Development and bioassay of transgenic Chinese cabbage expressing potato proteinase inhibitor II gene

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Lepidopteran larvae are the most injurious pests of Chinese cabbage production. We attempted the development of transgenic Chinese cabbage expressing the potato proteinase inhibitor II gene (pinII) and bioassayed the pest-repelling ability of these transgenic plants. Cotyledons with petioles from aseptic seedlings were used as explants for Agrobacterium-mediated in vitro transformation. Agrobacterium tumefaciens C58 contained the binary vector pBBBasta-pinII-bar comprising pinII and bar genes. Plants showing vigorous PPT resistance were obtained by a series concentration selection for PPT resistance and subsequent regeneration of leaf explants dissected from the putative chimera. Transgenic plants were confirmed by PCR and genomic Southern blotting, which showed that the bar and pinII genes were integrated into the plant genome. Double haploid homozygous transgenic plants were obtained by microspore culture. The pinII expression was detected using quantitative real time polymerase chain reaction (qRT-PCR) and detection of PINII protein content in the transgenic homozygous lines. Insect-feeding trials using the larvae of cabbage worm (Pieris rapae) and the larvae of the diamondback moth (Plutella xylostella) showed higher larval mortality, stunted larval development, and lower pupal weights, pupation rates, and eclosion rates in most of the transgenic lines in comparison with the corresponding values in the non-transformed wild-type line.

Key Words: transgenic Chinese cabbage, Pieris rapae, Plutella xylostella, insect resistance, proteinase inhibitor gene II (pinII), chimera.

Introduction

Chinese cabbage (Brassica campestris ssp. pekinensis), which is a widely grown vegetable crop in Asia, is increasing in popularity in the world. Insect pests, especially Lepidopteran insects such as cabbage worms (Pieris rapae) and larvae of the diamondback moth, cause severe damage in the production of Chinese cabbage and other crucifer crops by reducing the yield and quality of the crops. The use of synthetic insecticides to control the pests has raised concerns about food safety and environmental pollution (Jiang et al. 2008). Moreover, insects have developed resistance to many of the available insecticides (Shelton et al. 1993), which has made pest control more complex. Genetic breeding for pest resistance is a promising alternative to insecticides. However, conventional breeding of Chinese cabbage for obtaining plants with higher level of pest resistance is limited by germplasm resources and long breeding times. Therefore, it is desirable to develop insect-resistant crops through the direct introduction of foreign insecticidal genes into the cultivars (Ferry et al. 2006). The Bt cry1A gene, which expresses endotoxins from Bacillus thuringiensis, is the most popular and effective gene used in many transgenic plants, including some Brassica crops (Earle and Knauf 1999). However, diamondback moth populations with high level of resistance to Cry1A proteins have been collected from various field locations after exposure to Bt sprays (Shelton et al. 1993, Tabashnik et al. 1990). In addition, the cultivation of Bt crops is associated with the concern that constitutive expression of these insecticidal proteins may result in selection of insect-resistant populations (Eizaguirre et al. 2006). Plant-derived protease inhibitors (PIs) exert lower selection pressure on insect pests and are regarded as a viable alternative to Bt endotoxins in insect pest control (Ferry et al. 2006, Mosolov and Valueva 2008). PIs have been used to enhance the resistance of transgenic plants to insect pests because of their small size, abundance, stability and high specificity for a particular class of insect digestive enzymes (Maheswaran et al. 2007, Ussuf et al. 2001). PIs of the potato inhibitor II family, such as PINII, are the best-characterized plant serine PIs that possess reactive sites towards the 2 serine proteinases trypsin and chymotrypsin (Balandin et al. 1995, Choi et al. 2000, Keil et al. 1986, Sanchez-S et al. 1986). Serine protease are known to be predominant in the intestine of
Lepidoptera insects and account for up to 95% of the total proteolytic activity (Srinivasan et al. 2006). Transgenic plants expressing the pinII gene are expected to be well protected against insects of the order Lepidoptera (Lawrence and Koundal 2002, Mosolov and Valueva 2008). Some pinII transgenic plants have shown significant insect resistance in the form of increased mortality and developmental inhibition of the larvae fed on the transgenic leaves (Duan et al. 1996, Klopfenstein et al. 1998).

