB03 with replication origins of the pTi12 and the *ColE1*, the pGB03 *Sal*I fragment containing the RK2 replication origin was replaced by pBSXP containing both the replication

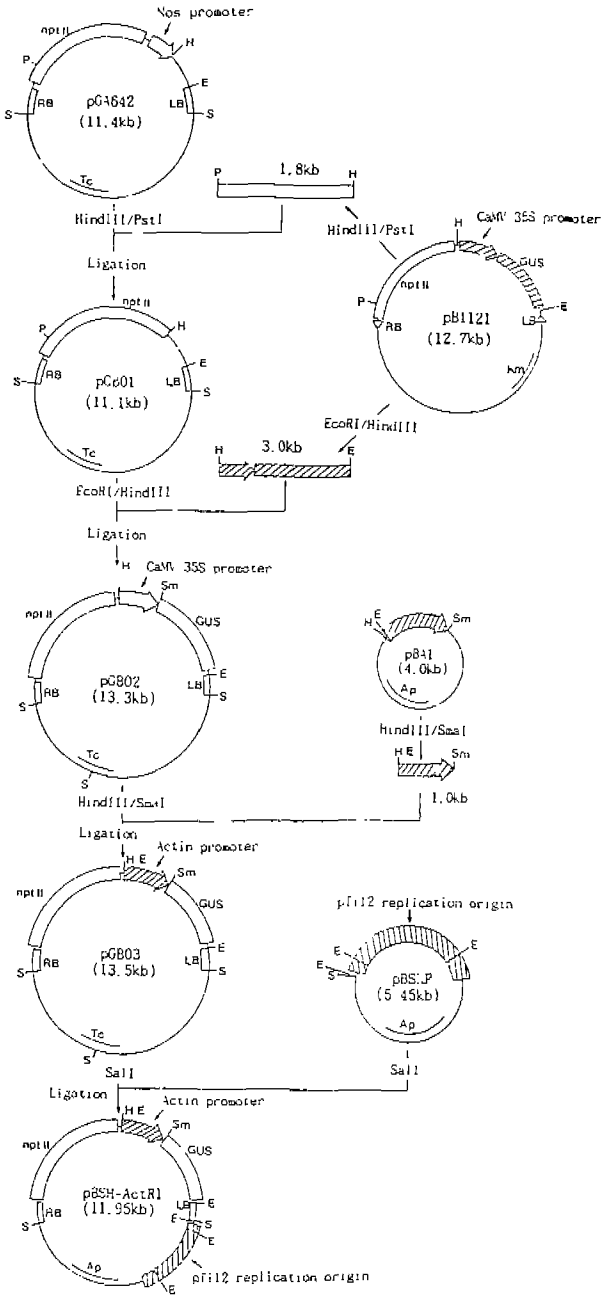

Fig. 2. Strategy for construction of pBSH-ActR1. Each abbreviation is as followes: LB, left border; RB, right border; nptII, neomycin phosphotransferase; GUS, β-glucuronidase; Ap, ampicillin resistent; Km, kanamycin resistent; Restriction sites are denoted by H, *Hid*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

origins of pTi12 and *ColE*1.

Transformation of rice explants

Japonica type rice cultivars (*Oryza sativa* L. cv. Chuchung, cv. Nakdong and cv. Seomjin) were tested for transformation. Rice cultivars were obtained from the Agricultural Research Center at Suwon, Korea. To transform the rice explants, we used a modified method of Li *et al.* (1992). Three to four day old seedlings were placed in a Petri plate containing a suspension of preinduced *A. tumefaciens*. In the presence of *A. tumefaciens*, each root and shoot was cut with a scalpel into two or four small pieces depending on the size of the seedling, and incubated for 10 min. After incubation, these fragments were transferred to a hormome- and antibiotic-free MS media (Murashige *et al.*, 1962) and incubated in darkness at 28℃ for 4 days.

