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TWO LEPIDOPTERAN CELL LINES STABLY TRANSFORMED BY THE ABC TRANSPORTER GENE *PDR5* SHOW TOLERANCE TO DIACETOXYSCIRPENOL

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SUMMARY

The pleiotropic drug resistance 5 gene (*pdr5*) encodes a multidrug membrane transporter and plays a very important role in the efflux of a broad range of chemicals in yeast cells. To study the possible function of *pdr5* in insect cells, two stably *pdr5*-transformed lepidopteran insect cell lines, Sf21 and CF-203, were developed. Transcripts of *pdr5* were detected in these two lines using Northern blotting and RT-PCR analysis. When cells were treated with the protein synthesis inhibitor diacetoxyscirpenol, the transformed Sf21 and CF-203 cell lines showed increased tolerance to this chemical. However, unlike in yeast cells, ecdysone agonist RH5992 could not be excluded by PDR5, probably because of low expression levels or imperfect incorporation of the recombinant protein in these transformed cell lines.

Key words: resistance; ecdysone; transformation; insecticide; CF-203 cell; Sf21 cell

INTRODUCTION

The pleiotropic drug resistance gene *pdr5* was isolated from yeast *Saccharomyces cerevisae* by Balzi et al. (1994). As a member of the ATP binding cassette (ABC) transporter, the PDR5 protein can efflux a broad range of substances, including anticancer drugs, ionophoric peptides, and steroids (Kolaczkowski et al., 1996; Golin et al., 2003). Because of its roles in the exclusion of chemicals that are variable in structure and mode of action, *pdr5* has been used to transform tobacco, resulting in resistance to mycotoxins in the transgenic plants (Muhitch et al., 2000).

The synthetic nonsteroidal ecdysone agonist tebufenozide (RH5992) is an insecticide that is very effective against the spruce

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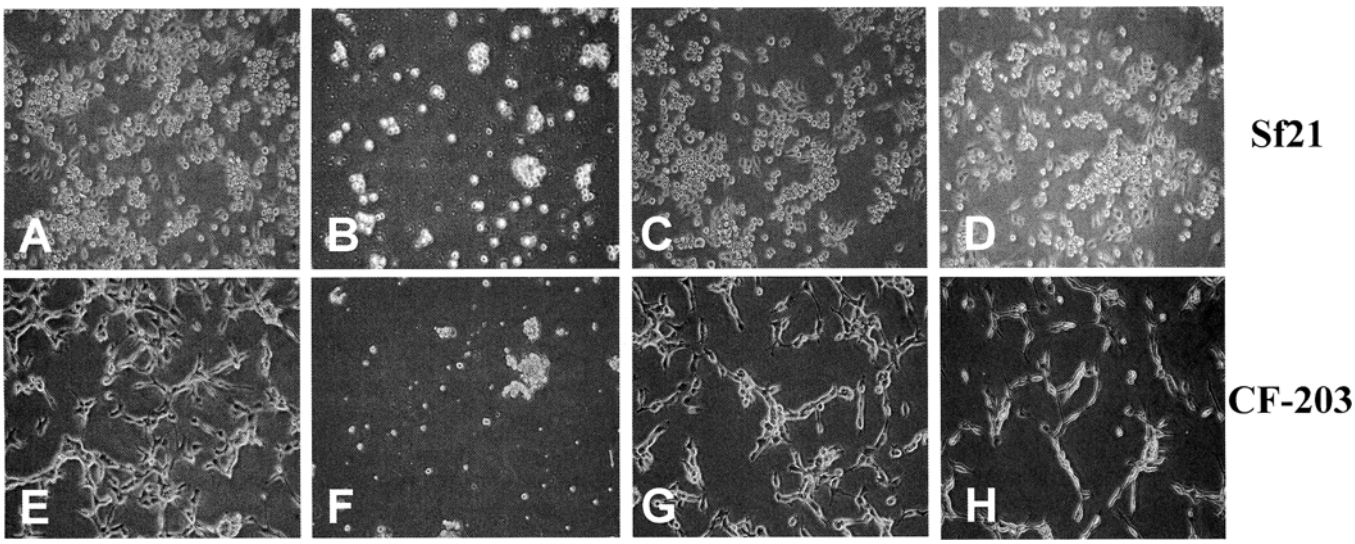


