Vol.51, n. 1 : pp.27-34, January-February 2008
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Restriction Enzyme Improves the Efficiency of Genetic Transformations in *Moniliophthora perniciosa*, the Causal Agent of Witches' Broom Disease in *Theobroma cacao*

Francis Julio Fagundes Lopes, Marisa Vieira de Queiroz*, Juliana Oliveira Lima, Viviane Aline Oliveira Silva and Elza Fernandes de Araújo
Departmento de Microbiologia; BIOAGRO; Universidade Federal de Viçosa; Campus Universitário; mvqueiro@ufv.br; Viçosa - MG - Brasil

ABSTRACT

The presence of restriction enzymes in the transformation mixture improved the efficiency of transformation in Moniliophthora perniciosa. The influence of the vector shape (linear or circular), the patterns of plasmid integration in genomic sites and the influence of the promoter used to express the gene marker were also analyzed. The addition of BamHI or NotI increased the number of transformants by 3-10-fold and 3-fold, respectively, over the control without added enzyme. The use of pre-linearized plasmid did not increase the transformation efficiency in comparison with the circular plasmid. However, the frequency of multi-copy transformants increased significantly. The transformation procedure here reported resulted in better production of protoplasts and transformation efficiency. In addition, the time necessary for the detection of the first transformants and the number of insertions were reduced.

Key words*:* *Moniliophthora perniciosa,* transformation, restriction enzyme

INTRODUCTION

Witches' broom disease in cacao is caused by the basidiomycete fungus *Moniliophthora perniciosa* (Stahel), (*Agaricales, Tricholomataceae*), formerly *Crinipellis perniciosa* (Aime and Phillips-Mora, 2005). *M. perniciosa* is one of the most important cacao pathogens. There is evidence that it has developed concomitantly with its host in the Amazon Basin (Pound, 1943). The biotype C has a hemibiotrophic life style. In the biotrophic phase the fungus invades young host tissues provoking a local response in the host tissues characterized by considerable swelling (Orchard et al., 1994). Within a period of three to nine weeks, for reasons yet unclear, the biotrophic mycelium is substituted or changes to the saprophytic phase (Calle et al., 1982). In spite of the economical importance of the disease, little is known about the genetic mechanisms underlying the host-pathogen interaction. In this sense, genetic transformation is a powerful instrument to study the pathogenicity mechanisms of this fungus at the molecular level. Transformation is widely employed in genetic studies in fungi and has been used in gene disruption (Cummings et al., 1999; Fujimoto et al., 2002; Jin et al., 2005; Seong et al., 2005), gene replacement (Oeser et al., 2002) and gene silencing (Warwar et al., 2000). Mutants have been demonstrated to be a valuable resource for

* Author for correspondence

genetic and molecular analysis. However, a successful mutant screening in fungi can be time-consuming and labor-intensive.
The efficiency of gene manipulation in fungi was significantly enhanced since the modification of the traditional transformation technique by the use of restriction enzyme-mediated integration (REMI). This procedure was first described in yeast (Schiestl and Petes, 1991). Since then, REMI has been successfully used to generate mutations at a relatively high frequency in some fungal species. The major advantage of this approach is that it permits random disruption of genes through plasmid insertions, which ones can subsequently be recovered by plasmid rescue in *Escherichia coli.* REMI depends on both an efficient transformation system and a way to link the insertion event with the mutation generated. By using REMI, several pathogenicity genes have been cloned and characterized in fungi so far (Chung et al., 2003; Kahmann and Basse, 1999; Riggle and Kumamoto, 1998; Tanaka et al., 1999).
A transformation system for *M. perniciosa* based on the selection for hygromycin B resistance was developed (Lima et al., 2003). In this system, the analyzed transformants showed a high number of plasmid insertions and the transformation efficiency was low (about 2 transformants/µg of plasmid DNA). Here we show that addition of some restriction enzymes in the transformation mixture may enhance the transformation efficiency and reduce plasmid integrations in the genome of *M. perniciosa.* Also, changes in the former protocol improved the production of protoplasts and resulted in less time-consuming procedures. The potential use of plasmid as mutagenic tool for *M. perniciosa* is shown by the isolation of a methionin mutant.