Because it was reported that monocotyledonous plants have not materials to induce the vir genes essential for transformation (Usami *et al.*, 1987), *A. tumefaciens* was induced prior to coculture with the rice explants. 50 mL of AB medium containing kanamycin was inoculated with a single colony of *A. tumefaciens* A281 harboring binary vector and cultured to an optical density (O.D.) of 0.9 at 600 nm. This culture was centrifuged and resuspended in 50 mL of induction medium to an O.D. of 0.45 and cultured to an O.D. of 0.9-1.2 as previously described by Li *et al.* (1992).

GUS expression of *A. tumefaciens* and plant tissue

So as to detect the GUS expression of *Agrobacterium*, 1 mL of overnight cultured *A. tumefaciens* (10^7 cell/mL) harboring binary vector was centrifuged and resuspended in 500 μL of GUS-staining solution (1 mM X-gluc, 100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100) and then incubated at 37℃.

The GUS expression of plant tissue was tested as follows. The cocultured explants were washed with sodium phosphate buffer (pH 7.0) three times, immersed in GUS-staining solution and then was incubated at 37℃ for 20 hours. The stained seedling tissues were examined by stereomicroscope.

RESULTS AND DISCUSSION

Construction of the plasmids, pBI-ActR1 and pBI-ActF1

We made binary vectors containing rice actin promoter for the purpose of rice transformation. Since we constructed in-frame translational chimeric gene fusion between the rice actin promoter and the GUS coding region, expression of the GUS gene in rice could be maximized. Because rice actin is a fundamental component and is actively expressed in rice, we chose actin promoter to express highly the GUS gene in rice. McElroy *et al.* (1990 and 1991) reported that CaMV 35S promoter activity was not constitutive and was low in transformed rice cells. Transient expression assays of transformed rice protoplasts showed that rice actin promoter is approximately 20 times more active than CaMV 35S promoter (McElroy *et al.*, 1991).

To minimize the size of 5′ noncoding region of rice actin, we constructed plasmids pBI-ActR1 and pBI-ActF1 containing 1.0 kb and 1.3 kb rice actin promoter respectively. When we measured promoter activities of recombinant plasmids, there was no difference in promoter activities (data not shown).

Construction of the plasmid, pBSH-ActR1

Both binary vectors, pBI-ActR1 and pBI-ActF1, have the RK2 replication origin which replicates in *E. coli* and in *A. tumefaciens* (Jefferson, 1987). However, the RK2 origin have two disadvantages. One is low copy number in *E. coli* (Spielman *et al.*, 1986b). The other is instability in *A. tumefaciens* (Close *et al.*, 1984). To overcome these drawbacks, pBSXP originated from pYWXP was joined with the nptII-Actin promoter-GUS cassette of pGB03. pYWXP has the *ColE*1 replication origin for *E. coli* and the pTi12 replication origin which is very stable in *A. tumefaciens* (Lee *et al.*, 1994). Therefore, pBSXP has a high copy number in *E. coli* and is stable in *A. tumefaciens*.

GUS expression in *A. tumefaciens*

To test the GUS activities in *A. tumefaceins* harboring the binary vectors, we assayed X-gluc stained bacterial cells. Fig. 3 shows *A. tumefaciens* stained with X-gluc for 5 h. When *A. tumefaciens* cells were incubated with X-gluc solution, these bacterial cells harboring pBI121 (CaMV 35S promoter-GUS) stained dark blue within 30 min. This means that CaMV 35S promoter-GUS works well in *A. tumefaciens*. However, *A. tumefaciens* cells harboring pBI-ActR1, pBI-ActF1 or pBSH-ActR1 (actin 5′ region-GUS) stained only light blue after 20 h. This means that the actin promoter-GUS works poorly in *A. tumefaciens*. There was no difference among the above-mentioned three recombinant plasmids in terms of extent of coloring.

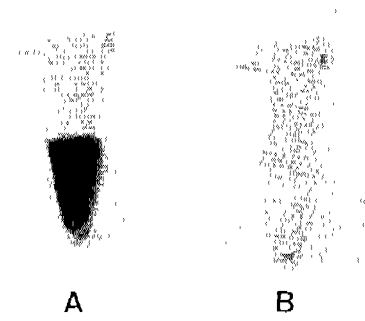

Fig. 3. *A. tumefaciens* A281 stained with X-gluc for 5 h at 37℃. Blue color indicates the expression of GUS in *A. tumefaciens*. A, *A. tumefaciens* A281 harboring pBI121; B, *A. tumefaciens* A281 harboring pBSH-ActR1.