FIG. 1. Phase contrast photomicrographs of transformed cells. The cells are cultured in the modified Grace's medium for Sf21 or Sf-900 medium for Cf203 in the absence or presence of 300 μg/ml of Zeocin. (*A*) The Sf21 cells culturing in the Grace's medium without Zeocin; (*B*) Sf21 cells in the Grace's medium with Zeocin; (*C*) pIZ/V5-His-transformed Sf21 cells with Zeocin; (*D*) pIZ/*pdr5*-transformed Sf21 cells with Zeocin; (*E*) Cf203 cells in the Sf-900 medium without Zeocin; (*F*) Cf203 cells in the Sf-900 medium with Zeocin; (*G*) pIZ/V5-His transformed Cf203 cells with Zeocin; (*H*) pIZ/*pdr5* transformed Cf203 cells with Zeocin. The photomicrographs were taken 3 d after culture for Sf21 and 5 d after culture for Cf203 cells. The pIZ/V5-His or pIZ/*pdr5*-transformed cells were cultured for three passages. Bars represent 30 μm.

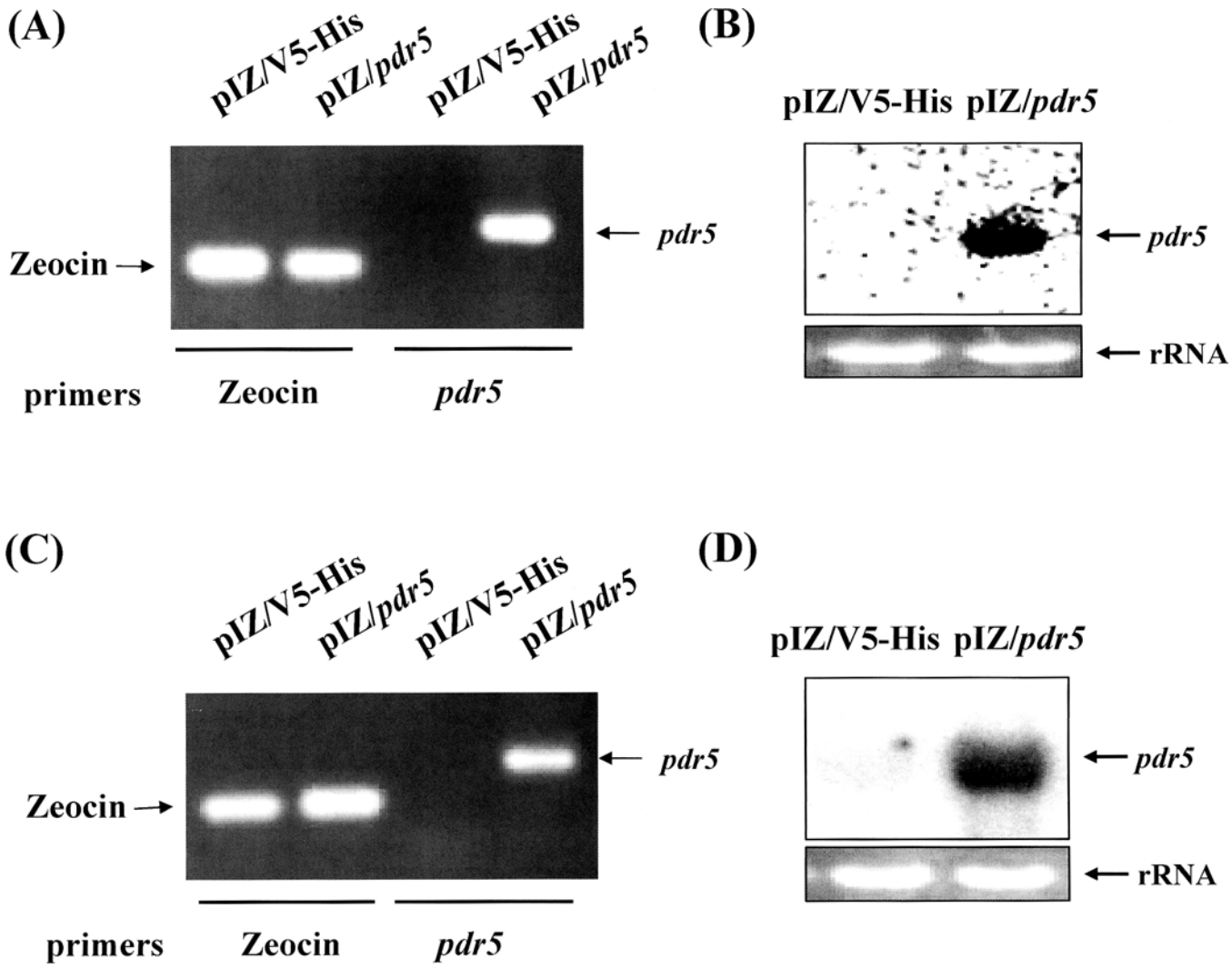


FIG. 2. Reverse transcription (RT)-PCR analysis (*A* and *C*) and Northern blotting analysis (*B* and *D*) of Zeocin and *pdr5* transcripts in the transformed Sf21 (*A* and *B*) and CF-203 (*C* and *D*) lines, respectively. The RNA was isolated from the cell lines that were transformed with pIZ/V5-His or pIZ/*pdr5*. PCR was conducted with Zeocin-specific primers or *pdr5*-specific primers. The Northern blots were probed with a fragment of *pdr5* DNA.

budworm, *Choristonuera fumiferana*, and other lepidopteran pests (Retnakaran and Oberlander, 1993; Retnakaran et al., 1995), but it is nontoxic to several other insect species belonging to the orders Diptera, Coleoptera, Homoptera, Orthoptera, and Hemiptera (Smagghe and Degheele, 1994; Slama, 1995; Dhadialla et al., 1998; Sundaram et al., 