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Original Paper

Expression of an enhancin gene from the *Trichoplusia ni* granulosis virus confers resistance to lepidopterous insect pests to rice

Mari Mori[1]*, Harushige Kitamura[1], Atsushi Kondo[2], Koji Dohi[3,4], Masashi Mori[3,4], Masanori Kaido[5], Kazuyuki Mise[5], Eiichi Shimojyo[6], Yoshifumi Hashimoto[6]

[1] Shiga Prefecture Agricultural Technology Promotion Center, Azuchi, Shiga 521-1301, Japan; [2] Shiga Prefecture Government, Kyo-machi, Otsu, Shiga 520-8577, Japan; [3] Ishikawa Prefectural University, Nonoichi-machi, Ishikawa 921-8836, Japan; [4] CREST, Japan Science and Technology Agency, Kawaguchi, Saitama 322-0012, Japan; [5] Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan; [6] Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585, Japan
* E-mail: mori-mari@pref.shiga.lg.jp Tel: +81-748-46-3083 Fax: +81-748-46-3567

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Abstract Transgenic plants that produce anti-insect substances are vital in improving crop yields and in reducing the environmental risks of chemical insecticides. Enhancin is a metalloprotease produced in occlusion bodies of the *Trichoplusia ni* granulovirus (TnGV). It is a key substance that enhances infection of the nucleopolyhedrovirus in lepidopteran insects. Rice (*Oryza sativa* L. cv. Nipponbare) protoplasts were cotransformed with pREX*Φ*VEF and pLTHyg, which respectively bear the chimeric enhancin gene and the hygromycin-resistance gene. Hygromycin-resistant regeneration plants were examined by genomic polymerase chain reaction and genomic Southern and northern blotting analyses to confirm the presence and expression of the enhancin gene. Fourteen transgenic plant lines harboring the enhancin gene were obtained, and stable inheritance and expression of the enhancin gene were confirmed in the second, third, and fourth plant generations. Feeding *Spodoptera exigua* larvae leaves of enhancin-expressing rice plants in the presence of *S. exigua* nucleopolyhedrovirus occlusion bodies enhanced infection of the virus. Further, the development of *Pseudaletia separata*, *S. exigua*, and *S. litura*, none of which are host insects of TnGV, was inhibited when these larvae were fed enhancin-expressing rice leaves. This indicates that expression of the enhancin gene confers resistance to lepidopteran insect pests in rice.

Key words: Baculovirus, enhancin, insect resistance, lepidopteran insects, transgenic rice.

Insect pests pose serious problems to the cultivation of agricultural crops. In modern agriculture, the extensive use of chemical insecticides has contributed to stable crop yields. However, the use of insecticides has resulted in the emergence of insect populations resistant to them. Insecticides also have environmental consequences and risks to human health. The breeding of crops resistant to insects is an effective pesticide-free option for controlling insect pests. Genetic engineering is expected to provide powerful tools for producing insect-resistant crops.

In the breeding of rice plants, several genes involved in resistance to hemipteran insects such as leafhoppers have been identified (Athal et al. 1971; Lakshminarayama et al. 1977; Siwi et al. 1977; Sidhu et al. 1979; Rezaul et al. 1982; Ghani et al. 1988; Kabir et al. 1988). However, the rice genes involved in resistance to lepidopteran pests have not been identified.

Enhancin is a protein isolated from the occlusion bodies (OBs) of the *Trichoplusia ni* granulovirus (TnGV) that enhances infection of the nucleopolyhedrovirus in *T. ni* larvae (Derksen et al. 1988; Gallo et al. 1991; Wang et al. 1994; Lepore et al. 1996). The TnGV enhancin gene has been isolated from the TnGV genome and sequenced (Hashimoto et al. 1991). Enhancin is a metalloprotease that degrades mucin, a major constituent of the peritrophic membrane in the larval midgut. It causes an increase in the permeability of the peritrophic membrane, which allows virus particles to infect epithelial midgut tissues (Wang and Granados 1997; Peng et al. 1999).