Chinese cabbage is known to be resistant to shoot regeneration and transformation (Narashimhulu and Chopra 1988). It is very genotype-dependent and has a transformation frequency lower than that of other Brassica species (Zhang et al. 2000). Although there are several successful transformation reports for Chinese cabbage, most of them did not involve the transfer of agriculturally important genes (Jun et al. 1995, Zhang et al. 2000). Cho has shown lepidopteran insect pest control in Chinese cabbage that was transformed with a synthetic cry1C gene (Cho et al. 2001), and Yu reported the development of turnip mosaic virus-resistant transgenic Chinese cabbage with the Nib replicase gene (Yu et al. 2007). No reports on the production and evaluation of transgenic Chinese cabbage with the potato pinII gene have been published so far. Furthermore, as Brassica crops are cross-pollinated, it takes about 8 years of continuous selfing to obtain homozygous transgenic plants. In this paper, we report our work on the effective development of homozygous pinII transgenic Chinese cabbage combining Agrobacterium-mediated in vitro transformation and microspore culture and the evaluation of the resistance to lepidopteran insect pests, including cabbage worms (Pieris rapae) and diamondback moths (Plutella xylostella).

Materials and Methods

Plant material and explants

The Chinese cabbage cultivar Beijing 80 (bred by Beijing Vegetable Research Center, China) was used as the plant material. Seeds were germinated in a sterile environment on solid MS basal medium (Murashige and Skoog 1962) in a 100-mL conical flask. Cultures were maintained at 22 ± 3°C under a 16-h photoperiod with a photosynthetic photon flux density of 45 μmol·m⁻²·s⁻¹ provided by cool white fluorescent lamps. Cotyledons with a 1–2 mm petiole were excised from 3-day-old seedlings and used as explants for plant transformation.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain C58 harboring a binary vector pBBBasta-pinII was constructed for the transformation (Fig. 1). The plasmid pBBBasta contained the phosphinotricin (PPT)-resistant bar gene driven by a CaMV 35S promoter (a donation from Dr. Robaglia, CEA-Cadarache, France). The pinII gene cassette was isolated from plasmid pTW (a donation from Professor Ray Wu, Cornell University, USA). Expression of the pinII gene was regulated by the

![Fig. 1. The map of the plasmid pBBBasta-pinII. PinII-CR represents the pinII gene; PinII-5' and PinII-3' represent the promoter and terminator, respectively; RAC15' represents the intron of the rice act1 gene.](image)

\[\text{pinII promoter in combination with the first intron of the rice act1 gene and terminated by the pinII 3' region (Duan et al. 1996).}\]

Genetic transformation and regeneration

The transformation and regeneration were performed according to a previously protocol (Lim et al. 1998). Agrobacterium colonies were grown in 30 mL of LB medium with 100 mg/L kanamycin, 50 mg/L rifampicin and 100 mg/L gentamicin at 28°C with shaking set at 200 oscillations/min until the OD₆₀₀ reached 0.8–1.0. The bacterial cells were then pelleted by centrifugation at 4000 × g for 15 min and resuspended gently in equal volume of the co-culture medium [COM; MS basal medium with 2 mg/L 6-benzyladenine (BA), 1 mg/L naphthaleneacetic acid (NAA) and 100 μmol/L acetylsyringone, pH 5.2]. The explants (cotyledons with a 1–2 mm petiole) were immersed in the Agrobacterium suspension for 15 min, blotted dry with sterile filter paper to remove excess bacteria and co-cultivated on 0.8% agar-solidified COM medium at 25°C for 3 d. The culture dishes were sealed with micropore tape. After co-cultivation, the explants were transferred to the Regeneration and Selection Medium 1 [RSM1; MS basal medium with 2 mg/L BA, 1 mg/L NAA, 4 mg/L silver nitrate (AgNO₃), 3.5 mg/L PPT, 300 mg/L carbenicillin (Cb) and 1.0% agar, pH 5.8]. The regenerated adventitious shoots were separated from the explants 3–5 weeks later and were transferred to the Growth and Selection Medium (GSM; MS basal medium with 0.02 mg/L BA, 0.01 mg/L NAA, 6 mg/L PPT, 100 mg/L Cb and 1.0% agar, pH 5.8).

