Notes

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Four Chitinase cDNAs from *Chenopodium amaranticolor*

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Chitinase hydrolyses the β-1, 4 linkages of *N*-acetyl-D-glucosamine polymers of chitin, a major component of many fungal cell walls [1]. The enzymes are induced by pathogen attack, and classified into one group of pathogenesis-related (PR) proteins [2], PR-3. A significantly higher level of chitinase activity was found in the resistant than the susceptible cultivar of *Brassica napus* after inoculation with *Phoma lingam* [3]. Transgenic tobacco plants constitutively expressing a bean chitinase gene under control of the cauliflower mosaic virus 35S promoter showed an increased resistance against infection of *Rhizoctonia solani* [4]. Such evidence indicates that chitinase may play an important role in plant defense against fungal and bacterial attacks.

The Chenopodiaceae, especially *Chenopodium amaranticolor* Coste & Reyn, has been known to be susceptible to many plant viruses [5], and it is widely used for a indicator host for diagnosis. Moreover, twenty-nine species of the Chenopodiales were shown to contain inhibitors of virus infection [6]. These specific characters of *C. amaranticolor* awaken our interest in the molecular defence mechanisms on pathogen- and viral attacks in the plant.

For the generation of the new transgenic plants with enhanced resistance to pathogen infection, trials for the isolation and characterization of useful genes were necessary. Then, we tried to isolate the ribosome-inactivating protein (RIP) gene, which has been shown to have antiviral activity [7], from *C. amaranticolor.* At the early stage of the screening, we obtained a putative chitinase gene, designated CAP9 (**Table 1**). We designed 17-mer oligonucleotides probes, 5′-GCXTCYGAYACCATXTG-3′ (X=C or T, Y=A, G, C or T), derived from a highly conserved amino acid sequences of RIP, QMVSEA [8]. A pSPORT cDNA library, prepared from poly (A)$^+$ RNA of *C. amaranticolor* leaves, was screened with the digoxigenin-labeled probes. A few clones that hybridized to the probes were obtained and the cDNA inserts were sequenced on both strands by automated dideoxy methods. One of these clones, CAP9, encoded a 29 kDa polypeptide, and it was characterized to a class IV chitinase from the identity, 56.7% to bean PR 4 chitinase [9], 55.0% to rapeseed ChB4 chitinase [3], and 62.1% to maize seed chitinase A [10]. Using a labeled CAP9 insert, we isolated three additive cDNA clones (CAM18, CAM19 and CAM24). They encoded 28-29 kDa polypeptides, and these amino acid sequences showed 96.7%, 80.7% and 95.3% identity to that of CAP9, respectively (**Fig. 1**). Each polypeptide contains a putative signal sequence, cysteine-rich domain and hinge region, as well as a potential catalytic sequence.

Expression of these four cDNAs in *Escherichia coli* cells induced by isopropylthio-β-galactoside led host cells to lyse immediately, indicating that the cDNA products had strong hydrolytic activity of bacterial cell walls. To characterize the gene products, we extracted soluble proteins from the leaves of *C. amaranticolor* with necrotic local lesions at three days after inoculation with cucumber mosaic virus, and from the healthy leaves treated with 2 mM salicylic acid for two days. Western blot analysis of the proteins using anti-CAM18 antibody, raised against the C-terminal polypeptides (154 amino acids) of CAM18 expressed in *E. coli* by pMAL-c2 vector system (New England Biolabs), showed that immunologically reactable proteins were highly induced in these leaves but not in healthy leaves (**Fig. 2**). These results indicate that the products of these four cDNAs belong to group 3 PR proteins, chitinases, which are induced by pathogen attack and salicylic acid-treatment as a

Table 1.
Characteristics of four chitinase cDNA clones from *Chenopodium amaranticolor.*

Clone Name	Length in nucleotides	Position of ORF	Size of Deduced Products	Accession Number
CAP9	1068	6 to 833	275 aa (*M*r 28,839)	D45181
CAM18	1052	25 to 846	273 aa (*M*r 28,620)	D45182
CAM19	952	18 to 821	267 aa (*M*r 28,428)	D45183
CAM24	1061	45 to 863	272 aa (*M*r 28,737)	D45184