The TnGV enhancin gene has been utilized in the production of transgenic plants resistant to lepidopteran insects. Transgenic tobacco plants supplemented with the

Abbreviations: Bt, *Bacillus thuringiensis*; *hph*, hygromycin phosphatransferase (hygromycin-resistance gene); OBs, occlusion bodies; PCR, polymerase chain reaction; SeNPV, *Spodoptera exigua* nucleopolyhedrovirus; TnGV, *Trichoplusia ni* granulovirus.
This article can be found at http://www.jspcmb.jp/

TnGV enhancin gene enhanced infection of the *Spodoptera exigua* nucleopolyhedrovirus (SeNPV) in the larvae of a lepidopteran insect, *S. exigua*, and inhibited the growth of *T. ni* (Hayakawa et al. 2000; Cao et al. 2002). However, the effect of transgenically expressed enhancin in other plants and to other lepidopteran insects is unknown.

In this study, the TnGV enhancin gene was introduced into rice plants to investigate the effect of enhancin on monocot plants and to produce a novel breeding material resistant to lepidopteran pests. The effect of the transgene on three lepidopteran insects, including a rice insect pest, was then examined. An insect bioassay revealed that transgenic rice plants expressing the enhancin gene inhibited the growth and development of lepidopterous larvae in *Pseudaletia separata*, *S. exigua*, and *Spodoptera litura*.

Materials and methods

Plasmids

Plasmids pREX*Φ*GUS and pLTRHyg (Figure 1) were gifts from Dr. H. Hirochika, National Institute of Agrobiological Sciences Japan. The plasmid pREX*Φ*GUS was a derivative of pE7133-GUS (Mitsuhara et al. 1996), and contains a modified cauliflower mosaic virus (CaMV) 35S promoter consisting of seven tandem repeats of enhancer-like elements and the 35S core promoter (E7 and P35S, respectively, in Figure 1), the 90 bp leader sequence of the rice stripe virus coat protein gene (*Φ*, in Figure 1; Hayano et al. 1990), the first intron of a gene for phaseolin (In, in Figure 1), and the polyadenylation signal of the gene for nopaline synthase (Tnos, in Figure 1). The plasmid pLTRHyg contains a hygromycin-resistance gene (*hph*, in Figure 1) under the control of the tobacco retrotransposon Tto1 promoter (PLTR, in Figure 1; Hirochika 1993) and Tnos.

Plasmid pREX*Φ*VEF was constructed as follows. The plasmid pREX*Φ*GUS was digested with *Bam*HI, blunted, and ligated with an *Xba*I linker to obtain pREX*Φ*GUS-XbaI. An *Xba*I-*Fba*I (blunted) fragment of pBI-Enh21 (VEF, in Figure 1; Hashimoto et al. 1991) encoding TnGV enhancin was cloned between the *Xba*I and SacI (blunted) sites of pREX*Φ*GUS-XbaI to create pREX*Φ*VEF.

Plant materials and transformation

Protoplasts were prepared from rice (*Oryza sativa* L. cv. Nipponbare) suspension culture as previously described (Kyozuka et al. 1987). The protoplasts were cotransformed with plasmids pREX*Φ*VEF and pLTRHyg using a polyethylene glycol (PEG)-mediated method (Hayashimoto et al. 1990) to produce Enh plants. Similarly, the protoplasts were transformed with only pLTRHyg to produce Hyg plants, which were used as a control. Plasmid pREX*Φ*VEF (40 μg), 20 μg of plasmid pLTRHyg, and 20 million rice protoplasts in 1 ml MaMg solution were used for cotransformation. The transformed protoplasts were cultured on an agarose bead (Shillito et al. 1983) with nurse cells (Kyozuka et al. 1987) for 10 days. They were then selected for hygromycin resistance on agar medium supplemented with 50 mg l^{-1} hygromycin B for three to four weeks. Hygromycin-resistant callus were transferred to a regeneration medium (N6 basal medium [Chu et al. 1975], 6% (w/v) sucrose, 1% (w/v) agarose [Type I, Sigma], pH 5.8) supplemented with 50 mg l^{-1} hygromycin B. Regenerated plants were transplanted into six-inch pots (Fujiwara Sci. Co.) and cultivated in a greenhouse under conditions of natural day length (30°C for 16 h during the day and 25°C for 8 h during the night).