Regeneration of young leaf explants to eliminate chimerism

Young green leaves were dissected from the plants grown in the GSM medium, and sliced pieces were incubated on Regeneration and Selection Medium 2 (RSM2; MS basal medium with 2 mg/L BA, 1 mg/L NAA, 4 mg/L AgNO₃, 7 mg/L PPT and 1.0% agar, pH 5.8) to induce adventitious shoot regeneration. After rooting, the plantlets were transferred to soil in pots and maintained in a greenhouse for further analysis.

Molecular analysis of the transgenic plants

Total genomic DNA was isolated from the leaf tissue of the putative transgenic plants and the control non-transformed plants by the cetyltrimethylammonium bromide method (Fulton et al. 1995). PCR was performed to identify the presence of the bar gene and the pinII gene. The primers used for bar gene amplification were as follows: 5'-
the plasmid pBBBasta-pinII was digested with EcoRI, which cut the T-DNA at a unique restriction site, to determine the number of independent loci of transgene integration. Digested genomic DNA was fractionated on a 0.8% agarose gel and electrophoresis. The total RNA was reverse-transcribed by a Bioteke super RT kit (Bioteke Corp., Beijing, China) according to the manufacturer's instructions. Quantitative and qualitative analyses of isolated RNA were assessed from the ratio of absorbance at 260 and 280 nm, and agarose gel electrophoresis. The total RNA was reverse-transcribed by a Biotek super RT kit (Biotek Corp.). A 165 bp pinII cDNA fragment was amplified by using the following pinII primers: 5′-CCACGTTCCAGAAGGAAGTCC-3′ (forward) and 5′-GTAGCCATATGGGATC GC-3′ (reverse) with an annealing temperature of 62°C. As an internal standard gene, the expression of *actin*, a 210 bp cDNA fragment, was analyzed with the following *actin* primers: 5′-CTGGACCTGCCTCAATCATA-3′ (forward) and 5′-GTGCTCAGTGGTG AAACAC-3′ (reverse).

Real-time qPCR amplification was performed using the LightCycler® 480 system (Roche Diagnostics). The reaction system was prepared in a 20 μL solution containing 2 μL of 20-fold-diluted cDNA, 0.2 μM of each primer and 1× SYBR® Premix Ex Taq™ (TaKaRa Bio Inc., Shiga, Japan). Relative mRNA levels were calculated as described previously (Miura et al. 2007). The relative mRNA level was calculated according to the following formulas: 2−ΔΔCt [ΔCt(X sample) = Ct(X sample, pinII) − Ct(X sample, actin), ΔΔCt = ΔCt(X sample) − ΔCt(calibrator)]. In our case, the calibrator was T80-3-6.

**Proteinase inhibitory activity assay**

The total protein was extracted from young leaves at rosette stages of the transgenic plants and non-transgenic plants (as control). The leaves were ground in liquid nitrogen and cold extraction buffer (100 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 2 mmol/L CaCl2, 4 mol/L guanidine thiocyanate, 14 μmol/L β-Mercaptoethanol) was added in proportion of 10 mL per g leaves (Maheswaran et al. 2007), then kept at 4°C for 8 hours. After centrifugation at 12,000 rpm for 35 min, the protein content was detected by Bradford protein assay (Bradford 1976) for the equality of the total soluble protein in different samples. Proteinase inhibitory activity was estimated spectrophotometrically based on the remaining proteolytic activity, after incubating a fixed amount of trypsin (bovine pancreatic trypsin, Sigma Chemical Co.) with different samples of protein extracts for 30 min, using benzoyl-L-arginine ethyl ester (BAEE) (Sigma) as the substrate (Fang et al. 2010). The inhibition rate was calculated as (A253f−A253i)/A253f, A253f means the initial absorbance of the sample at 253 nm and A253i means the end absorbance of the sample at 253 nm after incubating with the protein extracts for 30 min.

