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Higher-level accumulation of foreign gene products in transgenic rice seeds by the callus-specific selection system

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Transgenic rice plants that accumulate the hypocholesterolemic pentapeptide lactostatin (IIAEK) as a fusion protein with the endosperm seed storage protein glutelin were developed. Plants were selected from *Agrobacterium*-mediated transformants using a mutated-rice acetolactate synthase (*mALS*) marker protein expressed under the control of the rice callus-specific promoter (CSP) (CSP:*mALS*). Lactostatin accumulation levels were compared in mature seeds of 78 independent transgenic rice lines selected for either the CSP:*mALS* gene cassette or hygromycin phosphotransferase (HPT) under the control of the CaMV 35S promoter (35S:*HPT*). Transgenic rice seeds harboring a CSP:*mALS* gene cassette accumulated more (approximately 120–200% on average) lactostatin than those with a 35S:*HPT* gene cassette, and accumulation was largely independent of transgene copy number. Furthermore, transgenic rice seeds that were selected by HPT under the control of CSP (CSP:*HPT*) also accumulated more lactostatin (approximately 160% on average) than transgenic rice seeds with the 35S:*HPT* gene cassette. These results indicate that high-value bioactive peptides such as lactostatin can be produced at higher levels using callus-specific selection than by conventional constitutive selection. Specific expression of a marker gene at the selection stage may thus result in increased target peptide accumulation levels in seeds.

[**Key words:** *Agrobacterium*; Callus-specific promoter; Endosperm; *Oriza sativa* L.; Selectable marker; 35S promoter; Transgenic rice]

We have been developing human health-promoting transgenic rice plants in which bioactive peptides accumulate at pharmacoactive levels in the endosperm. Transgenic rice lines have been produced which express and accumulate the hypocholesterolemic peptide lactostatin (Ile-Ile-Ala-Glu-Lys) derived from milk beta-lactoglobulin (1), the anti-hypertensive peptide novokinin (Arg-Pro-Leu-Lys-Pro-Trp) derived from ovalbumin (2), and human intestinal L-cell glucagon-like peptide (3). Since small peptides of less than about 50 amino acids do not accumulate well in seeds, we have developed a system by which peptides accumulate in rice endosperm as a fusion with the major rice storage protein glutelin [up to about 60% of total endosperm protein (4)] under the control of an endosperm-specific promoter (1, 2, 5). To distinguish between rice cells with an integrated transgene and non-transformed cells, bacterial genes encoding hygromycin phosphotransferase (*HPT*), phosphinothricin acetyltransferase (*BAR*) or neomycin phosphotransferase (*NPT II*) have been used as selectable markers under the control of the constitutive CaMV 35S, maize ubiquitin-1 or rice actin promoters. However, selectable antibiotic resistance genes derived from *E. coli*, or other bacteria have been a focus of the debate over the safety of genetically modified crops.

Acetolactate synthase (ALS) is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids leucine, isoleucine, and valine. The sulfonylurea, imidazolinone and pyrimidinyl carboxy herbicides inhibit ALS activity as competitive inhibitors (6, 7), and a *mutated-ALS* (*mALS*) gene has been isolated from these herbicide-resistant mutant plants. The mALS enzyme has much lower affinity for these herbicides in transgenic plant cells, but has the same activity as ALS, and has been used to generate a number of herbicide-resistant transgenic plant species (8–11). Transgenic rice plants have been produced using *Arabidopsis mALS* under the control of the CaMV 35S promoter as a selectable marker (10). A native rice *mALS* allele was isolated from a pyrimidinyl carboxy herbicide (pyriminobac, pyrithiobac-sodium and bispiribac-sodium)-resistant rice mutant and was found to have a single amino acid change from serine to isoleucine in a conserved region (S627I, Kumiai Chemical Industry, Tokyo, Japan). Transgenic rice produced using the *mALS* gene as a selectable marker may be more acceptable to consumers, since this selectable marker gene is derived from the rice genome.

In this study, transgenic rice lines were produced using rice *mALS* under the control of the rice callus-specific promoter (CSP, Kumiai Chemical Industry, Tokyo, Japan) as a selectable marker gene. Since *mALS* transcripts driven by CSP are not detected in tissues other than callus, the food safety of transgenic rice seeds should be reduced

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as a consumer issue. Furthermore, accumulation levels of transgene products are increased in transgenic rice seeds by using the callus-specific selection marker system, as compared to selection with the conventional constitutive system. This is the first report demonstrating that accumulation levels of transgene products are enhanced by expressing a marker gene only at the callus selection stage.