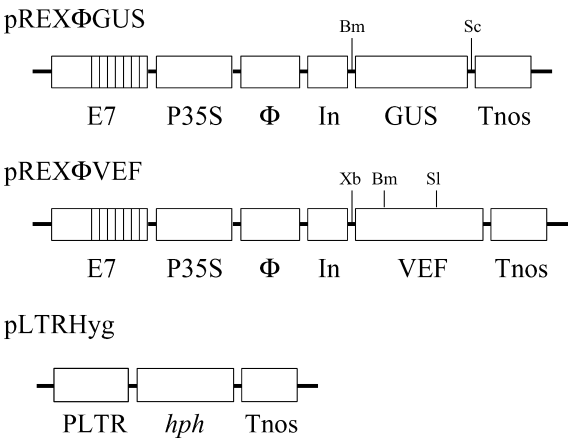

Figure 1. Schematic representation of plasmids used to produce transgenic rice plants. E7: Seven tandem repeats of enhancer-like elements of the CaMV 35S promoter. P35S: The CaMV 35S core promoter. *Φ*: Leader sequence of the ripe stripe virus coat protein gene. In: The first intron of a gene for phaseolin. GUS: β-Glucuronidase. Tnos: Polyadenylation signal of the nopaline synthase gene. VEF: TnGV enhancin gene. PLTR: Promoter of tobacco retrotransposon Tto1. *hph*: Hygromycin-resistance gene. Xb: *Xba*I. Bm: *Bam*HI. Sl: *Sal*I. Sc: *Sac*I.

DNA analyses

Rice plant genomic DNA and callus tissues were isolated using a CTAB method (Murray and Thompson 1980). The enhancin gene in the genomic DNA was detected with polymerase chain reaction (PCR) using primers Enh-up-fwd (5′-TCAGAGTCGGTGAGAATTGG-3′) and Enh-up-rv (5′-TTCGAATCGACAGTGTCTGC-3′), and primers Enh-dwn-fwd (5′-GAATAGGACATTGGCACGAC-3′) and Enh-dwn-rv (5′-CGACAGTCGATAACTGACTG-3′). These primers were expected to amplify the 543 bp and 690 bp regions of the DNA fragments corresponding to the upstream and downstream junction regions of the enhancin gene and vector,

respectively. The *hph* gene in the genomic DNA was detected by PCR using the primers hp-fwd (5′-ATGAAAAAGCCTGAACTCACCG-3′) and hp-rv (5′-GCATCTACTCTATTCCTTTGCC-3′), which were expected to amplify a 1.0 kbp region of the DNA fragment. Conditions for the PCR reaction consisted of annealing for 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C.

Southern blot analyses were carried out as described by Sambrook et al. (1989). A DIG-labeled probe used to detect the enhancin gene was prepared as follows. A *Bam*HI-*Sal*I fragment of pREX*Φ*VEF, which contains a 1.1 kbp fragment of the enhancin gene, was cloned between the *Bam*HI and *Sal*I sites of pBluescript KS+ (Stratagene) to create pBluescriptVEF. A DIG-labeled probe was transcribed from pBluescriptVEF using T7 RNA polymerase with the DIG RNA Labeling Kit (Roche). The enhancin gene was detected using the DIG Luminescent Detection Kit (Roche).