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CAP9     1:-----MSSIKQIFAILVAI-AC-M-S-CTMIAAQNCGCASGLCCSKYGYCGTTKAYCGDG 51
CAM18    1:-----MSSLGQIFAMLVAI-AC-M-S-CTMTTAQNCGCASGLCCSKYGYCGTTAAYCGDG 51
CAM19    1:-----MSSLGQIFAILMAI-TC-M-S-CTMIAAQNCGCASGVCCSQYGYCGNGDAYCGKG 51
CAM24    1:-----MSSLTQFFAIFMAI-AC-M-S-CTMTTAQNCGCASGLCCSKYGYCGTTAAYCGDG 51
ChB4     1:MNQSTITQNMALTKLSLVLFLCFLGLYSETVKSQNCGCAPNLCCSQFGYCGSTDAYCGTG 60
PR4      1:--------MGNKLVLVLVAVA-LVMG-PKNVSAQNCGCAEGLCCSQYGYCGTGEDYCGTG 50
ChiA     1:M-ANA-PRILALG-L-LAL-LC--AA-AGPAAAQNCGCQPNFCCSKFGYCGTTDAYCGDG 52
                                            *****    ***  ****    *** *

CAP9    52:CKQGPC-YSSTP--TTPSGGGGGGGASVQSLVTNAFFNGILNQAGSGCAGKSFYTRSAFL 108
CAM18   52:CKQGPC-YSSTP--TTPS--GGGGGASVQSLVTNAFFNGILNQAGSGCAGKSFYTRSAFL 106
CAM19   52:CQQGPC-YSS-----T-----GSGAVSVQSLVTDAFFNGIINQAGSSCAGKRFYTRSAFL 100
CAM24   52:CKQGPC-YSSTP--TTPS---GGGGASVQSLVTNAFFNGILNQAGSGCAGKSFYTRSAFL 105
ChB4    61:CRSGPCRS---PGGTPSPPGGGS----VGSIVTQAFFNGIINQAGGGCAGKNFYTRDSFI 113
PR4     51:CQQGPC----TTAS--PPPSNNV-NADI---LTADFLNGIIDQADSGCAGKNFYTRDAFL 100
ChiA    53:CQSGPCRSGGGGGGGGGGGGGGSGGANVANVVTDAFFNGIKNQAGSGCEGKNFYTRSAFL 112
           *  ***                          *  * ***  **   * ** ****  *

CAP9   109:NALGNYPQFGKGGSSDDTKREVAAFFAHVTHETGHFCYIEEI-AKS-TYC-QSSA-QWPC 164
CAM18  107:NALGNYPQFGKGGSSDDTKREVAAFFAHVTHETGHFCYIEEI-AKS-TYC-QSSA-QWPC 162
CAM19  101:NALGNYPQFGKGGSSDDTKREVAAFFAHVTHETGSFCYIEEI-SKS-TYC-NASA-TWPC 156
CAM24  106:NALGNYPQFGKGGSSDDTKREVAAFFAHVTHETGHFCYIEEI-AKS-TYC-QSSA-QWPC 161
ChB4   114:NAANTFPNFAN--S-V-TRREIATMFAHFTHETGHFCYIEEINGASRDYC-DENNRQYPC 168
PR4    101:SALNSYTDFGRVGSEDDSKREIAAAFAHFTHETGHFCYIEEIDGASKDYCDEESIAQYPC 160
ChiA   113:SAVNAYPGFAHGGTEVEGKREIAAFFAHVTHETGHFCYISEIN-KSNAYC-DASNRQWPC 170
            *      *          ** *  *** ***** **** **   *  **        **