MATERIALS AND METHODS

Plant materials and production of transgenic rice The low-glutelin mutant 'Koshihikari' rice (*Oriza sativa* L.) variety a123 (12) was used as a host for transformation. *Agrobacterium tumefaciens*-mediated transformation was carried out under the conditions as previously described (13, 14).

Vector construction All binary vectors used in this study were constructed by MultiSite Gateway LR clonase reactions (Invitrogen, Carlsbad, CA, USA). Destination-binary vectors containing a *mALS* gene cassette consisting of a callus-specific promoter, *mALS* coding region, and 10 kDa prolamin gene terminator (CSP:*mALS*); a *HPT* gene cassette consisting of a CaMV 35S promoter, *HPT* coding region and *Ag7* terminator (35S:*HPT*); and a callus-specific *HPT* gene cassette consisting of a callus-specific promoter, *HPT* coding region and *Ag7* terminator (CSP:*HPT*), were prepared from the original destination vectors pHGW, pHm24GW and pHm43GW (15). These modified destination-binary vectors were named CSP:*mALS*-GW, CSP:*mALS*-24GW, CSP:*mALS*-43GW, 35S:*HPT*-GW, 35S:*HPT*-24GW, 35S:*HPT*-43GW, CSP:*HPT*-GW, CSP:*HPT*-24GW and 35S:*HPT*-43GW, respectively. Each entry clone containing a gene cassette for endosperm-specific expression of the glutelin-lactostatin fusion protein (hexa-repeated lactostatin with QR spacers, 6*Lac*QR or dodeca-repeated lactostatin, 12*Lac*) was produced by insertion into the destination-binary vectors. Glutelin 2.3 kb GluB-1, 16 kDa and 10 kDa prolamin promoters were used as endosperm-specific strong promoters for expression of fusion proteins based on the results obtained from expression of GUS reporter gene in transgenic rice seeds (5). The QR spacer between bioactive peptide repeats allows the active peptide to be released more efficiently from the fusion protein by digestion in the mammalian small intestine than does a direct tandem repeat (16). Single, double and triple gene expression cassettes were introduced into each binary vector using LR clonase enzyme mix as descried by Wakasa et al. (1) (Fig. 1). We have previously demonstrated that multiple-gene expression cassette