MATERIALS AND METHODS

Microorganisms and culture maintenance

The strain CP02 of *M. perniciosa* (biotype C) used in this study was isolated from cacao and kindly provided by Prof. Gonçalo A. G. Pereira (UNICAMP, Campinas, Brazil). Wild type fungus was cultivated in PDA (potato-dextrose-agar) medium (MERCK), modified by the addition of 0.15% hydrolyzed casein, 0.2% yeast extract, 0.2% peptone, and 0.01% vitamin solution (0.2 mg biotin, 10.0 mg ρ-aminobenzoic acid, 50.0 mg pyridoxine, 50.0 mg thiamin, 100.0 mg nicotinic acid, 100.0 mg riboflavin, and 100 mL distilled water). The transformant strains were maintained in modified PDA medium containing 50.0 µg/mL of hygromycin B. The cultures were incubated at 27°C and periodically transferred (every 10 days) to a new medium.

Transformation vectors

The plasmid pBHg (8,182 pb) was kindly provided by Dr. Peter Romaine (University of Pennsylvania, USA) and contains a cassette with the gene coding for the hygromycin B phosphotransferase (*hph*) from *E. coli* fused to the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter from the basidiomycete *Agaricus bisporus* and the cauliflower mosaic virus terminator (35S-39). The plasmid pAN7-1 (6,756 bp), kindly provided by Dr. C.A.M.J.J. van den Hondel (Leiden, Netherlands), contains the *hph* gene from *E. coli* (Punt et al., 1987) joined to the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) and transcriptional terminator of a tryptophan synthase structural gene (*trp*C). The plasmid pHCP (5,791 pb) was constructed by the excision of the cassette that contains *hph* gene, *gpd* promoter and *trp*C terminator from pBHg with the restriction enzymes *Ssp*I and *Sma*I. The cassette was subcloned into the *Sma*I site of plasmid pBluescript SK+ (Stratagene).

Protoplast production and transformation of *M. perniciosa*

Discs of mycelia grown on modified PDA were subcultured in modified PG (Potato-glucose) liquid medium (infusion water from 500.0 g of potato, 10.0 g/L glucose, 2.0 g/L yeast extract, 2.0 g/L peptone, 1.5 g/L hydrolyzed casein, 1.0 mL/L vitamin solution) in Petri dishes for eight days at 27°C. Mycelia was collected and aseptically triturated in a microbiological blender with 50.0 mL of low sugar media containing: 0.17% (w/v) malt extract, 0.5% (w/v) yeast extract and 5.0% (w/v) glycerol MERCK (Rincones et al., 2003). Three milliliters of the suspension of grown mycelia were inoculated in 50.0 mL of liquid low sugar medium. The culture was incubated in a rotary shaker (200 RPM) for three days at 27°C. Two grams of fresh mycelia were blended with 10.0 mL osmotic stabilizer (0.8 M KCl, 10.0 mM phosphate buffer, pH 5.8), 150.0 mg Glucanex®

enzyme (Novo Nordisk), 100.0 mg BSA (bovine serum albumin) and incubated at 30 °C with agitation of 80 RPM for 3 hours. After removing the hyphal fragments by filtering, the protoplasts were centrifuged at 2,700 *g*, 4°C for 10 minutes. The protoplasts were then carefully resuspended in 5.0 mL of ST (1M sorbitol, 100 mM Tris-HCl) and centrifuged again under the conditions described above. This rinsing step was repeated three times for the removal of any traces of lytic enzyme. After the rinsing steps, the protoplast sediment was homogenized in STC (1M sorbitol, 100 mM Tris-HCl, 50.0 mM $CaCl_2$) to a final concentration of 10^8 protoplasts/mL. The protoplasts were kept on ice until the transformation step.