The reason for the weak activity of the actin promoter in *A. tumefaciens* is probably that the actin 5′ region has a low transcriptional efficiency in *A. tumefaciens* and/or the mRNA 5′ region of Act-GUS fused gene has a low binding affinity for ribosomes of *A. tumefaciens*. McElroy (1991) had reported that the translational initiation site (AAGATGG) in the actin promoter of pAct1-F is similar to eukaryotic consensus translation initiation site (ACCATGG) with the most highly conserved positions being purines at −3 (usually an A) and at +4 (usually a G). Also, the Act-GUS fusion has no similarity with Shine-Dalgarno sequence (Shine and Dalgarno, 1974). As noted by Stormo (1982), the bacterial ribosome binding site from +4 to +7 is GCUA or AAAA. However, the actin promoter-GUS fusion is GCTG. Therefore, it is concluded that Act-GUS fusion expresses well in rice, but poorly in *A. tumefaciens*.

Gus expression in rice explant

GUS expressions in seedling explants cocultured with *A. tumefaciens* harboring pBSH-ActR1 containing the actin promoter-GUS cassette were tissue-specific (Fig. 4). GUS expressions were seen in the

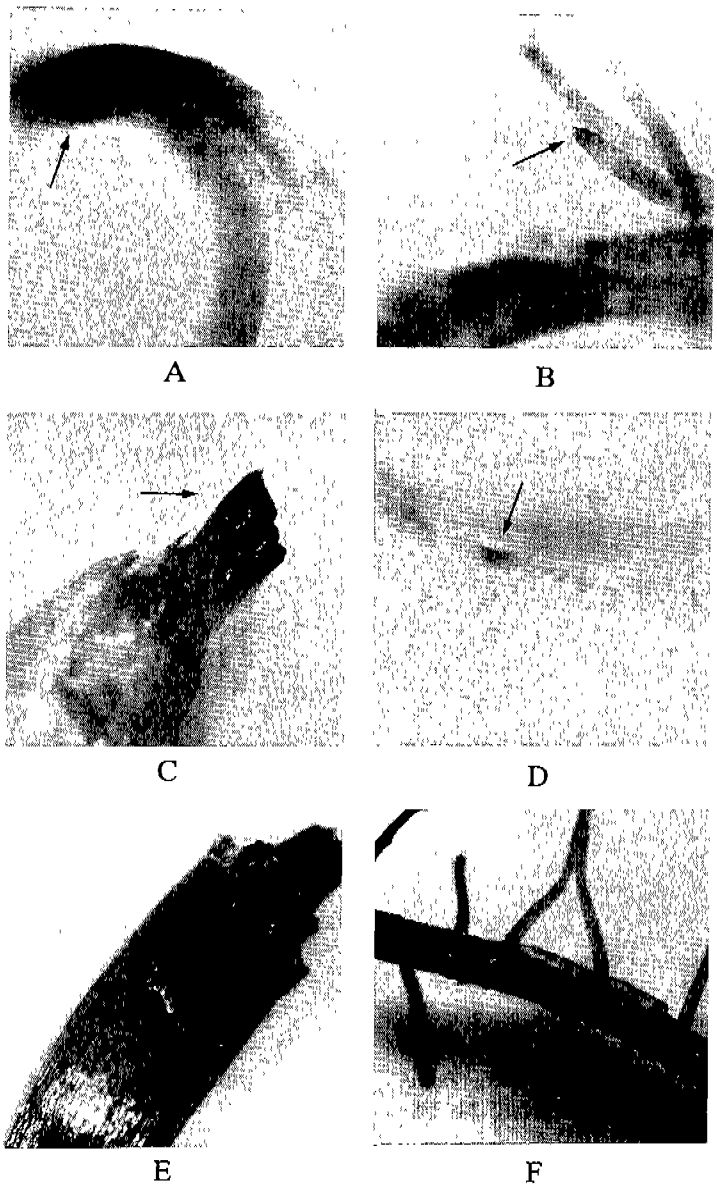

Fig. 4. GUS expression of rice explants (*Oryza sativa* L. cv. Nakdong). Each panel shows GUS expression in A, shoot apex; B, hypocotyl;, C, hypocotyl; D, root; E, root; F, root. A, B, D and E were rice explants infected by *A. tumefaciens* A281 harboring pBSH-ActR1, and C and F infected by *A. tumefaciens* A281 harboring pBI121. Arrow indicate GUS expression in plant tissue. Blue colors in C and F are the GUS expression of *A. tumefaciens* A281 harboring pBI121. The photograph was ×40 except panel F (×20).

meristematic regions of shoot apexes, hypocotyls and roots (Fig. 4A, 4B and 4D). However, GUS expression was not restricted to meristematic zones. Occasionally, GUS expression was detected in non-meristematic regions (Fig. 4E). These results are coincident with the results of Li *et al.* (1992) and of Liu *et al.* (1992) which used a binary vector pCaMV 35S promoter-GUS-INT. They did not detect GUS expression in bacteria because of the insertion of a modified plant intron into the gus gene.