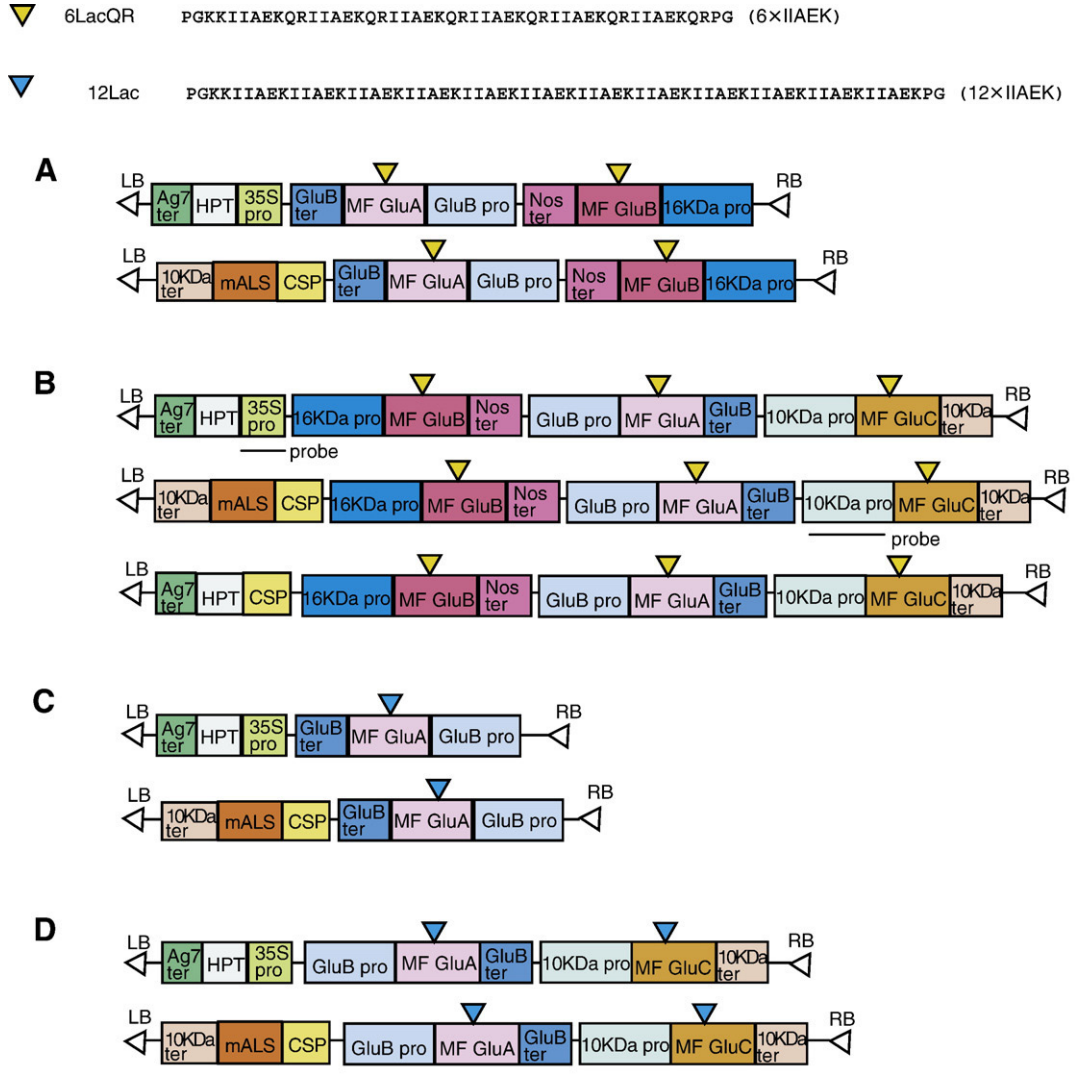

FIG. 1. Binary vector constructs. (A) 35S:*HPT* two-cassette 6*Lac*QR (upper) and CSP:*mALS* two-cassette 6*Lac*QR (lower); (B) 35S:*HPT* three-cassette 6*Lac*QR (upper), CSP:*mALS* three-cassette 6*Lac*QR (middle) and CSP:*HPT* three-cassette 6*Lac*QR (lower); (C) 35S:*HPT* single-cassette 12*Lac* (upper) and CSP:*mALS* single-cassette 12*Lac* (lower); (D) 35S:*HPT* two-cassette 12*Lac* (upper) and CSP:*mALS* two-cassette 12*Lac* (lower) are shown. Bars in panel B indicate the probe region for Southern or Northern blot analysis. Yellow and blue triangles indicate 6*Lac*QR or 12*Lac* insertion. The nomenclature and accession numbers of each binary vector (35S:*HPT* two-cassette 6*Lac*QR, CSP:*mALS* two-cassette 6*Lac*QR, 35S:*HPT* three-cassette 6*Lac*QR, CSP:*mALS* three-cassette 6*Lac*QR, 35S:*HPT* single-cassette 12*Lac*, CSP:*mALS* single-cassette 12*Lac*, 35S:*HPT* two-cassette 12*Lac*, CSP:*mALS* two-cassette 12*Lac* and CSP:*HPT* three-cassette 6*Lac*QR) are as follows: 35S, CaMV 35S promoter (accession No. AF485783); HPT, hygromycin phosphotransferase coding region (accession No. K01193); CSP, callus-specific promoter (Kumiai Chemical Industry, Tokyo); *mALS*, *mutated-ALS* coding region (Kumiai Chemical Industry); Ag7 T, *gene 7* terminator (accession no. AF242881); 10K Ter, 10 kDa prolamin gene terminator (accession no. X17074); 16 kDa pro, 16 kDa prolamin gene promoter (accession no. AY427574); GluB pro, *GluB1* promoter (accession no. AY427569); 10 kDa pro, 10 kDa prolamin gene promoter (accession no. AY427572); MFGluA, modified *GluA2* coding region and the 3′UTR (accession no. X05664); MFGluB, modified *GluB1* coding region and its 3′UTR (accession no. X54314); MFGluC, modified *GluC* coding region and its 3′UTR (accession no. AB016501); Nos ter, nopaline synthase gene terminator (accession no. AJ007624); GluB ter, *GluB1* terminator (accession no. X54314).

binary vectors make it possible to generate transgenic rice seeds which accumulate larger amounts of bioactive peptide than the single-gene expression cassette binary vector (1). Binary vector DNA was electroporated into *Agrobacterium tumefaciens* strain EHA 105.