The transformation was based on the method described by Yelton et al. (1984) and Balance and Turner (1985) with modifications. In the control experiment, the plasmid DNA (10 µ*g*), 50 µL of 25 % (w/v) PEG 6000 (prepared in SCT), and 150 µL of the protoplast suspension were incubated on ice for 20 minutes. Thereafter, 500 µL of the same 25% PEG 6000 solution were added and the mixture was kept at room temperature for 20 minutes. The transformation mixture was then plated on PDA medium with 0.5 M sucrose as osmotic stabilizer. REMI was carried out with 10 µg of pBHg and pHCP. The restriction enzymes used were *Bam*HI (10U) (PROMEGA) and *Not*I (10U) (Sigma). The variations in the transformation consisted in transformation with *Bam*HI-linearized plasmid (enzyme was heat-inactivated after the digestion) followed by addition of more enzymes at the moment of the transformation. Concomitantly, transformation was conducted with circular plasmid with the addition of enzymes to the plasmid immediately before incubation of the transforming DNA with the protoplasts. The protoplast regeneration controls were performed in PDA medium without stabilizer (negative control) and PDA with 0.5 M sucrose by plating different dilutions of the protoplast suspension. The plates were maintained at 27 °C for three days. Afterwards, the plates were covered (except for the regeneration controls) with a five milliliters layer of semi-solid PDA medium containing hygromycin B to attain a final concentration of 100 µg/mL. After seven days, the regeneration frequency of the protoplasts was calculated based on the control plates and after 15 days, some transformant colonies were transferred to modified PDA medium containing hygromycin B (50 µg/mL). At least three independent transformation experiments were conducted.

Screening for auxotrophic mutants

Hygromycin B resistant growing colonies from the transformation experiments were transferred to replica plates containing minimal medium (Pontecorvo et al., 1953) or PDA. The colonies were allowed to grow at 27°C for 30 days. The colonies showing arrested growth on minimal media (MM) were considered possible mutants and were picked for further analysis. The selected colonies were then inoculated in replica plates containing PDA, MM or MM supplemented with different nutrients (nitrogen bases, vitamins or aminoacids) in order to identify auxotrophic mutants. The analysis proceeded until the single required nutrient to restore the normal growth was determined (Holliday, 1956).

Stability test

Nine pBHg transformants were analyzed for the mitotic stability of the integrations. These transformants were cultivated in modified PDA medium without the selective agent during 40 days. Hyphal fragments were transferred every eight days to a new medium and after 40 days, the transformants were transferred back to selective medium containing hygromycin B (100 µg/mL) to determine if the colonies still retained the selection marker.

DNA extraction

Mycelium disks (approximately 75 mm) of the colonies were transferred to Petri dishes containing modified BG medium and hygromycin B (50 µg/mL). The dishes were kept at 27°C for seven days without agitation. The mycelia were harvested and rinsed with sterile distilled water to remove the excess of medium. The excess of liquid was removed with absorbent tissue and afterwards, mycelia were immediately frozen in liquid nitrogen. For the DNA extraction, the mycelia were ground in a liquid nitrogen-cooled porcelain mortar using a porcelain pestle. The subsequent procedures were based on the technique described by Specht et al. (1982).

Southern blot analysis

The analyses of plasmid integrations were performed according to the method described by Sambrook et al. (1989). The genomic DNA (about

4 μg) of each transformant was digested with appropriate restriction enzymes, separated by electrophoresis in 0.8% agarose gel and transferred to a nylon membrane (Stratagene®) by capillarity. DNA probe labeling and detection were performed using the "Gene Images Random Prime Labeling Module" and "Gene Images CDP-Star detection module" from Amersham Pharmacia Biotech. according to the manufacturer's instructions. The hybridizations were conducted under high stringency conditions (65°C/ 0.2X SSC/ 0.1% SDS). The linearized plasmids pBHg and pHCP were used as probes.

RESULTS AND DISCUSSION

Protoplasts isolation is one of the major bottlenecks in transformation experiments. The amount, size and stability of protoplasts or the presence of toxic compounds in protoplasts suspension and the type of regeneration medium used directly affect the regeneration rates. Efficiency of protoplast transformation directly depends on both the regeneration rates of the protoplasts and the destiny of the vector inside the cell.

A grinding step was introduced in comparison to the former protoplast production protocol (Lima et al., 2003). The fragmentation of the hyphae prior to the transfer to the low sugar media caused new and soft mycelia to develop from this fresh and physiologically very active subcultured mycelium. The following vigorous agitation of the soft mycelia growing in the low sugar medium broke the thin and new walled developing tips and further increased the amount of fresh fragments suitable for protoplastization. The new hyphal tips were more susceptible to the enzymatic digestion resulting in a high number of protoplasts (about 10^8 to 10^9 protoplasts/mL).