RNA analyses

Total RNA was extracted from rice plant leaves as follows. Leaves were homogenized in a buffer containing 100 mM glycine, pH 9.5, 100 mM sodium chloride, 10 mM Na_2EDTA, 1% sodium dodecyl sulfate, and 0.06% (w/v) bentonite. They were then extracted with phenol–chloroform, followed by ethanol precipitation. Northern blotting analyses were carried out as described by Mori et al. (2001). A DIG-labeled probe used to detect enhancin mRNA was prepared as described in the procedures of the Southern blot analyses.

Insect bioassay of nuclear polyhedrosis virus infection enhancement

Second stadium larvae of *S. exigua* and SeNPV strain #1 (Kondo et al. 1994) were used in this study. SeNPV OBs were prepared from *S. exigua* larvae infected with the virus as described by Hayakawa et al. (2000).

Rice plant leaves were lyophilized, powdered, and mixed with an artificial diet (Insecta LF, Nihon-Nosan-Kogyo) at a rate of 3% (w/w). The diet was made rod shaped by pushing it through a 1 ml disposable syringe. Rod-shaped artificial diet (5 mm) mixed with lyophilized leaves was fed to each larva. When SeNPV was fed to the larvae, 1 μl of suspension supplemented with 10^1, 10^2, 10^3, 10^4, 10^5, or 10^6 OBs was added to diet pellets placed in a 12-well plate. Second stadium larvae were individually placed in the plate wells. Sixty larvae were used for each bioassay with the respective OB doses. After 24 h, the larvae were transferred onto a new plate filled with fresh diet pellets and reared at 25°C in the dark. The LD_{50} of OB numbers per *S. exigua* larva was then calculated (Finney 1964). To evaluate the enhancement of baculovirus infection, the enhancement index $\log_{10}$ was calculated according to Hukuhara et al. (1987).

Insect toxicity bioassay using the artificial diet supplemented with enhancin-expressing rice leaves

The neonate larvae of *P. separata*, *S. exigua*, and *S. litura* were used to investigate the toxicity of rice plants. Rice plant leaves were lyophilized, powdered, and mixed with an artificial diet (Insecta LF, Nihon-Nosan-Kogyo). The mixed diet contained lyophilized leaves at 5% (w/w) for *S. exigua* and *S. litura*, and 20% (w/w) for *S. separata*. One larva was placed in each well of a 12-well plate with the diet pellets and reared at 25°C in the dark. Body weight, pupation rate, and emergence rate were monitored until the larvae fed with the control diet either emerged or died.

Insect toxicity bioassay using whole plants

Second stadium larvae of *P. separata* were used for whole plant bioassays. Rice plants were individually transplanted into six-inch pots (Fujiwara Sci. Co.). Five larvae were placed on each plant at the maximum branching stage, covered with a net, and reared in a greenhouse. Larvae bodyweights were measured 4 and 8 days after release.

Results

Production of transgenic rice plants expressing enhancin

Rice (*Oryza sativa* L. cv. Nipponbare) protoplasts were cotransformed with plasmids pREX*Φ*VEF and pLTRHyg (Figure 1), which respectively bear the enhancin and *hph* genes, using the PEG method. They were then cultured and selected for hygromycin resistance. Twelve hygromycin-resistant callus lines were obtained, among which five lines showed enhancin- and *hph*-specific bands following PCR analyses of the genomic DNA (data not shown). Transgenic plants regenerated from a transgenic callus were considered to belong to a transgenic plant line. Two such lines, designated Enh1 and Enh2, were obtained. They consisted of five (Enh1-1 to 1-5) and 10 (Enh2-1 to 2-10) sublines, respectively. Genomic PCR analyses revealed that all Enh plants other than Enh1-5 possessed the recombinant enhancin gene (data not shown). Genomic Southern analyses of Enh1 and Enh2 sublines other than Enh2-10 (T1 generation) showed that the pattern of hybridization bands was very similar among the Enh1 sublines Enh1-1 to 1-4 and the Enh 2 sublines Enh2-1 to 2-9, respectively (Figure 2).

