Rapid Communication

Stable transformation of a *Mamestra brassicae* (lepidoptera) cell line with the lepidopteran-derived transposon *piggyBac*

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Abstract

Cabbage moth cells were transfected with the vector pBac[3xP3-EGFPafm] and helper phsp-pBac. Seventeen percent of the transfected cells showed stable EGFP-expression. This indicates successful and stable transformation of *M. brassicae* cells with a *piggyBac*-derived vector. Genomic integration of *Bac*[3xP3-EGFPafm] in stably transformed cells was confirmed by Southern blots and inverse PCR. Since the integrations are stable, and transfection with pBac[3xP3-EGFPafm] alone did not yield in transformations, no cross-reacting transposase activity seems present in *M. brassicae* cells. Moreover, Southern blotting with a probe for *piggyBac* transposase indicated the absence of *piggyBac*-related elements in the genome of *Mamestra brassicae*. Due to the tissue specificity of the 3xP3-EGFP marker for eye and nervous tissues, it is intriguing that 3xP3-EGFP can successfully be used to identify stably transformed *M. brassicae* cells of cell line IZD-MB0503, which is hemocyte-derived. Sequence analysis of the insertion sites showed that *piggyBac* inverted repeats were adjacent to TTAA sequences on both termini in all the clones. The present results are particularly important as they suggest that *piggyBac* can be used for transgenesis of cabbage moth cells.

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1. Introduction

*Mamestra brassicae* is a cosmopolitan and polyphagous insect that lives in temperate regions of Eurasia on cruciferae plants. *M. brassicae* control in the field is performed using both chemical compounds and *Bacillus thuringensis* (Bt) toxins (Kwa et al., 1998). The low specificity of the used insecticides together with the diffusion of resistance to Bt endotoxins in Lepidoptera (Tabashnik et al., 1994) necessitates the design of new control strategies for *M. brassicae*. In view of results obtained with other pest crop insects (reviewed in Handler, 2001; Atkinson et al., 2001), genetic engineering techniques will allow the development of new strategies for *M. brassicae* control in the field.

Moreover, *M. brassicae* cell lines have a wide range of applications for the production of recombinant proteins, for studies on baculoviruses and for the analysis of apoptosis in insect cells (e.g. Wickham et al., 1992). Mutagenesis by insertion of transposable elements into the genome of such a cell line could be particularly interesting to identify and characterize the genes involved in apoptosis of insect cells.

Transposable elements have become an essential tool in the study of gene function in insects and their applications range from the use as mutagens to their use as tags for the identification of new genes. In particular, efforts have focused on the identification and molecular characterization of mobile elements that will serve as efficient gene vectors. Insect transformation is, in fact, an emerging technology that is likely to have a wide range of applications within insect biology and genetics (reviewed in Handler and James, 2000; Handler, 2001; Atkinson et al., 2001).

The successful improvement of this technology allowed the development of transgenic insects that could be useful to develop new control strategies for insects...
of agricultural and medical interest. In this regard, the transposable element piggyBac appeared particularly interesting since it has been used to achieve genetic transformation in several insect species of agricultural, economical and medical interest, such as Ceratitis capitata, Bactrocera dorsalis, Anastrepha suspensa, Musca domestica, Lucilia cuprina, Anopheles gambiae, An. stephensi, An. albimanus, Aedes aegypti, Pectinophora gossypiella, Bombyx mori, and Tribolium castaneum insects (reviewed in Handler, 2002).

The piggyBac element is a short inverted terminal repeat (ITR) transposable element, 2.5 kb long, with 13 bp ITR sequences and a single 2.1 kb ORF (Cary et al., 1989; Elick et al., 1996). It was originally discovered as a result of it causing few polyhedra mutations in baculoviruses that had been passed through the TN-368 cell line of cabbage looper moth Trichoplusia ni (Fraser et al., 1995). Subsequently, its ability to integrate in different lepidopteran and Anopheles cell lines (Fraser et al., 1995; Grossman et al., 2000) and in germ cells of numerous insects (reviewed in Handler, 2002) has been shown.