Some studies have reported that the transformation efficiency is strongly influenced by the promoter used to express the gene marker (Marmeisse et al., 1992; Ridder and Osiewacz, 1992). In order to investigate whether the promoter type could influence the transformation efficiency in *M. perniciosa,* the *gpd* promoters from *A. bisporus* (pBHg and pHCP) and *A. nidulans* (pAN7-1) were tested. The plasmid pBHg was used initially for *Agrobacterium*-mediated fungal transformation (Chen et al., 2000).The plasmid pHCP constructed in this work originated from pBHg. The transformation efficiency with circular pAN7-1 was 4 transformants/μg and the first transformant appeared after two weeks. The transformation efficiency with plasmids pHCP and pBHg varied from 4 to 24 transformants/μg of circular plasmid, respectively. The first pBHg and pHCP transformants appeared earlier than pAN7-1 ones (one week). A reasonable explanation for the delayed development of pAN7-1 transformants observed in the present study as well as by Lima et al. (2003) could be an inefficiency of utilization of the *gpd* promoter from *Aspergillus* (ascomycete) by the transcriptional machinery of *Moniliophthora*. On the other hand, *Agaricus* and *Moniliophthora* have a closer phylogenetic relationship (both are basidiomycetes). Therefore, the use of the *Agaricus gpd* promoter by *Moniliophthora* transcriptional machinery might be stronger. The stability test was carried out with nine pHCP transformants. All of them retained the selection marker for hygromycin B resistance after 40 days growing in non-selective medium. Lima et al. (2003) found the same for *Moniliophthora* pAN7-1 transformants during 25 days of growth. Therefore, Hygromycin B *M. perniciosa* transformants showed a high mitotic stability for the integrations.

Linear pBHg and pHCP did not differ in transformation efficiency in comparison to their circular form (Fig 1). The first report of the use of linear DNA for transformation was described for *S. cerevisiae* and resulted in higher transformation efficiency in relation to the circular DNA (Suzuki et al., 1983). In contrast, Herrera-Estrela et al. (1990) reported a significant decrease in the transformation efficiency for *Trichoderma* spp. when the linear vector was used. Similarly to *M. perniciosa*, the linearization of the transforming DNA in *Colletotrichum graminicola* did not influence the transformation efficiency (Thon et al., 2000). Levis et al. (1997) suggested that the increase in the transformation efficiency resulting from the linearization of the transforming DNA in cases where the vectors have homologous regions to the fungal genome was due to the fact that this procedure exposed these regions, favoring the integration by recombination in homologous sites. On the other hand, exposed extremities of a linear DNA fragment might be more susceptible to degradation by intracellular exonucleases. Goedecke et al. (1994) observed that different organisms might have a differentiated intracellular

exonuclease activity. This could reflect back on the transformation efficiency, especially in the case of transformation with linear vectors, since they are likely more susceptible to degradation by exonucleases. The differences observed in transformation efficiency resultant of the linearization procedure could, therefore, be a consequence of a differentiated intracellular exonuclease activity presented by each microorganism.

In preliminary assays to determine the best enzyme concentration for the transformation, an increase in transformation efficiency was found as the enzyme concentration raised up to about 10U. More than 10U did not lead to effective increase in transformation efficiency (data not shown). Therefore, 10U was established for the transformation using restriction enzymes.

When the restriction enzyme *Bam*HI was added to the transformation mixture, the mean increase in the transformation efficiency for the plasmid pBHg was more than three-fold and for pHCP, about ten-fold in comparison to the treatment without the enzyme. Transformation in the presence of the enzyme *Not*I led to a mean increase in the transformation efficiency of up to three times (Fig 1). There was no significant difference in the transformation efficiency in relation to the use of the circular or linear forms.