CAP9   165:NPNKQYYGRGPIQITWNYNYGAAGKSIGFDGINAPETVANNPVIAFKTAFWFWMNNVHSR 224
CAM18  163:NPNKQYYGRGPIQITWNYNYGAAGKSIGFDGINAPETVANNPVIAFKTAFWFWMNNVHSR 222
CAM19  157:NPSKQYYGRGPLQLTWNYNYGAAGRSIGFDGINAPETVANNPVTAFRTAFWFWMNNVHSI 216
CAM24  162:NPNKQYYGRGPIQITWNYNYGAAGKSIGFDGINAPETVANNPVIAFKTAFWFWMNNVHSR 221
ChB4   169:APGKGYFGRGPIQLSWNYNYGACGQSLNLNLLGQPELVSSNPTVAFRTGLWFWMNSV-RP 227
PR4    161:SSSKGYHGRGPIQLSWNFNYGPAGSANNFDGLGAPETVSNDVVVSFKTALWYWMQ--HVR 218
ChiA   171:AAGQKYYGRGPLQISWNYNYGPAGRDIGFNGLADPNRVAQDAVIAFKTALWFWMNNV-HG 229
                * **** *  ** ***  *          *  *       * *  * **

CAP9   225:IISGQGFGSTIRAVN-GGECGGGNTPAVNARVGYYTQYCKQLGVSPGNNLSC         275
CAM18  223:IVSGQGFGSTIRAVN-GGECGGGNTPAVNARVGYYTQYCKQLGVSPGNNLSC         273
CAM19  217:INSGRGFGATIRAIN-SIECNGGNTGAVNSRVQLYRQYCNQFGVSPGNNLSC         267
CAM24  222:IVSGQGFGSTIRAVN-GGECGGGNTPAVNARFGYYTQYCKQLGVSPGNNLSC         272
ChB4   228:V-LNQGFGATIRAING-MECNGGNSGAVNARIRYYRDYCGQLGVDPGPNLSC         277
PR4    219:PVINQGFGATIRAINGALECDGANPTTVQARVNYYTEYCRQLGVATGDNLTC         270
ChiA   230:V-MPQGFGATIRAINGALECNGNNPAQMNARVGYYKQYCQQLRVDPGPNLIC         280
                *** **** *   ** * *      *   *  ** *  *  * ** *
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Fig. 1 Comparisons of the deduced amino acid sequences of CAP9, CAM18, CAM19, CAM24, rapeseed ChB4 chitinase, bean PR4 chitinase and maize seed chitinase A.
Asterisks indicate amino acid residues that are identical in all chitinases. The underlined amino acids correspond to the nucleotides which most likely bound to the oligonucleotides probes.

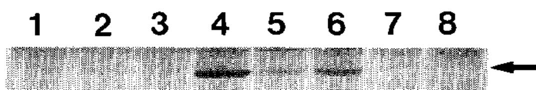

Fig. 2 Western blot analysis of *Chenopodium amaranticolor* and *Nicotiana tabacum* leaves.
SDS-soluble proteins were extracted from non-treated (lane 1), mock-inoculated (lane 2), mechanically wounded (lane 3) and cucumber mosaic virus-inoculated (lane 4) leaves of *C. amaranticolor*, and from leaf discs of *C. amaranticolor* (lanes 5 and 6) and *N. tabacum* (lanes 7 and 8) floated on distilled water (lanes 5 and 7) or 2 mM salicylic acid (lanes 6 and 8) for 2 days. A polyclonal antibody against CAM18 was used as the

self-defence response.

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Construction of Normalized cDNA Libraries from Plants