1998). In an attempt to study the mechanism of insect resistance against tebufenozide, we have demonstrated that PDR5 is able to exclude tebufenozide from yeast cells (Hu et al., 2001). To test whether yeast PDR5 can do the same in insect cells, two lepidopteran cell lines, Sf21 and CF-203, which are susceptible to tebufenozide, were transformed with the yeast *pdr5* gene. Diacetoxyscirpenol, a protein synthesis inhibitor, and RH5992 were used to test the possible function of PDR5 as a transporter in these transformed cell lines.

MATERIALS AND METHODS

Cell culture. Two lepidopteran cell lines, Sf21 and *C. fumiferana* FPMI-CF-203, were used in this study. The Sf21 line was derived from *Spodoptera frugiperda* ovarian cells. The CF-203 was derived from midgut tissues (Sohi et al., 1993); Sf21 cells were grown in Grace's insect medium supplemented with 10% FBS. The CF-203 cells were grown in SF900 medium supplemented with 5% FBS (Invitrogen Life Technologies, Carlsbad, CA). Cells were grown at 28° C in 25-cm² Falcon plastic flasks with 5 ml of the medium.

Construction of transformation vectors. The *pdr5* gene, cloned from genomic DNA of *Saccaromyces cerevisiae* strain YPH500 (Mahe et al., 1996), was cloned into pGEMT-easy cloning vector (Promega Corporation, Madison, WI) for sequencing. The open reading frame of the *pdr5* gene was then subcloned into the pIZ/V5-His expression vector (Invitrogen Life Technologies, Carlsbad, CA) between the XhoI and SacII sites. The *pdr5* insertion into the recombinant vector pIZ/ *pdr5* was confirmed using PCR and restriction endonuclease digestion. The vector was then used for development of stably transformed insect cells.