The expression of enhancin in the Enh plants was examined by northern blotting analyses of T1 plants. Enh2-3, Enh2-9, and Enh2-10 plants showed signals corresponding to enhancin mRNA (Figure 3). However,

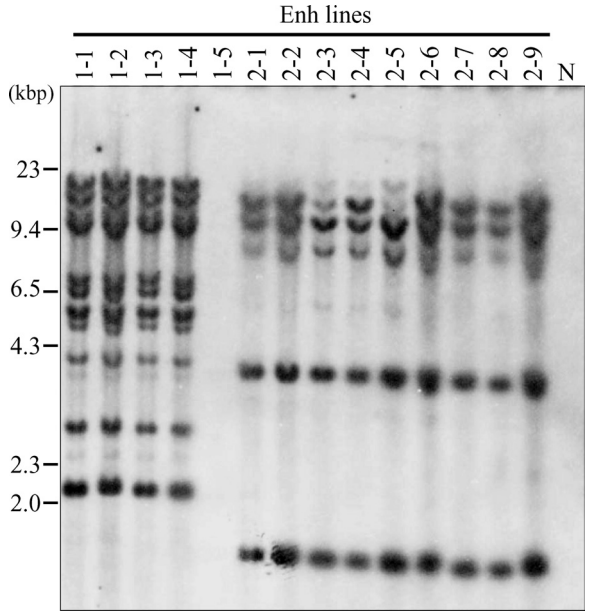

Figure 2. Southern blotting analyses of transgenic (T1 generation) and nontransformed rice plants. Genomic DNA (5 μg) was digested with *Xba*I, separated in 0.7% agarose gel, and subjected to Southern hybridization. A DIG-labeled RNA probe specific to the enhancin gene was transcribed from pBluescriptVEF using T7 RNA polymerase. N: Nontransformed plants.

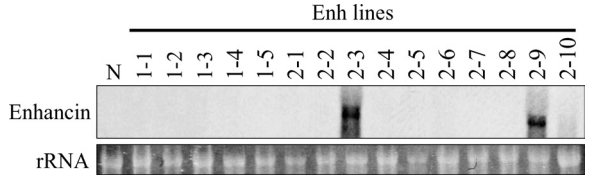

Figure 3. Northern blot analyses of total RNA extracted from transgenic rice plants (T1 generation). RNA (12 μg) was separated in 0.7% agarose gel in each lane and subjected to northern hybridization. A DIG-labeled RNA probe specific to enhancin mRNA was prepared as described in the legend of Figure 2. N: Nontransformed plants.

enhancin mRNA was not detected in the remaining Enh lines.

Morphological fertility and inheritance of enhancin-expressing transgenic rice

Morphology and fertility was observed in the Enh2-3 progenies that harbored the enhancin gene. Average culm lengths in T2, T3, and T4 generations of Enh2-3 plants were 49.8±0.8, 49.1±0.9, and 47.2±1.0 cm (mean±S.E., n=16), respectively, whereas the culm length for nontransformed plants was 53.9±1.2 cm (mean±SE, n=16). This indicates that the culm length of transgenic plants is slightly shorter than that of nontransformed plants. Panicle lengths of T2, T3, and T4 generations of Enh2-3 plants were 17.5±0.4, 17.4±0.4, and 18.3±0.3 cm (mean±S.E., n=16), respectively, and that of nontransformed plants was 17.9±0.3 cm (mean±S.E., n=16). Panicle numbers of T2, T3, and T4 generations of Enh2-3 plants were 11.4±0.3, 10.8±0.5, and 10.7±0.5 (mean±S.E., n=16), respectively, and that of nontransformed plants was 9.5±0.4 (mean±S.E., n=16). Progeny fertility was not significantly different between the transgenic and nontransformed plants (data not shown).

Inheritance of the enhancin gene was confirmed by genomic PCR and northern blotting analyses of Enh2-3 progenies. The enhancin-specific genomic PCR band was detected in 43 of the 64 plants in the second generation (T2) of Enh2-3. Enhancin mRNA was detected in 26 of the T2 plants. Inheritance of the enhancin gene was also confirmed in T3 and T4 plants (data not shown).