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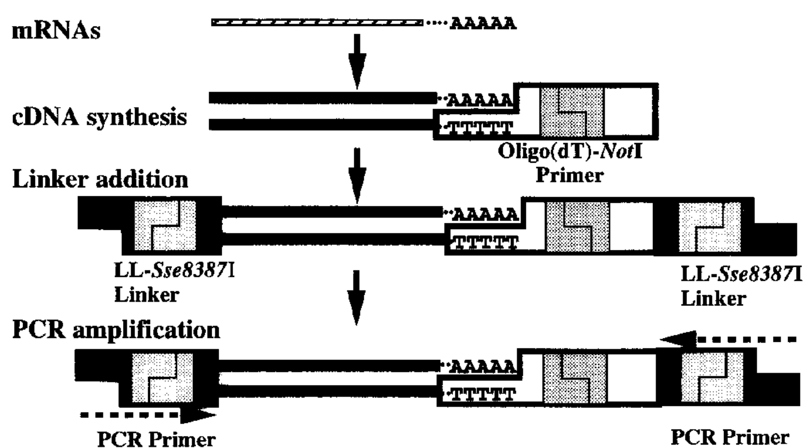
The ratio of cDNA clones for each gene in cDNA libraries reflects its abundance of mRNA in tissue preparations. It is technically difficult to isolate genes expressed at very low levels by differential screening and by expression screening, especially when the amount of available tissue is limited. On the other hand, in normalized cDNA libraries, all the genes expressed in prepared tissues are present as cDNA clones at almost equal representation. Although the normalized cDNA library has the disadvantage that a relatively complicated procedure and PCR steps are involved in the protocol to construct these libraries and that the ratio for abundant clones is reduced in normalized libraries, normalized cDNA libraries are a good alternative to isolate rarely expressed genes efficiently by differential screening.

Normalization of cDNA molecules is based on denaturing-annealing kinetics of double stranded DNA molecules (**Fig. 1**). Reassociation of nucleic acid molecules is highly sequence-specific under appropriate conditions. Abundant species of DNA molecules tend to anneal in relatively short periods of incubation time, although rare molecules tend to remain as single-stranded DNA. Single stranded DNA can be separated



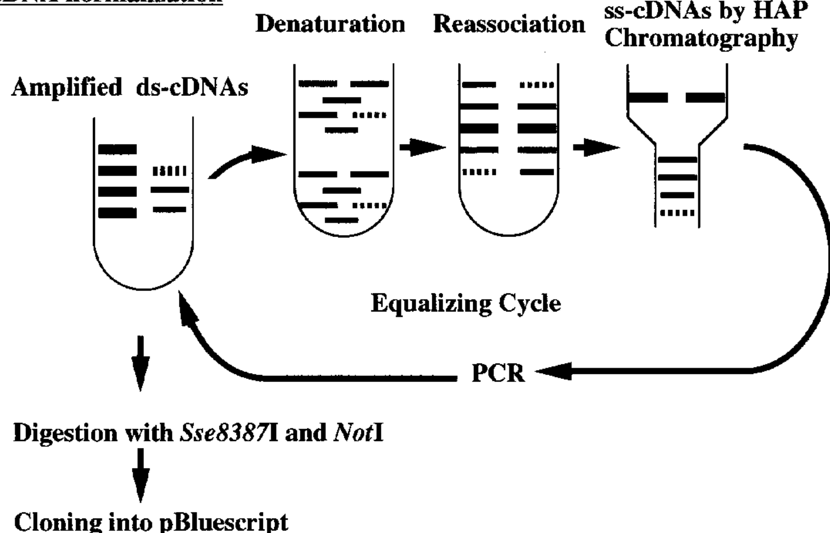


Fig. 1 Scheme of cDNA normalization.

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from double stranded DNA by hydroxylapatite column chromatography. Collected single-stranded DNA molecules are converted to double stranded DNA by PCR and cloned into widely used plasmid vectors to construct cDNA libraries. The difficulty of normalization is to maintain the size distribution of the cDNAs. Improved methodology has been developed to construct a normalized cDNA library [1, 2] In this issue, the author describes a procedure to construct normalized cDNA libraries.

1. *cDNA synthesis*

(1) RNA preparation

In general, it is important to use highly purified RNA to synthesize cDNA. Total RNA from plant tissues is prepared by the AGPC (Acid Guanidium thiocyanate-Phenol-Chloroform) method [3] with the modification that plant materials are ground in the presence of phenol. Although extraction buffer is routinely prepared in laboratories, RNA extraction kits, such as Isogen (Nippon Gene, Tokyo), are commercially available. Poly(A)$^+$ RNA is purified by oligo dT column chromatography or commercially available kits such as Oligotex^{TM}-dT30 Super (Takara Shuzo, Kyoto).