Transformation of insect cell lines with pIZ/pdr5. The Sf21 and CF-203 cells were transformed with pIZ/*pdr5* expression vector in LIPOFECTIN (Invitrogen). Fifteen microliters of LIPOFECTIN Reagents were diluted in 100 μl of serum-free medium and kept at room temperature for 30 min. Two micrograms of DNA were diluted in 100 μl of serum-free medium. These solutions were combined and incubated at room temperature for 10 min. The LIPOFECTIN Reagent-DNA mixture was added to 1.8 ml serum-free medium and then used to incubate 2×10^5 cells/ml in a 25-ml flask for 12 h at 28°

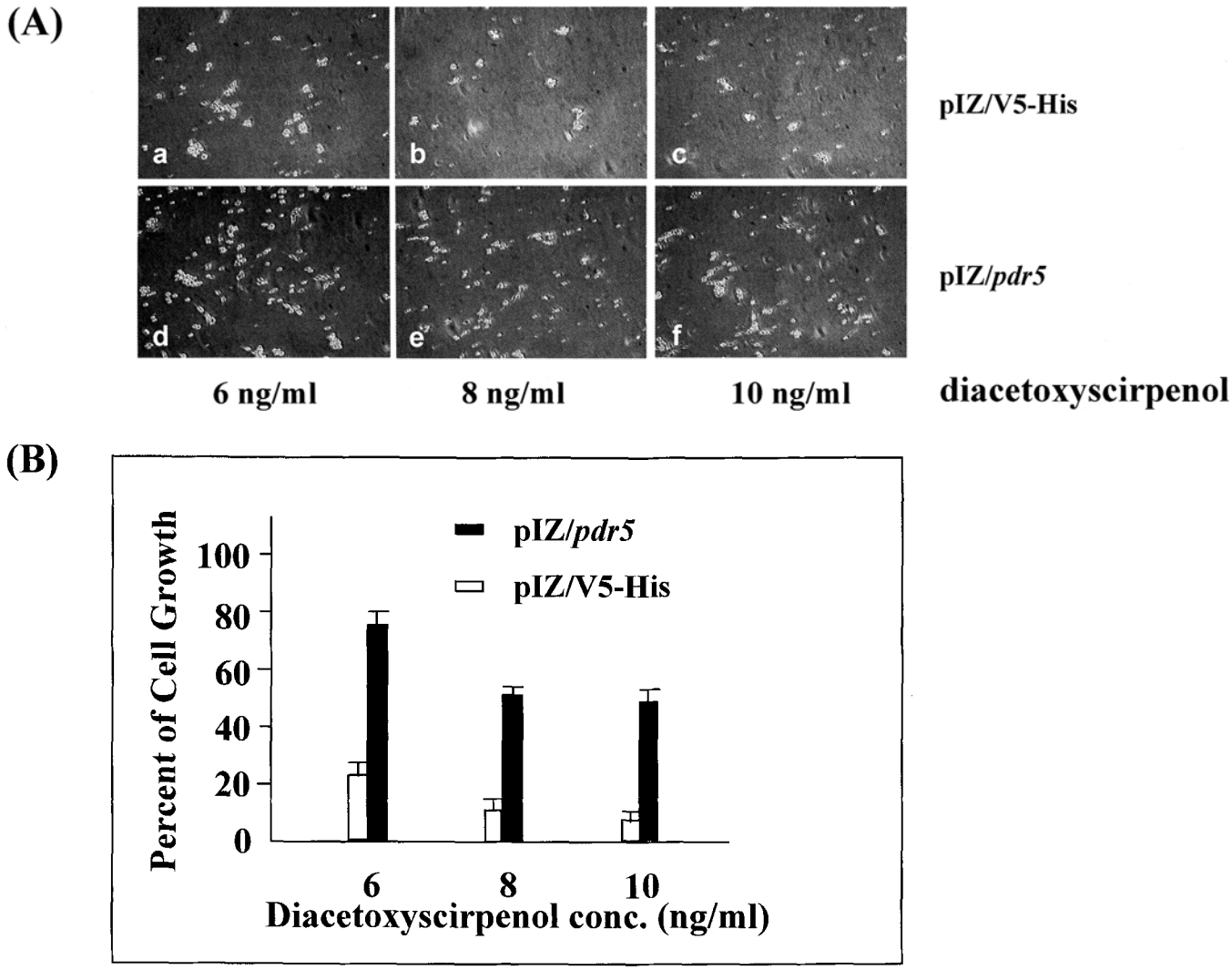


FIG. 3. Effect of diacetoxyscirpenol on morphology (*A*) and cell growth (*B*) of Sf21 cells transformed with pIZ/V5-His or pIZ/*pdr5*. Diacetoxyscirpenol was added at 6, 8, and 10 ng/ml on the first day of the cultures. The cells were photographed after 2 d of culture. The surviving cells were harvested and counted after 2 d of culture. Data points represent a mean from three replicates ± SD.