Following insect bioassay experiments, we chose Enh2-3, in which strong enhancin expression was observed, and Enh1-2, in which detectable enhancin mRNA was not observed, but the enhancin transgene was present. T2 progenies of Enh1-2 and Enh2-3 with the enhancin gene were selected according to genomic PCR analyses, and used for the experiments.

Enhancement of SeNPV infection in S. exigua *larvae fed enhancin-expressing rice leaves*

Various amounts of SeNPV OBs were added to the diet supplemented with rice leaves and fed to *S. exigua* larvae for 24 h to infect them with the virus. The larvae were subsequently reared with the diet supplemented with rice leaves, and the number of larvae that died before pupation was counted to calculate the LD_{50} of the OBs. Larvae fed with Enh2-3 plants were killed by SeNPV infection with significantly lower amounts of SeNPV OBs than larvae fed with nontransformed plants or Hyg plants (vector control), which showed no detectable enhancin DNA, but possessed the transgene. The LD_{50} of OBs was 7.25×10^5 in larvae fed the diet supplemented with nontransformed plants, 2.40×10^5 in those fed the diet with Hyg plants, and 1.82×10^4 in those fed the diet with Enh2-3 plants. The enhancement index calculated from the above result was 0.48 for Hyg plants and 1.60 for Enh2-3 plants. This indicates that enhancin-expressing transgenic rice leaves enhanced the infection of SeNPV in the larvae.

Transgenic rice plants expressing enhancin inhibited the development of P. separata*,* S. exigua*, and* S. litura

Larvae of *P. separata*, *S. exigua*, and *S. litura* were reared on an artificial diet supplemented with transgenic rice plant leaf powders. Feeding a diet supplemented with transgenic rice plants harboring *hph*, which was used as a vector control, did not affect larval bodyweight, pupation, and emergence rates in any of the insect species examined (Table 1). *P. separata* larvae fed the diet supplemented with Enh2-3, which strongly expressed enhancin, had significantly lower bodyweights

Table 1. Growth of lepidopteran larvae fed an artificial diet supplemented with leaves of high enhancin-expressing (Enh2-3), low or no enhancin-expressing (Enh1-2), vector control (Hyg), and nontransformed rice plants.

Insects	Days after feeding	Mean body weight per larva (mg)±SE			
		Nontransformed	Hyg plant	Enh1-2	Enh2-3
P. separata (n=30)	8	41±1	38±1	38±2	28±2
	12	401±24	357±24	358±28	247±21
	16	759±31	764±33	687±50	627±38
S. litura (n=20)	12	165±19	161±32	134±27	126±17
S. exigua (n=48)	6	NT[a]	18±1	13±1	13±1
	10	NT	157±7	147±9	144±11

[a] Not tested.

than larvae fed the diet supplemented with nontransformed and Hyg plants (Table 1). The growth of larvae fed the diet supplemented with Enh1-2, in which detectable enhancin mRNAs were not observed, was slightly lower than that of the control (Table 1).

The metamorphic development of insects fed the diet supplemented with Enh1-2 or Enh2-3 leaves was investigated. Three independent experiments produced similar results, and typical results are shown in Figure 4. Compared with the controls, *P. separata* larvae fed the diet supplemented with Enh1-2 or Enh2-3 had delayed pupation and significantly lower emergence rates (Figure 4A). Similar results were obtained in the larvae of *S. litura* (Table 1, Figure 4B). *S. exigua* larvae fed diets supplemented with Enh1-2 or Enh2-3 plants had delayed pupation and emergence, but their bodyweight was not affected, (Table 1, Figure 4C). These results suggest that biologically active enhancin was expressed in both Enh1-2 and Enh2-3 plants.