**Insect-feeding bioassays**

Insect-feeding bioassays were performed using detached leaves. Expanding leaves were detached from parallel sites on the transgenic and control plants. The detached leaves were dipped in 0.1% sodium hypochlorite for 5 min, rinsed well with sterilized water, and then blotted dry using tissue paper. The leaves were placed on moistened sterile filter paper in petri dishes with a diameter of 9 cm. Freshly hatched larvae were used for a feeding assay. Three larvae of cabbage worms (*Pieris rapae*) per dish for 12 replications and 10 larvae of diamondback moth (*Plutella xylostella*) per dish for 5 replications were inoculated on the leaves separately. The leaves were replaced with fresh leaves every 2 days. The mortality and developmental status of the larvae were observed and recorded. Pupae were weighed and maintained.
until adults emerged, at which time sex was determined.

Data are presented as means and their standard deviation. To evaluate the differences among the transgenic plants studied, one-way ANOVA was used to analyze all data. Probability values of $P < 0.05$ was considered statistically significant.

**Results**

*Transformation and regeneration of Basta-resistant plantlets*

Explants of Chinese cabbage were sensitive to *Agrobacterium* infection, and the cut edges of 30–50% of the explants showed various degrees of browning on cocultivation medium for 3 d. After co-cultivation, the healthy explants were transferred onto the RSM1 with 3.5 mg/L PPT and a callus began to form at the cut ends of 40–60% of the explants within 2 weeks. The adventitious shoots emerged after 2–4 weeks (Fig. 2A) and were isolated from the explants and transferred onto the GSM medium with 6 mg/L PPT after growing up to at least 4–5 mm in length. Most of the young shoots were able to sustain vigorous growth on the new selective medium, but some became etiolated and died 1 week later, which indicated that they must be escapes. We noticed that in a few cases, some leaves of the formed plants turned yellow on the GSM medium in an additional 2–4 weeks, which suggested that these plants were chimeras.

*Elimination of chimerism and establishment of transgenic plants in the greenhouse*

Green leaves were dissected from the chimeras, cut into small pieces and incubated on the RSM2 medium with 7 mg/L PPT for regeneration. Gemmiform structures were formed after 3–4 weeks and many of them were shown to be abnormal (Fig. 2B). Some intact shoots with uniform green leaves could be obtained after several subcultures of the regenerated gemmiform structures on the GSM medium, and no yellow leaves were subsequently observed. Plants with roots were obtained in the rooting medium (MS basal medium with 0.01 mg/L NAA and 7 mg/L PPT) and transferred to the greenhouse.

When the plants had acclimated and were growing vigorously, 750 mg/L PPT solution was smeared on 1 leaf of each plant. After 3 days, the treated leaves of the regenerated plants were alive and green, while the leaves of the control wild-type plants turned brown and died gradually (Fig. 2C). This showed that the *bar* gene had been inserted into the regenerated plants.