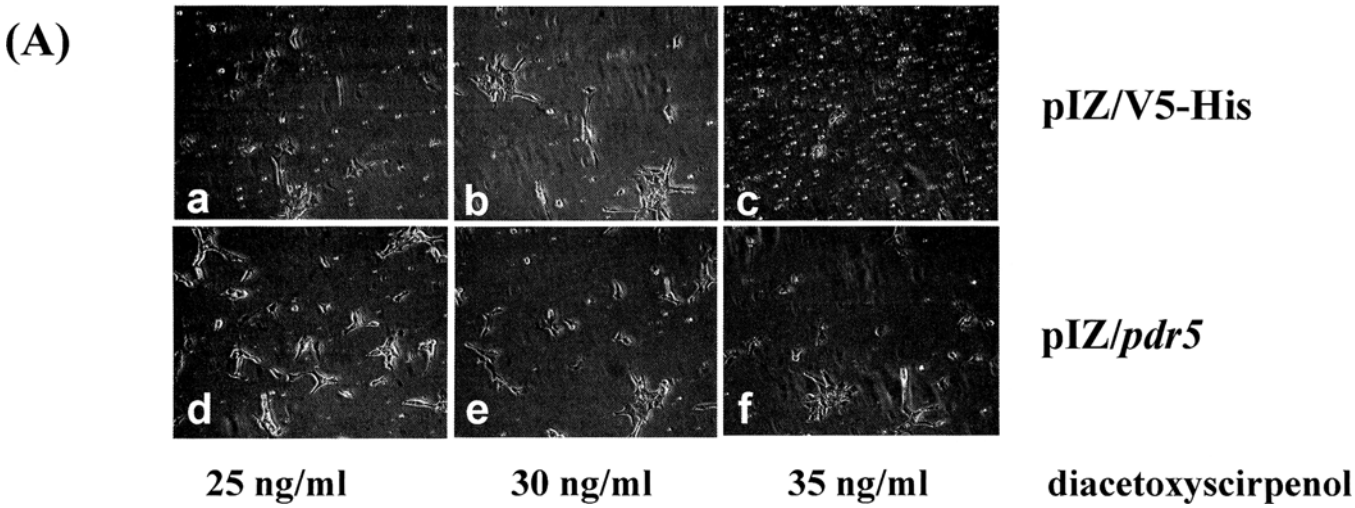
C. The DNA-containing medium was replaced with 5 ml of fresh medium containing serum. Zeocin® (Invitrogen) was added to the medium until the final concentration of 300 μg/ml was reached. The cells were cultured at 28° C until the cells grew to 70–80% confluency. After three passages in the presence of Zeocin, the cell lines were used for analysis of *pdr5* expression.

Northern blot and RT-PCR analysis. Total RNA was extracted using TRIzol Reagent according to the manufacturer's instructions (Invitrogen). For Northern blotting, 10 μg of total RNA were separated on formaldehyde agarose gels and then transferred to a nylon membrane. The blots were prehybridized in a prehybridization solution (Rapid-hyb buffer, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) for 4 h and then hybridized in a hybridization solution (prehybridization solution plus ^{32}P-dCTP labeled *pdr5* DNA probe) at 65° C for at least 12 h. After hybridization, the membranes were washed twice in 2× SSC plus 0.1% SDS at 42° C for 15 min, twice with 0.5× SSC plus 0.1% SDS at 55° C for 15 min, and once with 0.1× SSC plus 0.1% SDS at 65° C for 15 min.

For RT-PCR, mRNA was amplified using the Superscript First Strand Synthesis System for RT-PCR according to the manufacturer's instructions (Invitrogen). The primers for amplifying *pdr5* and Zeocin genes were GCGTGGTTTCGGTATTGGTATG (*pdr5* forward primer), AATGACTCCCTCACAGTGGCAG (*pdr5* reverse primer), ATGGCCAAGTTGACCAGTGCCGTT (*Zeocin* forward primer), and GTCCTGCTCCTCGGCCACGAAGTG (*Zeocin* reverse primer). Template DNA was denatured at 94° C for 3 min, followed by 35 cycles of 94° C for 45 s, 60° C for 1 min, and 72° C for 2 min for each cycle. The PCR products were separated in 0.9% agarose gels and stained with ethidium bromide.