Seed protein extraction and Western dot blot analysis Mature seeds from independent T_1 primary transgenic rice lines were individually ground into fine powder with a multi-beads shocker (Yasui Kikai, Tokyo). For total protein extraction, 500 μL of extraction buffer (50 mM Tris–HCl, 8 M Urea, 4% SDS, 20% glycerol, 5% 2-Mercaptoethanol, 1% BPB) was added to seed powder and vortexed for more than 1 h at room temperature. The mixture was centrifuged at 12 000 ×*g* for 20 min at room temperature, and the crude soluble protein sample was decanted into a new tube. Four positive seed extracts per independent transgenic line selected by Western blot using anti-LacQR (NH_2-CKKIIAEKQRIIAEKQR-COOH) or anti-Lac (NH_2-CIIAEKIIAEKIIAEK-COOH) antibody were pooled and used for quantitation. For relative comparison of lactostatin accumulation, equal amounts of the protein extracts were spotted onto a nitrocellulose membrane (Whatman, Dassel, Germany). The lactostatin in each dot was detected immunologically with anti-LacQR or anti-Lac antibody and quantified with NIH ImageJ (National Institutes of Health, Washington DC, USA).

Southern blot analysis Genomic DNA was extracted from young leaves with CTAB (17). Five micrograms of genomic DNA were digested with *Xba*I, fractionated by electrophoresis on a 0.8% agarose gel, and transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Probes were prepared from purified PCR products of the *HPT* coding region or the 10 kDa Prolamin gene promoter region. Southern hybridization was carried out with a Gene Images AlkPhos Direct Labelling and Detection System kit (GE Healthcare, Buckinghamshire, UK).

Northern blot and reverse transcription (RT)-PCR analysis *HPT* mRNA was detected by both Northern and RT-PCR analyses, but *mALS* mRNA was detectable only by RT-PCR, because it would be extremely difficult to discriminate transgenic *mALS* from endogenous *ALS* based on their separation on an agarose gel. However, the *mALS* synthetic gene is composed of the *mALS* coding region and the 10 kD prolamin terminator, so *mALS* mRNA can be specifically amplified by using a *mALS* coding region forward primer and a 10 kD prolamin terminator reverse primer.

Total RNA was extracted from callus, leaf, stem, root and young seed tissues as previously described (18). For Northern hybridization, 5 μg of total RNA were electrophoresed in a 1.2% formaldehyde-containing agarose gel. Blotting and Northern hybridization were done as with Southern blots.

For RT-PCR, first-strand cDNA was synthesized from 5 μg of total RNA using Superscript III (Invitrogen, CA, USA) and oligo-dT primer. Portions of *mALS* cDNA (a 300 bp region between the *mALS* coding region and the 10 kDa prolamin gene terminator), *ALS* cDNA (a 450 bp region between the *ALS* coding region and terminator) or *HPT* cDNA (a 321 bp of the *HPT* coding region) were amplified with 25 or 33 cycles of polymerase chain reaction using Ex Taq polymerase (Takara, Tokyo) and specific primer sets for *mALS* (forward primer: 5′-aggttttacaaggcgaatagg-3′; reverse primer: 5′-ctagagtacatgtaaccaacg-3′), *ALS* (forward primer: 5′-aggttttacaaggcgaatagg-3′; reverse primer: 5′-catgccaagcacatcaaacaag-3′) and *HPT* (forward primer: 5′-gcgacgtctgtcgagaagtttctg-3′; reverse primer 5′-ttcggtttcaggcaggtcttgc-3′). To normalize for differences in starting RNA concentrations, actin mRNA was also determined in each sample (forward primer: 5′-tccatcttggcatctctcag-3′; reverse primer: 5′-gtacccgcatcaggcatctg-3′).

RESULTS AND DISCUSSION

The low-glutelin mutant 'Koshihikari' rice variety a123 lacks three glutelin seed storage proteins [GluA1, GluA2, and GluB4 (12)], making it an ideal host for the accumulation of large amounts of transgene products in seeds because there is more storage capacity for the accumulation of engineered products than in wild type (19).

In order to reconfirm the callus specificity of CSP as a selectable marker in a123, Northern blot and RT-PCR analyses were used to detect transcripts of the 35S:*HPT*, CSP:*mALS*, and CSP:*HPT* transgene constructs using total RNA extracted from calli, leaves, stems, roots and young seeds (Fig. 2). *HPT* gene expression under the control of the constitutive CaMV 35S promoter produced high levels of transcript in callus, leaf, stem and root, but was not detected in developing seed (Fig. 2A). However, RT-PCR revealed that *HPT* was distinctly expressed in developing seeds of transgenic rice with 35S as the promoter (Fig. 2D). This difference in expression of *HPT* between Northern analysis and RT-PCR is due to that the latter method can detect much lower level compared with the former analysis. *HPT* under the control of the callus-specific promoter, however, was only expressed in callus (Fig. 2C, D). *mALS* was also expressed in a strictly callus-specific manner when directed by the callus-specific promoter (Fig. 2B), which is in remarkable contrast