Insect toxicity of whole rice plants

To investigate whether enhancin in living tissues affects the growth of lepidopteran insect pests, *P. separata* larvae were reared on whole plants of Enh2-3 that expressed enhancin from 4 to 8 days. The average bodyweight of larvae fed Enh2-3 plants was 19.6% to 23.1% lower than that of larvae fed nontransformed plants. Values were 148±17 and 340±36 mg (mean± S.E., n=30) for larvae fed transgenic rice plants for 4 and 8 days, respectively, and 184±17 and 442±26 mg (mean±S.E., n=30) for larvae fed nontransformed plants. The pupation of larvae fed Enh2-3 plants was significantly delayed compared with the control (data not shown). These results indicate that transgenic enhancin-expression confers the ability to inhibit the development of *P. separata* larvae in rice.

Discussion

Feeding *S. exigua* larvae transgenic rice plants expressing enhancin facilitated infection of the nuclear polyhedrosis virus. This indicates that the enhancin produced in transgenic rice plants enhances infection of the virus. Other than a slightly shorter plant length, which is often observed in rice plants regenerated from protoplasts (Ogura et al. 1987; Abdullah et al. 1989; Kawata et al. 1992), the morphological characters of the transgenic plants were not significantly different from those of nontransformed plants. Moreover, the enhancin gene was inherited in the progenies to at least the fourth generation. These results suggest that enhancin expression does not affect the growth and fertility of rice plants.

When fed artificial diets supplemented with the enhancin-expressing rice plants Enh2-3, the growth, pupation, and emergence of *P. separata*, *S. exigua*, and *S. litura* were inhibited. Growth inhibition was also observed in *P. separata*, an insect pest of rice plants, when fed whole plants of enhancin-expressing rice. These results suggest that enhancin-expressing rice plants inhibit the growth of various species of lepidopteran insect pests. Although detectable enhancin mRNA was not observed in Enh1-2, these plants significantly affected the emergence rates of *P. separata*. This suggests that the metamorphic development of *P. separata* is sensitive to extremely low amounts of enhancin.

It has been reported that transgenic tobacco plants expressing TnGV enhancin inhibited the development of *T. ni*, a TnGV host insect (Cao et al. 2002). However, we found that the development of *P. separata*, *S. exigua*, and *S. litura* was inhibited by TnGV enhancin expressed in transgenic rice plants, even though these insects are not TnGV hosts. These results indicate that the enhancin derived from TnGV had an effect in suppressing the growth of lepidopteran insects regardless of viral host specificity. TnGV enhancin can possibly degrade mucin in the larval midgut of these insects to inhibit their growth. The TnGV enhancin gene could be used to provide resistance against lepidopteran insects in various plant species other than rice, both in monocots and dicots.

Transgenic rice plants that expressed enhancin significantly delayed and inhibited the development of lepidopteran insect pests. Although this inhibition effect was not very severe, it might significantly affect the