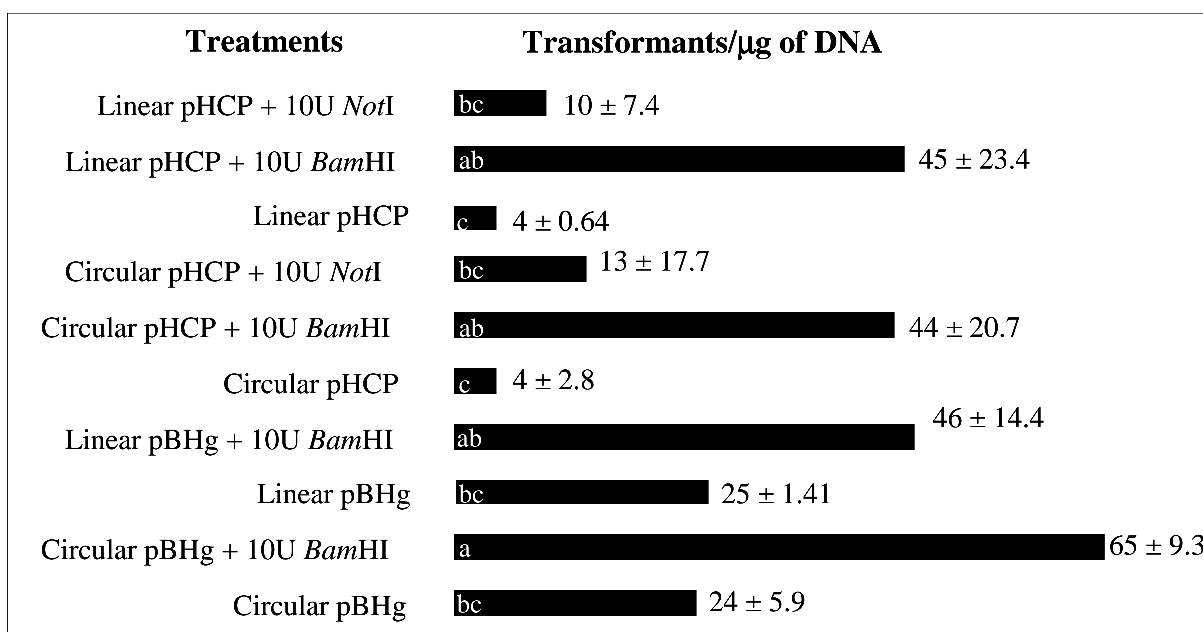

Figure 1 - Transformation efficiency of *M. perniciosa* in different conditions (circular or linear vectors and presence or absence of restriction enzymes). The numbers are average of at least three independent experiments. Treatments with different letters differ statistically at 5% of significance through the test of Scott-Knott.

*Not*I enzyme increased the transformation efficiency. However, it was less effective than *Bam*HI, possibly by the fact that there might be less *Not*I sites (8pb recognition site) than *Bam*HI (6pb recognition site) in the fungal genome. Manivasakam and Schiestl (1998) suggested that the effects shown by different restriction enzymes in promoting integration events might depend on the ability of the enzyme to enter the cell. If it enters, it might be inactive in the cell environment or degraded. Each organism is a different system that should be thus separately analyzed.

In order to investigate at molecular level the effects of each treatment over the integration events, genomic DNA of pBHg and pHCP transformants was cleaved with *Xho*I and *Sal*I, respectively. Both the enzymes chosen for this analysis cut once in the vectors. Concerning the pBHg insertions, two transformants had a single integration (Fig 2, lanes 1 and 4), three had two insertions in distinct regions (Fig 2, lanes 2, 7 and

11), two had two integrated tandem copies (Fig 2, lanes 3 and 12), and five had more than two copies inserted in different positions (Fig 2, lanes 5, 6, 8, 9 and 10). The Southern blot hybridization of the genomic DNA from transformants carrying pHCP showed that eight transformants had a single plasmid insertion in the genome (Fig 3, lanes 6, 8, 12, 14, 15, 16, 19 and 24). Six of these eight transformants originated from treatments where restriction enzyme was added. Nine transformants had two copies of the integrated vector (Fig 3, lanes 2, 10, 11, 13, 17, 18, 20, 22 and 23) and seven transformants had more than two copies (Fig 3, lanes 1, 3, 4, 5, 7, 9 and 21). Comparing the treatments where circular (5 to 8) and linear (1 to 4) pHCP plasmid were used, one can notice that in the latter the transformants had more integrations. The same is valid for pBHg plasmid. Few integrations were observed in the treatment where circular pHCP was used together with enzyme *Bam*HI. In this treatment, three transformants had a single integration (Fig 3, lanes, 14, 15 and 16) and one had two integrations (Fig 3, lane 13). Therefore, the linearization of the vector was unnecessary and the use of circular plasmid was thus significantly effective when enzyme *Bam*HI was added to the transformation mixture. Concerning the experiments where enzyme *Not*I was used, 75% of the transformants had single or double insertions. On the other hand, when *Not*I was not added, 75% of the transformants had multiple integrations (Fig 3). Enzyme *Not*I was, therefore, effective in promoting less plasmid insertions, but not as effective as enzyme *Bam*HI in raising the transformation efficiency. All the integration occurred randomly at different genomic sites. In order to use the transformation as a mutagenic method, the randomness of the integrations is important, since every gene under such condition, is likely to be inactivated.