Effect of diacetoxyscirpenol on cell growth. Cells were set up at an initial concentration of 2 × 10^5 cells/ml in their respective media, and different concentrations of diacetoxyscirpenol (Sigma Chemical Company, St. Louis, MO) were added to the cultures as indicated in *Results*. The attached cells were harvested and counted with a Coulter particle counter (Beckman counter, Inc., Miami, FL) at d 2 posttreatment for Sf21 cells and at d 3 for CF-203 cells.

RH-5992 retention assay. The Sf21 and CF-203 cells were set up at concentration of 4 × 10^5 cells/ml in a 5-ml flask. The ^{14}C-labeled RH-5992 at a specific activity of the ^{14}C-RH-5992 of 23.1 mCi/g was provided by Rohm and Haas Research Laboratory (Spring House, PA). Five microliters of 1 mM ^{14}C-RH-5992 (300,000 dpm) were added to the cell cultures at a final concentration of 1 μ*M*. The cells were harvested at 2, 6, 12, and 24 h posttreatment. The cells were washed three times with 1× PBS and centrifuged at 14,000 rpm for 5 min. The cells were suspended in 200 μl of 1.5 *M* NaOH. The cells were then added to 10 ml of scintillation solution. Intracellular amounts of ^{14}C-RH5992 were quantified by liquid scintillation (Beckman LS 6000 SE). Counts were standardized to dpm/10^6 cells.



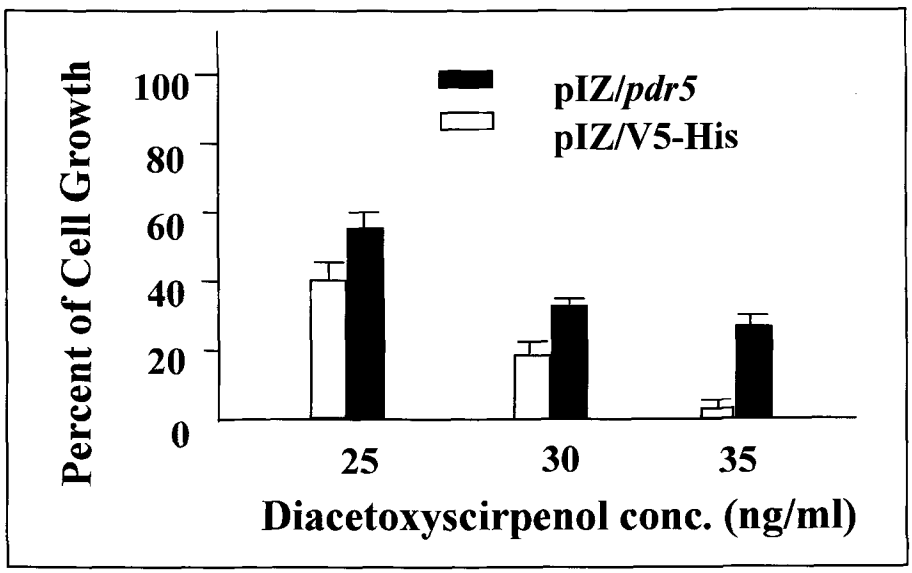


FIG. 4. Effect of diacetoxyscirpenol on morphology (*A*) and cell growth (*B*) of CF-203 cells transformed with pIZ/V5-His or pIZ/*pdr5*. Diacetoxyscirpenol was added at 25, 30, and 35 ng/ml on the first day of the cultures. The cells were photographed after 3 d of culture. The surviving cells were harvested and counted after 3 d of culture. Data points represent a mean from three replicates ± SD.