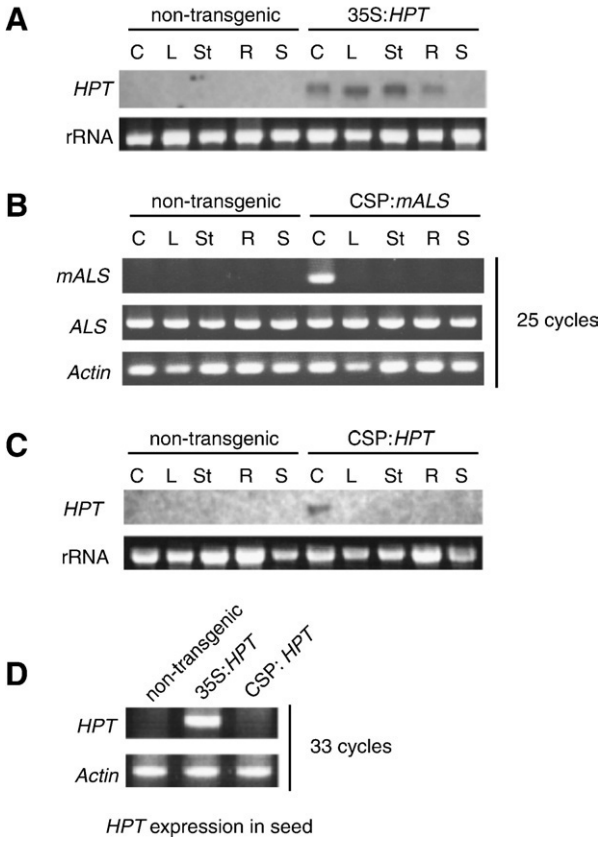

FIG. 2. Expression analyses of selectable marker genes in organs of transgenic plants. Transcriptional signals derived from 35S:*HPT* (A), CSP:*mALS* (B, upper panel) and CSP:*HPT* (C) Expression of endogenous *ALS* is in panel B, middle panel. Panel D indicates the differences in expression between the 35S:*HPT* and CSP:*HPT* in each transgenic seed tissue using RT-PCR. C, callus; L, leaf; St, stem; R, root; S, seed.

to the constitutive expression of the native *ALS* and actin genes (Fig. 2B).

The accumulation of lactostatin as a fusion protein with glutelin in constructs with either the 35S:*HPT* or CSP:*mALS* selectable gene cassettes was compared by dot blot Western analysis using anti-LacQR or anti-Lac antibody [35S: *HPT* two-cassette 6*Lac*QR versus CSP:*mALS* two-cassette 6*Lac*QR (Fig. 1A), 35S:*HPT* three-cassette 6*Lac*QR versus CSP:*mALS* three-cassette 6*Lac*QR (Fig. 1B), 35S:*HPT* single-cassette 12*Lac* versus CSP:*mALS* single-cassette 12*Lac* (Fig. 1C), and 35S:*HPT* two-cassette 12*Lac* versus CSP:*mALS* two-cassette 12*Lac* (Fig. 1D)]. Seeds of 78 independent transgenic rice lines (T_1) expressing each of the transgene sets included in the vector cassettes were selected for this investigation. Only transgenic rice lines expressing all of the two or three genes in the double or triple cassette constructs were used, and transgenic rice plants incompletely expressing transgenes were excluded. The average accumulation level for the 35S:*HPT* two-cassette 6*Lac*QR or 35S:*HPT* single-cassette 12*Lac* vector was adopted as an arbitrary standard unit for accumulation levels. A comparison of the relative mean accumulation levels in transgenic rice lines for each construct indicated that CSP:*mALS* two-cassette 6*Lac*QR was approximately 1.40-fold higher than 35S:*HPT* two-cassette 6*Lac*QR (Fig. 3A). In the three-cassette 6*Lac*QR series, the CSP:*mALS* construct accumulated lactostatin at approximately 1.25-fold that of the 35S:*HPT* construct (Fig. 3B). Furthermore, in the single-cassette and two-cassette 12*Lac* constructs, 12*Lac* products in the CSP:*mALS* construct resulted in approximately 2.08- and 1.19-fold increases over the 35S:*HPT* constructs (Fig. 3C, D). Thus, higher levels of lactostatin could consistently be obtained using CSP:

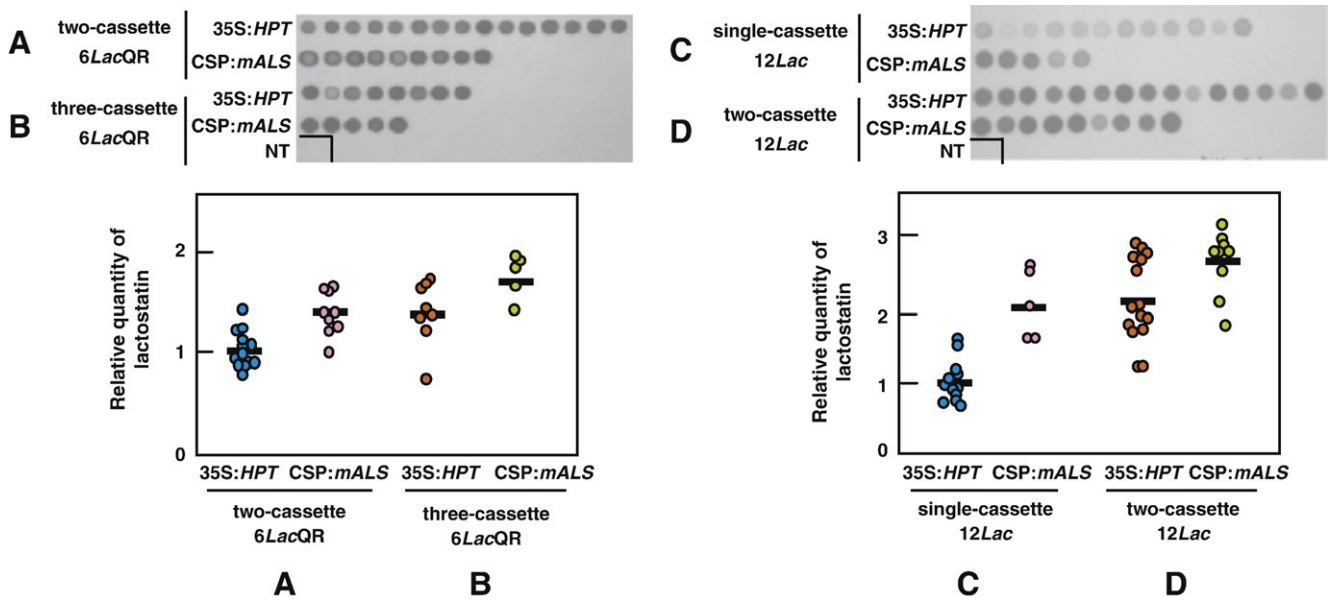

FIG. 3. Relative lactostatin accumulation as a glutelin fusion protein. Equal amounts of protein extracts derived from four positive seeds per transgenic line were spotted onto a nitrocellulose membrane. The lactostatin in each dot was detected immunologically with anti-LacQR (A, B) or anti-12Lac antibody (C, D). The mean accumulation levels of a 35S:*HPT* two-cassette 6*Lac*QR vector (A) and 35S:*HPT* single-cassette 12*Lac* vector (C) were set as arbitrary standard units. NT was shown non-transgenic a123 seed protein as negative control.

mALS constructs than with conventional 35S:*HPT* constructs. Furthermore, it is notable that in four independent comparison experiments, not only average of accumulation level but also maximum and minimum accumulation levels are inconsistently higher in CSP:*mALS* constructs than in 35S:*HPT* constructs (Fig. 3). However, increases in lactostatin ranged from 1.19- to 2.08-fold for individual constructs. We have already demonstrated that the signals of transgene products in transformants of the 6*Lac*QR series could be easily detected by CBB straining of SDS-PAGE gels (1), but the 12*Lac* series in this set of experiments did not accumulate enough product to be visualized, indicating that higher amounts of transgene products can be accumulated with the 6*Lac*QR series construct (1). Taken together, it may be that transgene products of the two-cassette 6*Lac*QR, three-cassette 6*Lac*QR and two-cassette 12*Lac* series reach some maximum production or accumulation level, but the single-cassette 12*Lac* series do not. Since the single-cassette 12*Lac* series had sufficient capacity to accumulate transgene products in seed, it may be substantially affected by differences in the marker gene used (i.e., 2.1-fold, as compared with the other construct series, ranging from 1.25-to 1.4-fold). In order to ascertain whether transgene product accumulation can be improved with callus-specific expression of a selectable marker, transgenic rice plants containing the callus-specific and CaMV 35S *HPT* selectable marker genes were generated (6*Lac*QR) and their relative lactostatin accumulation levels were compared. When 6*Lac*QR levels in mature seed were compared between 35S:*HPT* and CSP:*HPT* three-cassette 6*Lac*QR constructs (Fig. 1B), the latter construct accumulated approximately 1.62-fold higher levels of 6*Lac*QR than 35S:*HPT* (Fig. 4), indicating that constitutive expression of *HPT* by the CaMV 35S promoter had an inhibitory effect on the expression of foreign gene products.