Isolation of *M. perniciosa* mutants is laborious. The mutations may be masked by the wild type phenotype since the mycelium develops most of the time from multinucleate protoplasts. However, a mutant impaired in methionin biosynthetic pathway could be isolated from a total of 500 colonies screened for detrimental growth on minimal medium lacking methionin. This mutant was investigated for the plasmid integration patterns. Southern blot analysis confirmed the presence of two copies of pBHg inserted in different genomic sites (result not shown). Unfortunately, it was not possible to rescue the plasmid to confirm if the mutation was due to gene knockout. After successive rounds of transfers, the mycelia deteriorated and the mutant was lost.

Based on the results, the addition of restriction enzyme in the transformation mixture was effective in enhancing the transformation efficiency and reducing the number of plasmid integrations in the *M. perniciosa* genome. These results represent important steps toward improving the transformation system of *M. perniciosa* aiming insertional mutagenesis with plasmids.

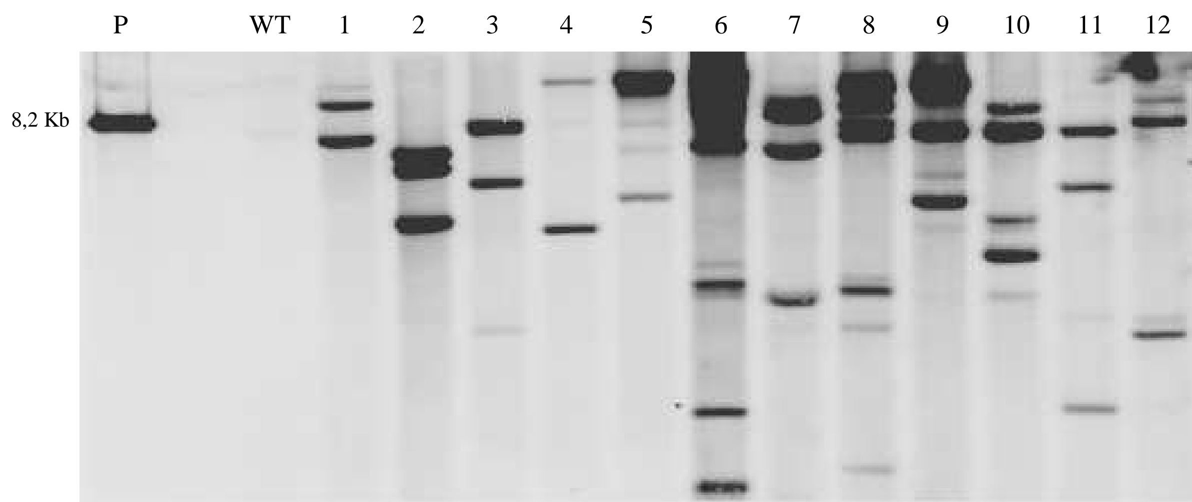

Figure 2 - Southern blot hybridization of genomic DNA from wild type and transformants. The genomic DNA was digested with *Xho*I and pBHg was used as probe. (P) Linearized pBHg, (WT) wild type, (1-4) transformants with circular pBHg, (5-8) linear pBHg, (9-12) circular pBHg + 10U *Bam*HI.