RESULTS

Development of stably transformed cell lines. Two cell lines, Sf21 and CF-203, were transformed with the expression vector pIZ/*pdr5* and the control vector pIZ/V5-His, respectively (Fig. 1). Normal cells that were not transformed died and floated in the media when Zeocin was present at 300 μg/ml (Fig. 1*B* and *F*). The surviving cells were subcultured for an additional three passages in the presence of Zeocin to develop the stably transformed cell lines. The four transformed cell lines, pIZ/V5-His-Sf21 (Fig. 1*C*), pIZ/*pdr5*-Sf21 (Fig. 1*D*), pIZ/V5-His-CF-203 (Fig. 1*G*), and pIZ/*pdr5*-CF-203 (Fig. 1*H*) were developed after three-passage selection in the Zeocin-containing media, in which most cells of these four lines could be maintained and grew normally.

Transcripts of the Zeocin gene were detected using RT-PCR in both of the pIZ/V5-His- and pIZ/*pdr5*-transformed Sf21 (Fig. 2*A*) and CF-203 (Fig. 2*C*) cells, respectively. However, transcripts of *pdr5* were detected using RT-PCR (Fig. 2*A* and *C*) and Northern blotting (Fig. 2*B* and *D*) only in the pIZ/*pdr5*-transformed cell lines. These results indicate that the two lines (pIZ/*pdr5*-Sf21 and pIZ/*pdr5*-CF-203) were resistant to Zeocin and expressed the *pdr5* gene.

Effects of diacetoxyscirpenol on cell growth. The multidrug transporter PDR5 is responsible for the efflux of a large range of substrates through the yeast cell membrane (Golin et al., 2003). To test whether the transformed cell lines (pIZ/*pdr5*-Sf-21 and pIZ/*pdr5*-CF-203) that were expressing *pdr5* gained this activity in the exclusion of toxic compounds, we used diacetoxyscirpenol, a protein synthesis inhibitor (Ueno et al., 1973) as an indicator to perform the assay. In these two transformed insect cell lines, if *pdr5* was expressed into an active protein, which was properly incorporated into the cell membrane, it should be capable of excluding diace-

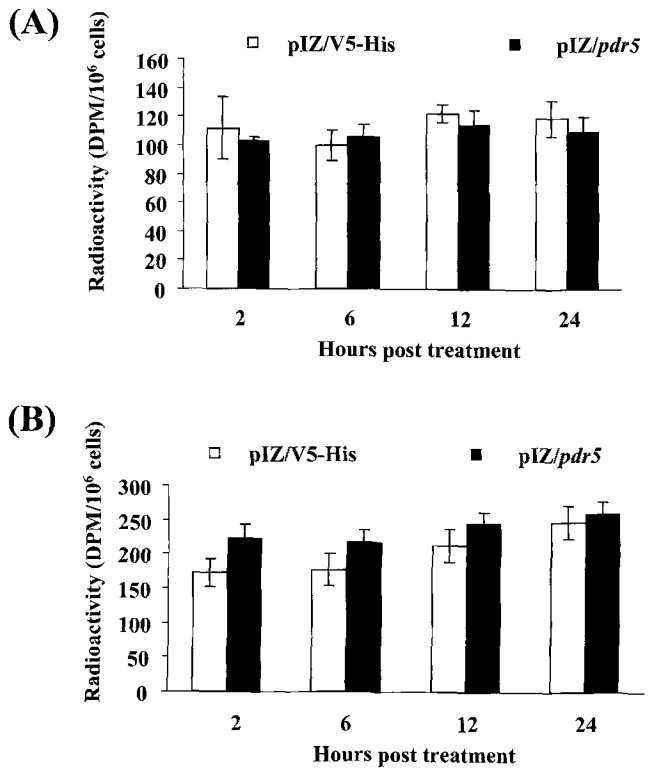


FIG. 5. Retention of ^{14}C-RH5992 in the *pdr5*-transformed Sf21 (*A*) and CF-203 cell lines (*B*). The cells were incubated in their respective media containing 10^{-6} M ^{14}C-RH5992 and then harvested at 2, 6, 12, and 24 h posttreatment. The cells were washed three times with 1× PBS and were suspended in 200 μl of 1.5 *M* NaOH. Intracellular amounts of ^{14}C-RH5992 were quantified by liquid scintillation.