In the present paper, we report that the piggyBac transposable element from the cabbage looper T. ni can be used for the effective and stable transformation of a M. brassicae cell line and that the 3xP3-EGFP marker gene (Berghammer et al., 1999; Horn et al., 2000, 2002) can be used to identify such lines. This represents the first successful transformation of a cabbage moth cell line.

2. Materials and methods

The IZD-MB-0503 cell line from the insect M. brassicae (Lepidoptera) (ATCC number: CRL-8003) was used. The cells were cultured in Ex-Cell 405 medium (JRH Biosciences, KS, USA) at 26 °C.

Genomic DNA extraction from the IZD-MB-0503 cell line was performed according to Mandrioli (2002). Electrophoresis, transfer of the DNA from agarose gel to nylon membrane and Southern blotting were performed following Mandrioli (2002).

Construction of the pBac[3xP3-EGFPa fm] vector is described in Horn and Wimmer (2000). The helper plasmid, phsp-pBac, contains the piggyBac transposase sequence under the control of the Drosophila hsp70 promoter (Handler and Harrell, 2001).

Transfection of the cell with both helper phsp-pBac and pBac[3xP3-EGFPa fm] plasmids were performed using DOTAP liposomes (Roche) following manufacturer protocols. After a 48 h incubation after transfection, transposase expression was induced by heat-shock (1 h at 37 °C). Twelve hours after heat-shock, EGFP-fluorescent cells were isolated from non-fluorescent ones using a flow cytometer equipped with a cell sorter.

Primers PLF (5’-CTTGACCTTGCCACAGAGGACT ATTAGAGG) and PRR (5’-AGTCAGTCAGAAACA ACTTTGCCACATATC) were used to PCR-amplify the EGFP cassette, whereas inverse PCRs was performed according to Hediger et al. (2001). PCR fragments were separated in a 1.2% TAE agarose gel, purified with the Agarose Gel Extraction kit (Roche), cloned, and sequenced. The amplified fragments were cloned with the ‘pGEMT-easy cloning kit’ following the Promega protocols. Sequencing was performed at the ‘CRIBI-Sequencing Centre of the University of Padua’.

3. Results and discussion

When cabbage moth cells had been transfected with both pbac[3xP3-EGFPa fm] as well as helper plasmids and transposase expression had been induced by heat-shock, 17% of the transfected cells resulted in stable EGFP expression and could therefore be considered successfully transformed with a piggyBac-derived vector (Fig. 1B). In order to evaluate if piggyBac-integration was due to the presence of the helper plasmid or to a phenomenon of cross-mobilization, the presence of endogenous transposase able to mobilize piggyBac in M. brassicae cells was assessed by transfecting cells with
the pBac{3xP3-EGFPafm} vector alone (Fig. 1A). No stable EGFP-expressing cells were obtained and only transient EGFP expression could be observed. Moreover, the presence of piggyBac-like elements was evaluated in the cabbage moth genome by Southern blots using the DIG-labeled piggyBac transposase gene as a probe. No hybridization signal was observed even when low stringency conditions were tried. This indicates the absence of piggyBac-related elements from the M. brassicae genome.

To verify stable integration of Bac{3xP3-EGFPafm} into the genome, genomic DNA was extracted from stable EGFP-expressing and control M. brassicae cells not expressing EGFP. This DNA was initially tested by PCR with primers PLF and PRR and by Southern blotting. PCR amplified DNA was found in all the EGFP expressing cell lines, whereas no PCR products were recovered from non-fluorescent cells (Fig. 2, lanes 1 and 2). These results strongly suggested that successful transformation of M. brassicae cells occurred, since fluorescence of transformed cells was correlated to the presence of the EGFP gene. To further verify these results, M. brassicae genomic DNA from transformed and non-transformed cells were digested with restriction enzymes and labelled with the EGFP probe obtained by digesting the pBac{3xP3-EGFPafm} plasmid with BamHI and NorI. All the EGFP expressing cell lines showed a 750 bp band after simultaneous digestion with BamHI and NorI corresponding to the EGFP cassette (Fig. 3A, lane 3). Moreover, 2–5 bands were detected in the EGFP-expressing cells when digested with only one of the restriction enzymes (Fig. 3A, lanes 1 and 2). This indicates that the EGFP construct was inserted into several different genomic sites and that piggyBac was indeed an effective vector for M. brassicae cell line transformation.