(2) cDNA synthesis

The cDNA is synthesized as described by Gubler and Hoffman [4] using cDNA synthesis kit such as cDNA synthesis plus (Amersham). The first strand of cDNA was primed with 100 units of AMV reverse transcriptase and with 2.5 μg of *Not* I primer-adapter (CAATTCGCGGCCGCT15, Promega) as oligo dT primer for 1 μg of poly(A)$^+$ RNA as illustrated in **Fig. 1**. Since DNA denaturing and annealing kinetics is involved in the normalization procedure, partial cDNA up to 1 kb in length is suitable to avoid potential bias by message size difference of each gene in the normalization steps. For this purpose, AMV reverse transcriptase is more convenient than MuLV reverse transcriptase because AMV reverse transcriptase has much higher RNaseH activity to synthesize relatively short cDNA. An excess amount of primers and enzyme are also included in the first strand cDNA synthesis, although these conditions are never recommended to synthesize 'good' cDNAs of longer size.

The double stranded cDNA is purified by phenol/chloroform extraction and ethanol precipitation followed by size fractionation with a Sup-Rec2 microconcentrator (Takara Shuzo, Kyoto), or by the use of size exclusion chromatography such as Sephadex G50.

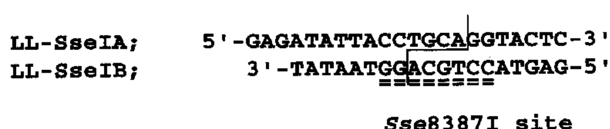

Fig. 2 Oligonuceotide sequence used for 'lone linker' and for PCR primer.

2. *Linker ligation and the amplification of DNA by PCR*

To amplify cDNA fragments of a highly complex mixture without bias, cDNA was ligated to a "lone linker" that is a synthetic linker with one non-palindromic protruding end, and one blunt end. This linker sequence is useful not only for PCR amplification but also for unidirectional cloning after normalization steps.

(1) Synthesize two oligonucleotide linkers to contain an *Sse*8387I site in its sequence and to have non-palindoromic 5′ protruding and blunt ends. This type of linker is called a 'lone linker'. [5] The nucleotide sequence of lone linker is shown in **Fig. 2**. [1]

(2) Phosphorylate 5′ end of LL-SseIB with T4 polynucleotide kinase and ATP. Incubate for 1 hr at 37°C The reaction mixture of 40 μl contains:

- 10 μg Linker LL-SseIB
- 1×T4 polynucleotide kinase buffer
- 1 mM ATP
- 40 units T4 polynucleotide kinase

(3) Ligate linkers to the end of synthesized cDNA. Incubate for 24 hours at 12°C. The reaction mixture of 20 μl contains:

- 1 μg cDNA
- 2 μg Linker LL-SseIA
- 2 μg Linker LL-SseIB (phosphorylated)
- 1×T4 DNA ligase buffer
- 1 mM ATP
- 100 units T4 DNA ligase

(4) Set PCR reaction using LL-SseIA as a primer. The reaction mixture of 100 μl contains:

- 1 ng cDNA
- 1×PCR buffer with 1.5 mM $MgCl_2$
- 1 μM Primer LL-SseIA
- 200 μM dNTPs
- 1 unit AmpliTaq

(5) Add mineral oil.

(6) Amplify cDNAs using a step cycle program of 94°C for 2 min., 50°C for 2 min., and 72°C for 4 min. for 25 cycles by a thermal cycler (PJ2000, Perkin-Elmer). "Hot start" method is highly recommended to reduce the PCR artifacts.

(7) Check the amplification by agarose gel electrophoresis Since all cDNA species are amplified, smearing profile of DNA should be observed. Usually PCR reaction up to 1 kb in size is efficient enough.

(8) Remove mineral oil.

(9) Remove excess primers with a Sup-Rec2 microconcentrator (Takara Shuzo, Kyoto) and precipitate DNA using ethanol.