*Molecular analysis of the transgenic plants*

The results of PCR amplification of the plant genome revealed the presence of the 1.5 kb *pinII* (Fig. 3A) and 0.4 kb *bar* (Fig. 3B) gene fragments in the regenerated plants and the absence of these in the wild-type plants (Fig. 3). Genomic DNA samples from selected transgenic and control plants were digested with the restriction enzyme EcoRI (Fig. 4A) and subjected to Southern blot hybridization to determine the copy number of the inserted foreign genes. No hybridization signal was observed for the control plant (Fig. 4B, lane 2). The herbicide resistant regenerated plant 80-1 had 2 hybridization fragments (10.5 kb and 8.0 kb) (Fig. 4B, lane 3), which suggested the integration of 2 copies of the transgene. The regenerated plants 80-2, 80-3 and 80-4 each showed a single hybridization fragment and the bands were almost at the same position, which suggested the single-copy insertion of the transgene and the same origin of these three regenerated plants (Fig. 4B, lanes 4–6). The hybridization
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The signal was not detected for the regenerated plant 80-5, which indicated that it could be an escape (Fig. 4, lane 7). The identified transgenic plants were named respectively as T80-1, T80-2, T80-3, T80-4 and they were the T0 generation.

DH homozygous transgenic plant production

Microspores were isolated from the flower buds of T80-1 and T80-2 transgenic plants. Embryoids were formed in NLN medium without any selective reagents in about 14 days. Intact plantlets were obtained from torpedo-shaped and cotyledonary embryoids after growing them in the plant-inducing medium (MS basal medium with 0.1 mg/L BA and 3% sucrose) for another 14 days. These plants were separated into transformants and non-transformants according to the presence of Basta resistance when subcultured on plant growth medium with 5 mg/L PPT. The leaves of transformants stayed green while the leaves of non-transformants turned yellow and wilted (Fig. 5).

Quantifying pinII expression levels using qRT-PCR

Amplification curves of the potato pinII gene of the Beijing 80 control plant indicated that there is no endogenous potato pinII gene in its genome while amplification curves of its actin gene were normal. All the amplification curves of both T80-3-6 and T80-2-6 illustrated the expression of a foreign potato pinII gene. Thus, these results prove the existence and the expression of the transferred pinII gene in the DH plants and show that the pinII mRNA level of T80-2-6 is 1.6 times higher than that of T80-3-6 (Fig. 6).

Protease inhibitory activity in the transgenic line

BAEE is a substrate to detect protease activity. The differences of protease inhibitory activity can be displayed as the reduction of protease activity after adding the test sample. In our experiments, the inhibition rate was used to express the reduction of protease activity, and calculated and displayed in Fig. 7. It showed that the protease activity inhibition rate of DH transgenic lines T80-3-6 and T80-2-6 were nearly 2.5 times higher than those of the cross F1 generation C80-2 (transgenic line T80-2 × Beijing 80) and C80-3 (transgenic line T80-3 × Beijing 80). The protease activity inhibition rate of T80-2-6 was higher than that of T80-3-6, which was also consistent with the results of qRT-PCR. As in the control sample, the reduction of protease activity was very low, the high inhibition rate of the other samples relatively reflected the existence and the content of the foreign PINII protein in the transgenic plants.

Bioassays of insect feeding of transgenic plants

Cabbage worm resistance was determined by performing detached leaf assays on primary transformants (T0). As can be seen in Fig. 8, the mortality of cabbage worm larvae increased gradually with the increasing number of feeding days and reached the highest level, 78.3%, at the adult stage. On day 12, six larvae were at the second or third instar. These larvae would eventually die because they could not molt and grow into the next instar, whereas all larvae that fed on wild-type leaves progressed to the fifth instar. Finally, the pupation rate of larvae fed on transgenic leaves was only
38.89%, which was significantly lower \((P < 0.05)\) than the pupation rate of 69.44% for the larvae fed on wild-type leaves (Table 1). However, there were no significant differences in the eclosion rate and the sex ratio between larvae fed on transgenic and control leaves \((P < 0.05)\). We also observed that all the larvae fed on transgenic aged leaves died after 6 d, while only 19.04% of those fed on the transgenic young leaves died (Table 2). The differences between the aged and the young leaves in the wild-type leaf group suggest that the transgenic aged leaves were more toxic than the transgenic young leaves.