No hybridization signal was observed with the EGFP probe on Southern blots of cells not expressing EGFP (Fig. 3B).

In order to identify the piggyBac insertion sequences in the cabbage moth cells, inverse PCR was performed using DNA extracted from EGFP-expressing cells. piggyBac left and right flanking sequences were separately amplified as described (Hediger et al., 2001), and five amplified fragments (Fig. 2, lanes 3 and 4) were cloned and sequenced for each terminal repeat. Analysis of the sequenced clones showed that piggyBac inverted repeats were adjacent to a TTAA sequence on both the termini in all the clones (Table 1). Moreover, sequence analysis performed using alignment with GCG software did not reveal further significant similarities among the insertion sequences amplified by inverse PCR. These results allowed us to conclude that piggyBac insertion into the M. brassicae genome was due to a specific transposase-mediated mechanism. These data are in full agreement with those reported for An. gambiae, A. suspensa, Spodoptera frugiperda and M. domestica (Elick et al., 1996; Grossman et al., 2000; Handler and Harrell, 2001;
Table 1

<table>
<thead>
<tr>
<th>Clone</th>
<th>Left end</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>tagctgctagatagactctagTTAA - piggyBac</td>
</tr>
<tr>
<td>L2</td>
<td>gtagctacgctagatagTAA - piggyBac</td>
</tr>
<tr>
<td>L3</td>
<td>aactactactactactTTAA - piggyBac</td>
</tr>
<tr>
<td>L4</td>
<td>gacgctggctagctgctggTTAA - piggyBac</td>
</tr>
<tr>
<td>L5</td>
<td>cgcgccccctacagacatagcaTTAA - piggyBac</td>
</tr>
</tbody>
</table>

Hediger et al., 2001) and strongly reinforce the hypothesis that the TTAA sequence is the integration consensus sequence of piggyBac. The structure of piggyBac integration sequence has been contradictory since Cary et al. (1989) suggested the existence of a larger piggyBac consensus sequence consisting of YYTTTT/TTAAAYAG (Y = pyrimidine, R = purine, / = insertion sequence). The same hypothesis has been proposed in An. gambiae (Grossman et al., 2000). Our results do not support this but reinforce the existence of a short TTAA piggyBac insertion sequence.

The present results indicate that the marker 3xP3-EGFP can be successfully used to identify stably transformed M. brassicae cells of line I2Z-MB-0503. This is particularly intriguing as this cell line is supposed to be of hemocyte origin (Miltonburger and David, 1976), but the 3xP3-EGFP marker is supposedly tissue specific for eye and nervous system tissues (Horn et al., 2000). It is possible that the 3xP3-EGFP marker itself does not express EGFP stably in this cell line. This would explain why plasmid-based marker gene expression is only very transiently detected. However, after insertion of the marker gene into the genome, enhancer trap effects might allow for strong expression of EGFP. Enhancers close enough to the 3xP3-EGFP insertion might activate the basal promoter of 3xP3 (Horn et al., 2000) and thus mediate stable EGFP expression.

In any case, the availability of a highly efficient transformation vector based on piggyBac will be particularly interesting for the study of gene expression in the M. brassicae I2Z-MB-0503 cell line. This will allow for the quick evaluation of promoter strengths in a genomic environment. Expression libraries constructed in piggyBac vectors could be functionally screened in the M. brassicae cell line. In particular, it will be interesting to integrate gene constructs that could be used to perform RNA-interference experiments. Furthermore the stable integration suggests that piggyBac could also be employed for transgenesis of cabbage moth eggs and embryos.

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