Accurate accumulation levels were estimated by comparing with purified recombinant protein (Glutelin acidic subunit-lactostatin fusion protein) expressed in *E. coli* cells. Transgene products ranged from approximately 5% to 15% of total seed proteins, indicating that accumulation levels already reached a maximum level throughout all transgenic lines. Taken together, such variation levels of transgene product (1.2- to 2.1-fold) observed in this research are sufficiently significant.

Gene dosage and co-suppression have substantial influence on transgene expression (20, 21). Transgene copy numbers in independent transgenic rice lines expressing recombinant product were investigated as a variable in transgene expression. Southern blots of either the 35S:*HPT* three-cassette 6*Lac*QR or CSP:*mALS* three-cassette 6*Lac*QR show that one to three transgene copies were integrated into the genomes of most of the transgenic rice lines, but some lines had six or eight copies (Fig. 5). There is no significant difference in mean transgene copy number between 35S:*HPT* and CSP:*mALS* three-cassette 6*Lac*QR constructs. In keeping with the phenomenon of co-suppression, a transgenic rice line with eight transgene copies had the lowest accumulation level among the CSP:*mALS* three-cassette 6*Lac*QR series. An inverse correlation between transgene copy number and expression level has been reported when a reporter [e.g., β-glucronidase (*GUS*), Green Fluorescent Protein (*GFP*)] or the selectable

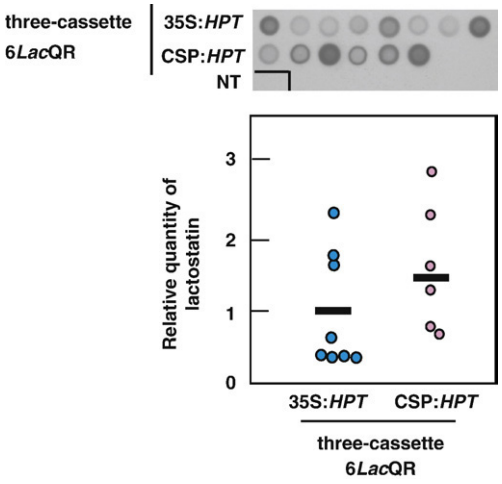

FIG. 4. Relative lactostatin accumulation as a glutelin fusion protein (continued). The lactostatin in each dot was detected immunologically with anti-LacQR. The mean accumulation level of a 35S:*HPT* three-cassette 6*Lac*QR vector was set as a standard unit. NT was shown non-transgenic a123 seed protein as negative control.

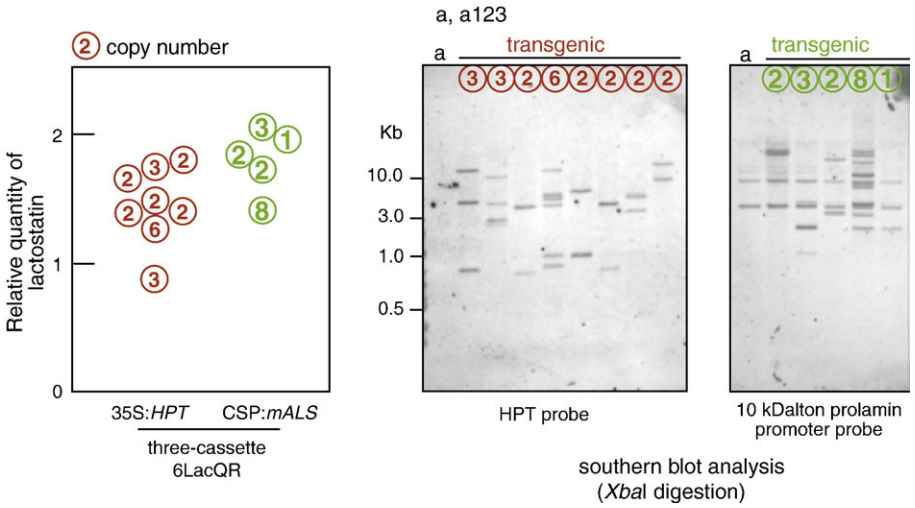

FIG. 5. Relationship between lactostatin accumulation and transgene copy number. Transgenic rice containing an 35S:*HPT* three-cassette 6*Lac*QR (red) and CSP:*mALS* three-cassette 6*Lac*QR (green) series were used with Southern blots to indicate the transgene copy number of each transgenic line.