toxyscirpenol from the cells, and therefore, the transformed insect cells could have enhanced resistance to diacetoxyscirpenol. The results indicated that the *pdr5*-transformed pIZ/*pdr5*-Sf21 had more surviving cells in the presence of diacetoxyscirpenol at 6–10 ng/ml than the control (pIZ/V5-His-Sf21) cells at day 2 postincubation (Fig. 3*A*). The cell numbers of the pIZ/*pdr5*-Sf21 lines were 76.2%, 52%, and 48.5% of the original cell intensity in the treatments of 6, 8, and 10 ng/ml diacetoxyscirpenol, respectively, whereas the cell numbers of the pIZ/V5-His-Sf21 line were 22.6%, 13%, and 8.8%, respectively (Fig. 3*B*), indicating that the *pdr5*-transformed cells have a higher resistance to diacetoxyscirpenol than the nontransformed cells. Similar results were also found in the CF-203 cell line (Fig. 4). More cells were surviving at d 3 postincubation in the presence of diacetoxyscirpenol than in the pIZ/V5-His-CF-203 cells (Fig. 4*A*). These were more surviving and growing cells of the pIZ/*pdr5*-CF-203 line than of the nontransformed cell line (Fig. 4*B*). These results indicate that the transformed cell lines could resist diacetoxyscirpenol more efficiently than the control line.

Retention of ^{14}C-RH5992 in the transformed cells. To test whether these transformed cell lines expressing *pdr5* mRNA could exclude ^{14}C-labeled RH5992, as reported in yeast cells (Hu et al., 2001), we measured the accumulation of ^{14}C-RH5992 in these transformed cells. If PDR5 functioned properly, retention of ^{14}C-RH5992 would be lower in the transformed cells than in the nontransformed cells because PDR5 is expected to pump the ^{14}C-RH5992 out of the cells. The results showed that there was no significant difference in retention of ^{14}C-RH5992 between *pdr5*-transformed and nontransformed Sf21 cells (Fig. 5*A*) or between *pdr5*-transformed and nontransformed CF-203 cells (Fig. 5*B*), indicating that although the *pdr5* gene was expressed, it could not exclude RH5992 in the transformed lines.

DISCUSSION

Pleiotropic drug resistance genes, like *pdr5*, have been extensively studied in yeast and can mediate the transport of many compounds including steroids (Balzi et al., 1994; Mahe et al., 1996). Our previous study demonstrated that yeast ABC transporter PDR5 could efficiently and actively modulate the efflux of the ecdysone agonist RH5992 in yeast (Hu et al., 2001). Our study was to establish stably transformed insect cell lines that express the yeast *PDR5*, which we can then be used as a working model system to study the exclusion mechanism in insects. To achieve this purpose, we transformed with the yeast *pdr5* two lepidopteran cell lines that normally do not exclude ecdysone compounds. The *pdr5* gene appeared to be expressed into mRNA, as detected using Northern blotting and RT-PCR analysis. However, we did not detect any exclusion of RH5992 in these transformed insect cells. The expressed PDR5 transporter protein in transformed cells was not detectable in SDS-PAGE using Coomassie staining (data not shown), probably because the level of expression was too low. Because there is not an antibody to PDR5, we could not examine protein expression by Western blotting analysis.

To test whether the yeast *PDR5* could function in excluding other compounds, we examined the capability of the transformed cell lines to exclude the protein synthesis inhibitor diacetoxyscirpenol. Diacetoxyscirpenol is an effective inhibitor of protein synthesis in animal cells (Ueno et al., 1973). It can increase resistance to diacetoxyscirpenol when expressed in transgenic tobacco (Muhitch et al., 2000). The results of our study show that the *pdr5*-transformed cells have a higher resistance to diacetoxyscirpenol than the control, implying that *pdr5* may have functioned in the exclusion of diacetoxyscirpenol in the transformed cells. Thus, the *pdr5*-transformed Sf21 and CF-203 cells that gained resistance to diacetoxyscirpenol may be a useful system for studying the biological function of the transporter in resistance against toxic compounds.

We notice that there is a difference in exclusion between RH5992 and diacetoxyscirpenol. This difference may due to structural folding of the recombinant protein. The gene was originally isolated from yeast cells, and its gene product may not be perfectly incorporated into the membranes of the transformed insect cells, resulting in a partial loss of the transport activity to RH5992. This needs further investigation.

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