3. *cDNA normalization*

Reassociation reactions are performed by a modification of the procedures described by Patanjali *et al.* [6] and by Ko [7]. The single-stranded cDNA fraction is separated from double-stranded cDNA by column chromatography at 60°C on hydroxylapatite [8].

(1) Suspend the amplified DNA (20 μg/ml) in a 50 μl reaction mixture containing [0.3 M sodium phosphate (pH 7.0), 0.4 mM EDTA, 0.04% SDS] and add mineral oil to avoid concentrating DNA during incubation.
(2) Denature in a boiling water bath for 5 min.
(3) Incubate at 65°C for 24 hours.
(4) Chill on ice quickly.
(5) Add 0.5 ml of [10 mM Na-PO_4 (pH 7.0), 0.1% SDS].
(6) Hydrate the hydroxylapatite (Bio-Gel HTP, Bio-Rad) in [10 mM Na-PO_4 (pH 7.0), 0.1% SDS] and heat the hydroxylapatite suspension for 30 min. in boiling waterbath to reduce non-specific binding of DNA to the hydroxylapatite.
(7) Keep the hydroxylapatite at room temperature and remove the buffer over the settled hydroxylapatite. Resuspend the hydroxylapatite with [10 mM Na-PO_4 (pH 7.0), 0.1% SDS] and warm at 60°C.
(8) Mount the water-jacket column on a stand and keep at 60°C by circulating 68°C water.
(9) Pour 0.5 ml of the hydroxylapatite suspension into the column and wash with 10 ml of pre-warmed [10 mM Na-PO_4 (pH 7.0), 0.1% SDS].
(10) Load 0.5 ml of DNA to the column.
(11) Elute the single stranded DNA by washing 4 tims with 0.5 ml of [160 mM Na-PO_4 (pH 7.0), 0.1% SDS]. Collect fraction separately in microtubes.
(12) Concentrate and wash the eluted single-stranded cDNA with TE using Sup-Rec 2, extract with phenol/ chloroform, and precipitate by ethanol.
(13) Estimate the amount of DNA in each fraction by ethidium bromide staining.
(14) Amplify DNA using LL-SseIA as a primer. The reaction mixture of 100 μl contains:

1 ng cDNA
1×PCR buffer with 1.5 mM $MgCl_2$
1 μM Primer LL-SseIA
200 μM dNTPs
1 unit AmpliTaq

(15) Add mineral oil.
(16) Amplify cDNAs using a step cycle program of 94°C for 2 min., 50°C for 2 min., and 72°C for 4 min. for 25 cycles using a thermal cycler (PJ2000, Perkin-Elmer) with the "Hot start" method.
(17) Check the amplification by agarose gel electrophoresis Since all cDNA species are amplified, smearing profile of DNA should be observed as seen before amplification.
(18) Remove mineral oil.
(19) Remove excess primers with a Sup-Rec2 microconcentrator (Takara Shuzo, Kyoto) and precipitate DNA by ethanol.
(20) Repeat step (1) to (19). [9]
(21) Repeat step (1) to (13). [9]

4. *Cloning into plasmid vector*

The amplified DNA contains the recognition sequence by *Not*I and *Sse*8387I at its ends. The digested cDNA fragments can be cloned unidirectionally into vectors, such as pBluescriptII KS+after digestion with *Not*I and *Pst*I to construct a cDNA library. Resulting junctions between pBluescript vector and cDNA insert are recognized by '8 base-cutter', *Not*I and *Sse*8387I.

(1) Digest DNA with *Not*I and *Sse*8387I in appropriate buffers.
(2) Remove excess linkers by Sup-Rec2 microconcentrator.
(3) Ligate to plasmid vector.
(4) Introduce ligated DNA to *E. coli* cells with high competency and make a library.

5. *Troubleshooting*

(1) Particular DNA is amplified in PCR.

Although the amplification of particular DNA species has not been observed in PCR against *Arabidopsis* cDNA using the primer under the conditions described above in the author's laboratory, it is very important to follow the precise conditions in the PCR reaction and normalization steps. Great care must be paid to incubation temperature since normalization utilizes a kind of subtractive hybridization.