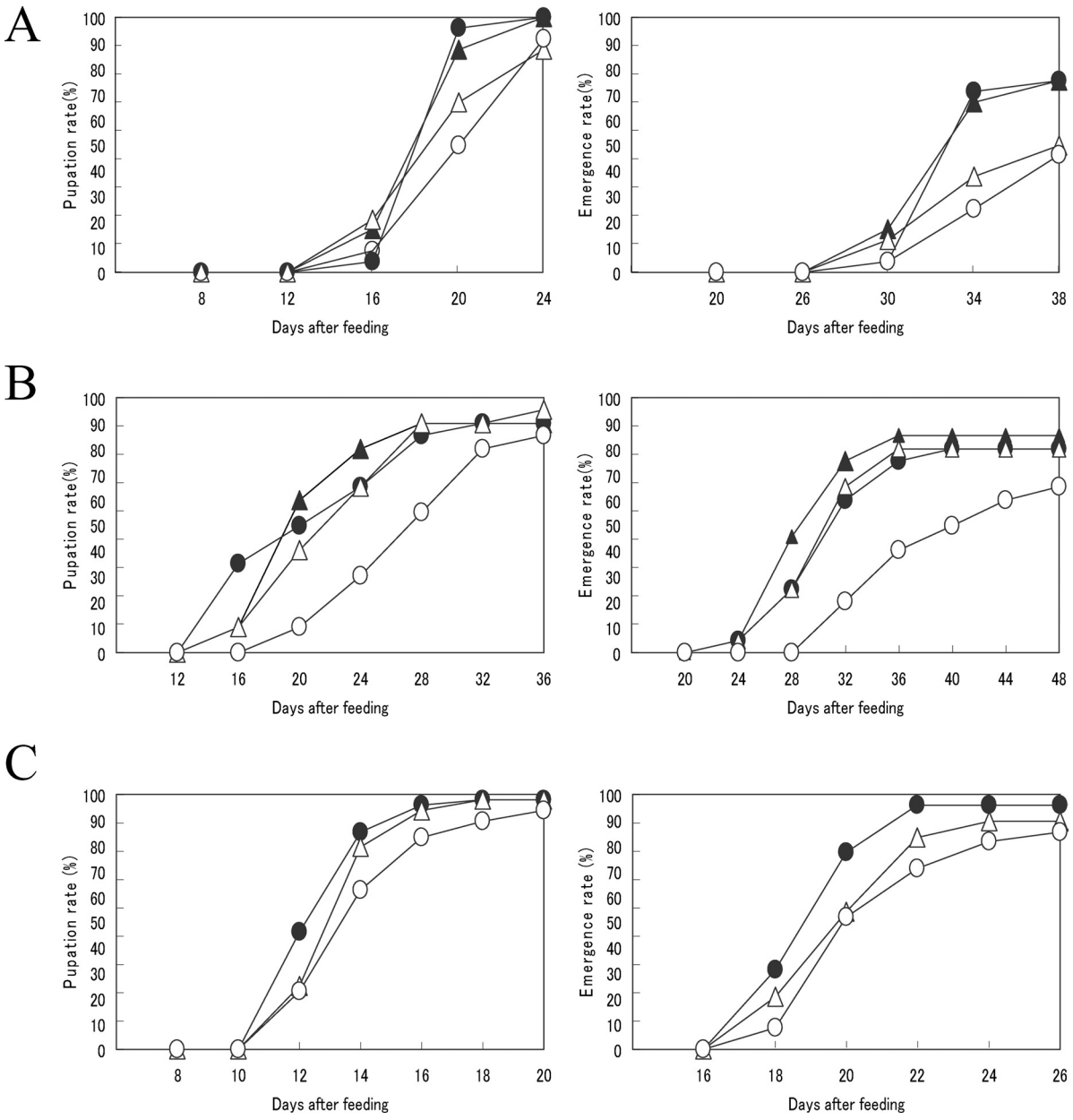

Figure 4. Pupation and emergence rates of lepidopteran larvae fed an artificial diet supplemented with enhancin-expressing rice plants. ▲: Nontransformed. ●: Hyg plants. △: Enh1-2 plants (T2 generation). ○: Enh2-3 plants (T2 generation). (A) *P. separata*, $n=30$. (B) *S. litura*, $n=20$. (C) *S. exigua*, $n=48$.

population of insect pests under field conditions by decreasing their fitness or making their life cycles longer. Combinations of enhancin genes with other insect-resistance genes might provide crops with more efficient resistance against insect pests. The efficiency of insecticides supplemented with the *Bacillus thuringiensis* (Bt) toxin might be increased in enhancin-expressing crops because purified enhancin significantly increases the toxicity of Bt formulations to six species of lepidopteran insects (Granados et al. 2001). Enhancin-expressing rice plants may also facilitate an effect of bioinsecticides supplemented with a modified polyhedrosis virus. Therefore, enhancin-expressing rice plants, which have no deleterious characters, should have excellent potential as a novel breeding material. Transgenic rice plants expressing enhancin could destroy insect pests without the extensive use of chemical insecticides and contribute to stable yields.

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