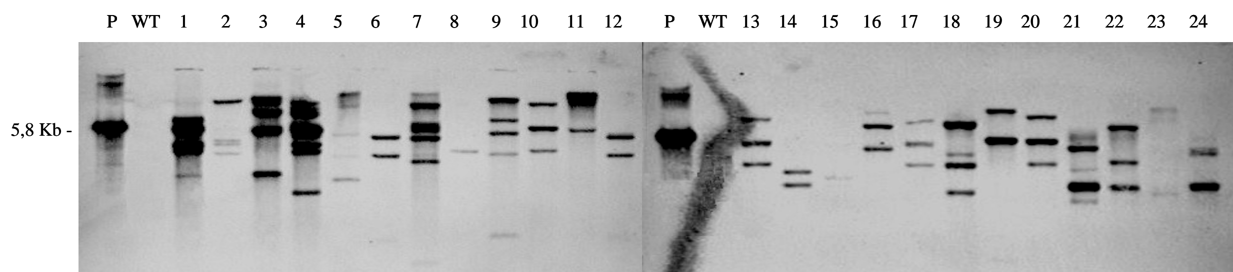

Figure 3 - Southern blot hybridization of genomic DNA from wild type and transformants. DNA was digested with *Sal*I and pHCP was used as probe. (P) pHCP, (WT) wild type, 1-4 linear pHCP, 5-8 circular pHCP, 9-12 linear pHCP + 10U of *Bam*HI, 13-16 circular pHCP + 10U of *Bam*HI, 17-20 linear pHCP + 10U of *Not*I, 21-24 circular pHCP + 10U of *Not*I.

ACKNOWLEDGEMENTS

The authors wish to thank CAPES (Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the research grants and financial support for this work.

RESUMO

A presença de enzima de restrição na mistura de transformação aumentou a eficiência da transformação em *Moniliophthora perniciosa*. A influência da forma do vetor (linear ou circular), o padrão de integração do plasmídeo nos sítios genômicos e a influência do promotor usado para expressar o gene marcador foram também analisados. A adição de *Bam*HI ou *Not*I aumentou o número de transformantes 3-10 vezes e 3 vezes, respectivamente, em relação ao controle sem a adição da enzima. O uso de plasmídeos pré-linearizados não aumentou a eficiência da transformação quando comparado à eficiência obtida com plasmídeos circulares. No entanto, a freqüência de transformantes multi-cópias aumentou significativamente. Juntos os procedimentos reportados aqui resultaram em processos mais eficientes de produção de protoplastos e transformação, onde o tempo necessário para o aparecimento dos transformantes e o número de inserções múltiplas foi reduzido.

REFERENCES

Aime, M.C. and Phillips-Mora, W. (2005). The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia*, 97(5), 1012–1022.

Balance, D. J. and Turner G. (1985), Development of a high frequency transforming vector for *Aspergillus nidulans*. *Gene*, **36,** 321-331.

Calle, H., Cook, A. A. and Fernando, S. Y. (1982), Histology of witches´ broom caused in cacao (*Theobroma cacao*) by *Crinipellis perniciosa. Phytopathology*, **72**, 1479-1481.

Chen, X., Stone, M., Schlagnhaufer, C. and Romaine C. P. (2000). A fruiting body tissue method for efficient *Agrobacterium*-mediated transformation of *Agaricus bisporus*. *Appl. Environ. Microbiol.,* **66**, 4510-4513.

Chung, K. R., Ehrenshaft, M., Wetzel, D. K. and Daub, M. E. (2003), Cercosporin-deficient mutants by plasmid tagging in the asexual fungus *Cercospora nicotianae*. *Mol. Genet. Genomics,* **270**, 103-113.

Cummings, W. J., Celerin, M., Crodian, J., Brunick, L. K. and Zolan, M.E. (1999), Insertional mutageneses in *Coprinus cinereus*: use of a dominant selectable marker to generate tagged, sporulation-defective mutants. *Curr. Genet.*, **36**, 371-382.

Fujimoto, D., Shi Y, Christian, D., Mantaguihan. J. B. and Leung, H. (2002), Tagging quantitative loci controlling pathogenicity in *Magnaporthe grisea* by insertional mutagenesis. *Physiol. Mol. Plant. Pathol.*, **61**, 77-88.

Goedecke, W., Pfeiffer, P. and Vielmetter, W. (1994), Nonhomologous DNA end joining in *Schizosaccharomyces pombe* efficiently eliminates DNA double-strand-breaks from haploid sequences. *Nucleic Acids Res.*, **22**, 2094-2101.