(2) Single stranded DNA is not quantitatively recovered by hydroxylapatite column chromatography.

The quality of hydroxylapatite varies among suppliers and lots. Test the condition for elution of single stranded DNA using heat-denatured and native lambda DNA digested with restriction enzymes such as *Alu*I. It is important to maintain constant column temperature. All buffers for the hydroxylapatite column chromatography should be pre-warmed in a waterbath. Column temperature can be measured by inserting thermometer into the column. The waterbath temperature used for circulating hot water with a small pump is adjusted at 68°C to maintain column temperature at 60°C.

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[9] When the author constructed a normalized cDNA library, normalization was evaluated by colony hybridization and DNA sequencing against a straight cDNA library (S library) and normalized cDNA libraries (E1, E2 and E3 libraries) with different number of normalization cycles [1]. The normalized cDNA library that was processed three times for equalization (E3), was normalized well.

Erratum

H. Anzai, Y. Ishii, M. Shichinohe, K. Katsumata, C. Nojiri, H. Morikawa and M. Tanaka, **Transformation of Phalaenopsis by Particle Bombardment**, *Plant Tissue Culture Letters*, **13**, 265-272 (1996).

Figure 2 in page 268 should be printed in color as below.

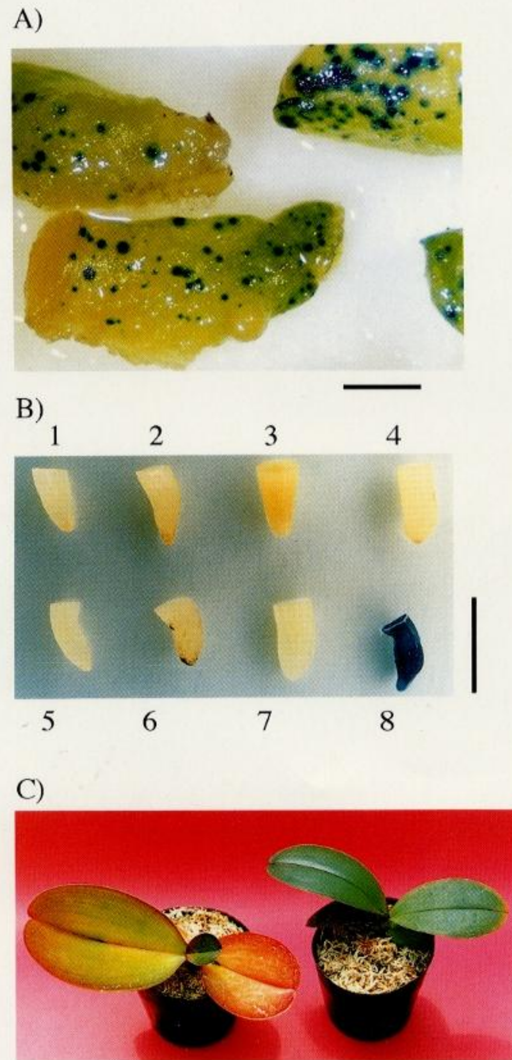

Fig. 2 Histochemical assay of GUS activity in phalaenopsis and herbicide application.

A : Transient GUS expression of PLB 24 hr after bombardment with pBI221 by 15 times pumping. Bar: 2 mm

B : Stable GUS expression in the root tips of transgenic plantlets. 1, untransformed plant; 2-8, transgenic plantlets. Bar: 10 mm

C : Untransformed control (left) and transgenic (right) plants were sprayed with HERBIE™. Bar: 5 cm

Table 2. Effect of promoter on the transient GUS expression in phalaenopsis PLBs.

Plasmid	Promoter	Number of blue spots*1
None		0.0
pBI221	CaMV 35S	98.0±42.2
pAHC27	maize ubiquitin	61.3±14.8
pActI-F	rice actin	13.7± 7.6

Ten PLBs and 15 times pumping were used for each treatment.

*1 Average of three experiments.