Six independent DH transgenic lines derived from the microspore culture of the transgenic plants T80-2 and T80-3 were selected for evaluating the resistance to the diamondback moth. The total mortality of the diamondback moth larvae fed on different DH transgenic lines ranged from 50–90%, which was significantly higher than that for larvae fed on wild-type leaves \((P < 0.05)\). There were also significant differences among the different independent DH transgenic lines. The highest larval mortality was 90% for the T80-2-6 line and the lowest was only 50% for the T80-3-2 line. Larvae died mainly at the first and fourth instars (Fig. 9). The pupal weight, pupation rate and eclosion rate were significantly different between most of the DH transgenic lines and the wild-type leaves \((P < 0.05)\) (Table 3).

**Discussion**

Explants of cotyledons with a 1–2 mm petiole from 3–4-day-old seedlings had strong regeneration capacities and were also relatively easier to infect and transform by *Agrobacterium* than other tissues and organs of Chinese cabbage (Zhang et al. 2000, Zhao et al. 2006). Kanamycin was used as the selective agent in most similar studies, but it was not an ideal selector for the transformation of Chinese cabbage because of the high frequency of escapes at a low kanamycin concentration (Takasaki et al. 1997, Zhao et al. 2006) and a high lethal effect at a high concentration (Zhao et al. 2006). Cho used hygromycin as a selective agent and eliminated the regeneration of any non-transgenic escapes (Cho et al. 2001). Basta was used in this experiment and was shown to be an effective agent for Chinese cabbage transformation. In the course of screening of transformed cells and induction of adventitious buds, the selection pressures of Basta were increased gradually, and this not only permitted the effective relief of the growth pressure of the initially transformed cells but also eliminated the escapes to a certain extent.

In our experiment, we noticed the formation of chimeras on the GSM and intact green plants were obtained by the regeneration of green leaf parts that were dissected from the chimeras. The regeneration of chimeras has also been reported in other works and green fluorescent protein was used to display this phenomenon visually (Bastar et al. 2004, Rakosy-Tican et al. 2007). Site-specific integration effects, reductions in the code protein synthesis, or gene silencing may be the cause of the chimeras observed (Rakosy-Tican et al. 2007).

**Table 1.** Effects on the reproduction of cabbage worms fed with leaves of transgenic (T0) or wild-type Chinese cabbage

| Leaf type          | Pupation rate (%) | Eclosion rate (%) | Female : Male |
|--------------------|-------------------|-------------------|---------------|
| Transgenic leaves  | 38.89 ± 6.75*     | 78.57 ± 7.05      | 7:4           |
| Wild type          | 69.44 ± 8.19      | 84.00 ± 6.06      | 13:8          |

Data are mean ± standard deviation * Significantly different from the wild type control \((P < 0.05)\).

**Table 2.** The larvae mortality (%) of cabbage worms fed with leaves of transgenic (T0) or wild-type Chinese cabbage plants of different ages

| Feed days | Transgenic aged leaf | Wild type aged leaf | Transgenic young leaf | Wild type young leaf |
|-----------|----------------------|---------------------|-----------------------|---------------------|
| 3         | 76.19                | 14.28               | 9.51                  | 0.00                |
| 6         | 100.00               | 19.04               | 19.04                 | 4.75                |

Fig. 8. The larvae mortality of cabbage worms during feeding with *pinII* transgenic or wild-type leaves of Chinese cabbage.

Fig. 9. The larvae of the diamondback moth feeding on transgenic leaves of Chinese cabbage. A living larva is on the left and a dying, growth-stunted larva is on the right.
The microspore culture technique was first established by Nitsch and Norrel (1973) in order to study the development of microspore-derived embryos. Lichter (1982) first reported the successful application of microspore culture technique to *Brassica napus*. Now, microspore doubled haploid (DH) methodology is employed in many *Brassica napus* breeding programs around the world as an alternative/supplement to conventional methods of homozygous line production (Koprna et al. 2005). In this paper, six independent DH transgenic lines were obtained by microspore culture in a short time.