To know whether the tissue-specific GUS expression in explants of seedlings is due to the contaminated Agrobacterium, seedling explants were infected with *A. tumefaciens* harboring pBI121 (containing CaMV 35S promoter). The infected explants were stained in the entire region as shown in Fig. 4C and 4F. The entire staining of seedling explants is due to the GUS expression in the contaminated *Ag-*

robacterium tumefaciens. Therefore, the tissue-specific GUS expression of explants which were infected by A. tumefaciens harboring pBSH-ActR1 was not the result of *A. tumefaciens* infection but the result of transformation of plant tissue.

Additionally, we tested the GUS expression with the different tissues and the different rice cultivars. Shoot apexes were more sinsitive than roots and hypocotyls. 25-35% of shoot apexes, 10-20% of hypocotyls and 0-5% of roots were transformed by *A. tumefeciens*. These results are similar to those of Li *et al.* (1992). The differences among three cultivars were not striking. *Oryza sativa* cv. Nakdong showed slightly higher activity than *Oryza sativa* cv. Seomjin in GUS expression

ACKNOWLEDGEMENTS

We thank Waggen Zhang, David McElroy, Dr. Ray Wu for the gift of plasmid pAct-F and Ha-Bun Lee for photographing the seedling explants. This work was support by a research grant (1996-1997) from the Korean Ministry of Education.

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(Received October 2, 1995)

Agrobacterium tumefaciens KU12로부터 분리한 pTi12의 Replication Origin과 벼의 Actin 유전자 프로모터를 이용한 벼의 Binary Vector 제조

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적 요

벼의 형질 전환을 위해 binary vector인 pBI-ActR1, pBI-ActF1 그리고 pBSH-ActR1을 플라스미드 pGA642, pTi12의 replication origin 그리고 벼의 actin 유전자 프로모터 부위를 이용해 제조하였다. pBI-ActR1, pBI-ActF1 그리고 pBSH-ActR1의 크기는 각각 12.9 kb, 13.2 kb 그리고 11.95 kb이며, 벼의 actin 유전자 프로모터에 결합된 GUS 구조 유전자를 가지고 있는 이들 vector들은 형질전환된 벼 세포에서 고도로 발현될 수 있다. 발아된 후 3-4일된 벼의 유식물체 조각을 pBI-ActR1, pBI-ActF1 혹은 pBSH-ActR1을 가지고 있는 *Agrobacterium tumefaciens*와 함께 배양한 후 유식물체 조각에서의 GUS 발현정도를 분석하였더니 이들 binary vector에 의한 벼의 형질전환은 조직특이적이었다. 묘의 정단과 뿌리 그리고 하배축의 정단분열조직 부위가 이들 binary vector에 의해 형질전환되었다.

주요어 : binary vector, 식물 형질전환, *A. tumefaciens*, 벼

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