marker gene *NPT II* are introduced under the control of the CaMV 35S promoter (20, 21). However, there is no clear correlation in our results between copy number and lactostatin expression in transgenic rice with one to six copies of the transgene. Because endogenous glutelin genes constitute a small gene family of about 10 copies per haploid genome (4) and are highly expressed in the endosperm during seed maturation (5), they may have a high threshold for transgene silencing by transcriptional and post-transcriptional gene silencing. Thus, our transgenic rice plants may not exhibit any clear correlation with suppression because the bioactive peptides are expressed as part of the native protein glutelin. There is a possibility that transgenic rice plants containing very high transgene copy numbers (i.e., >8) might be excluded from the quantitative analysis sample due to gene silencing. However, it is clear that higher accumulation of lactostatin in transgenic rice seeds with the CSP:*mALS* three-cassette 6*Lac*QR can be accounted for by the differences in marker gene cassettes rather than by transgene copy number. One plausible explanation is that there is a significant difference in the promoters directing individual marker gene products, because the CaMV 35S promoter directs constitutive expression of selection marker genes in transgenic rice, whereas CSP has little expression in any tissues other than callus at the selection stage. Although the *HPT* expression signal from the 35S:*HPT* construct was not detected in seed by Northern hybridization, it was clearly shown by RT-PCR that *HPT* was expressed in developing seed (Fig. 2D), suggesting that even low levels of expression during grain development from the 35S:*HPT* construct might have an inhibitory effect on foreign gene expression. Alternatively, high-level expression by the CaMV 35S promoter in the leaf, stem and root organs may also decrease net vegetative growth or yield due to use of energy for constitutive expression of marker gene or its detrimental effect on plant growth (22). In contrast, expression of *mALS* or *HPT* under the control of the callus-specific promoter would have less effect on the accumulation of lactostatin as a fusion protein with glutelin than *HPT* directed by a constitutive promoter. As shown in Fig. 4, lactostatin was higher with the CSP:*HPT* marker gene than with 35S:*HPT*, suggesting that specific expression of a marker gene by CSP reduces the inhibitory effects of the CaMV 35S constitutive promoter, which results in an increase of transgene product. When gene of interest is driven under the control of constitutive promoter such as CaMV 35S or ubiquitin, it has been reported that gene product expressed in tissue other than the target tissue has problematical effect on plant growth (23, 24). Thus, growth of transgenic plant with 35S:*HPT* may be slightly inhibited or retarded, which may be directly or indirectly related to slight reduction of accumulation of introduced gene product. On the other hand, transgenic plants with CSP:*mALS* or CSP:*HPT* may save own source energy as compared to those with 35S:*HPT*. For example, nutrients such as amino acids, sucrose or minerals in seed tissue are transported from the other vegetative sink tissues by translocation (25). Transgenic plant with 35S:*HPT* may spend nutrients more than plant with CSP:*mALS* or CSP:*HPT*, resulted in a slight reduction of the accumulation levels of lactostatin for 35S promoter construct. Further work will be required to examine whether these explanations are true or not, and what unknown mechanism may be involved in higher-level accumulation observed for CSP.

Taken together, expression of selectable marker proteins under the control of CSP for the production of bioactive peptides in seeds generally works better than constitutive expression as a selectable marker. The advantages of CSP were evaluated in heterozygous T_1 transgenic rice seeds for the five pairs of construct (35S:*HPT* two-cassette 6*Lac*QR versus CSP:*mALS* two-cassette 6*Lac*QR, 35S:*HPT* three-cassette 6*Lac*QR versus CSP:*mALS* three-cassette 6*Lac*QR, 35S:*HPT* single-cassette 12*Lac* versus CSP:*mALS* single-cassette 12*Lac*, 35S:*HPT* two-cassette 12*Lac* versus CSP:*mALS* two-cassette 12*Lac* and 35S:*HPT* three-cassette 6*Lac*QR versus CSP:*HPT* three-cassette 6*Lac*QR) used for comparison. However, in order to obtain transgenic plant with higher accumulation steady, some prospective T_1 lines should be selected among the T_1 population, because these accumulation levels in T_1 seeds may be usually influenced by physiological conditions of mother plants. The next logical set of experiments would be to examine the influence of constitutive promoters on seed development as an indicator of the greater effect that foreign gene expression can have on tissues that show promise as vectors for pharmacoactive products.

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