The expression of the transferred *pinII* gene was identified by PCR, genomic southern blotting and qRT-PCR. The contents of the PINII protein were reflected by the inhibition rate of protease activity in the transgenic and control samples. Specific PINII protein quantification should be done by western blotting.

The effect of insect resistance of the *pinII* transgenic Chinese cabbage was confirmed by the bioassay performed using the larvae of cabbage worms and diamondback moths. We did not evaluate the size of the adult after eclosion in this experiment. A strong correlation between pupal weight and reproductive fitness has been reported for *E. postvittiana*, with less heavy pupae resulting in smaller adults that had reduced reproductive fitness (Danthanarayana 1975). Therefore, the smaller pupae that developed from the larvae fed with transgenic Chinese cabbage are likely to show reduced reproductive fitness, which will impact the next generation by reducing the population size. Further work is required to follow the effect of the transgenic food on the progenies of cabbage worms and diamondback moths and on the size of subsequent moth populations.

In conclusion, the use of *Agrobacterium*-mediated *in vitro* transformation and microspore culture yielded homozygous transgenic Chinese cabbage in a short period. Basta solution spraying proved the herbicide resistance of these transgenic plants as all progenies were herbicide resistant (data not shown) and suggested that no *bar* gene silencing existed in the case of twin copies of the DH transgenic plants. T80-2-6 and T80-3-6 had better agronomic characters, higher level expression of *pinII* gene. Thus, enrolling the elite homozygous transgenic lines into the hybrid-breeding program may be profitable.

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**Table 3. Resistance assay of the homozygous DH transgenic lines to diamondback moths**

| Lines    | Mortality (%) | Pupae weight (mg) | Pupation rate (%) | Eclosion rate (%) | Total mortality (%) |
|----------|--------------|-------------------|-------------------|------------------|---------------------|
|          | 1st instar   | 2nd instar   | 3rd instar   | 4th instar | Pupal stage |
| control  | 25.00 0.00 6.67 0.00 3.57 3.70 5.70 | 96.43 ± 3.57 a | 96.30 ± 3.70 a | 35.00 ± 5.92 a | |
| T80-2-1  | 47.50 14.29 11.11 18.75 46.15 | 3.83 ± 0.30 d | 81.25 ± 6.72 c | 53.85 ± 6.20 d | 82.50 ± 5.27 d |
| T80-2-6  | 52.50 42.11 0.00 45.45 33.33 | 3.73 ± 0.25 d | 54.55 ± 4.84 c | 66.67 ± 9.01 d | 90.00 ± 4.80 e |
| T80-2-8  | 42.50 17.39 15.79 25.00 41.67 | 3.64 ± 0.29 d | 75.00 ± 7.77 d | 58.33 ± 5.37 d | 82.50 ± 6.38 d |
| T80-3-2  | 14.29 2.78 2.86 17.65 28.57 | 4.72 ± 0.39 b | 82.35 ± 6.82 c | 71.43 ± 4.43 b | 50.00 ± 4.95 b |
| T80-3-5  | 55.00 5.56 0.00 11.76 6.67 | 4.36 ± 0.30 c | 88.24 ± 4.78 b | 93.33 ± 6.67 a | 75.00 ± 8.26 c |
| T80-3-6  | 62.50 40.00 0.00 22.22 28.57 | 4.84 ± 0.21 b | 77.78 ± 4.09 c | 71.43 ± 7.49 b | 87.50 ± 4.08 de |

Note: the data are presented as means ± standard deviation. In the same column, means sharing the same letters denote nonsignificant differences while different letters denote statistically significant differences with *P* < 0.05. T80-2-1, T80-2-6 and T80-2-8 are independent transgenic lines from the microspore culture of transgenic plant T80-2. T80-3-2, T80-3-5 and T80-3-6 are independent transgenic lines from the microspore culture of transgenic plant T80-3.
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