Holliday, R. (1956), A new method for the identification of biochemical mutants of micro-organisms. *Nature*, **178**, 987.

Herrera-Estrella, A., Goldman, G. H. and Van Montagu, M. (1990). High-efficiency transformation system for the biocontrol agent *Trichoderma* spp. *Mol. Microbiol.*, **4**, 839-843.

Jin, X., Mo, M. H., Wei, Z., Huang, X. W. and Zhang, K. Q. (2005), Transformation and mutagenesis of the nematode-trapping fungus *Monacrosporium sphaeroides* by restriction enzyme-mediated integration (REMI). *J. Microbiol.*, **43**, 417-23.

Kahmann, R. and Basse, C. (1999), REMI (Restriction Enzyme-mediated integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. *Eur. J. Plant Pathol.*, **105**, 221-229.

Levis, C., Fortini, D. and Brygoo, Y. (1997), Transformation of *Botrytis cinerea* with the nitrate reductase gene (*nia*D) shows a high frequency of homologous recombination. *Curr. Genet.*, **32,** 157-162 .

Lima, J. O., dos Santos, J. K., Pereira, J. F., de Resende, M. L. V., de Araújo, E. F. and Queiroz, M. V. (2003), Development of a transformation system for *Crinipellis perniciosa*, the causal agent of witches´broom in cocoa plants. *Curr. Genet.*, **42**, 236-240.

Manivasakam, P., and Shiestl, R. H. (1998), Nonhomologous end joining during restriction enzyme-mediated DNA integration in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **18**, 1736-1745.

Marmeisse, R., Gay, G., Debaud, J. C. and Casselton, L. A. (1992), Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum. Curr. Genet.,* **22**, 41-45.

Oeser, B., Heidrich, P. M., Muller, U., Tudzynski, P. And Tenberge, K. B. (2002), Polygalacturonase is a pathogenicity factor in the *Claviceps purpurea/*rye interaction. *Fungal Genet. Biol.*, **36**, 176-186.

Orchard, J., Collin, H.A., Hardwick, K. and Isaack, S. (1994), Changes in morphology and measurement of cytokinin levels during the development of witches´ broom on cocoa. *Plant Pathol.,* **43**, 65-72.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D., and Bufton, A. W. (1953), The genetics of *Aspergillus nidulans*. *Adv. Genet*., **5**, 141-238.

Pound, F. J. (1943), Cacao and witches´broom disease (*Marasmius perniciosus*). A report on a recent visit to the Amazon region of Peru, September 1942-february 1943. Department of agriculture of Trinidad and Tobago. Yuille´s printerie, St. Augustine, Trinidad, 14p

Punt, P. J., Oliver, R. P., Dingemanse, M. A., Pouwels, P. H. and van den Hondel, C. A. M. J. J. (1987), Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene*, **56**, 117-124.

Ridder, R. and Osiewacz, H. D. (1992), Sequence analysis of the gene coding for glyceraldehyde-3-phosphate dehydrogenase (*gpd*) of *Podospora anserina*: use of homologous regulatory sequences to improve transformation efficiency. *Curr. Genet*., **21**, 207-213.

Riggle, P. J. and Kumamoto, C. A. (1998), Genetic analysis in fungi using restriction-enzyme-mediated integration. *Curr. Opin. Microbiol*., **1**, 395-399.

Rincones, J., Meinhardt, L. W., Vidal, B. C. and Pereira, G. A. G (2003), Electrophoretic karyotype analysis of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. *Mycol. Res.,* **107**, 452-458.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schiestl, R. H. and Petes, T. D. (1991), Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*, **88,** 7585-7589.

Seong, K., Hou, Z., Tracy, M., Kistler, H. C. and Xu, J. R. (2005), Random insertional mutagenesis identifies genes associated with virulence in the wheat scab fungus *Fusarium graminearum. Phytopathology,* **95,** 744-750.

Specht, C.A., DiRusso, C.C., Novotny, C.P. and Ullrich, R.C. (1982), A method for extracting high-molecular weight deoxyribonucleic acid from fungi. *Anal. Biochem*., **119**, 158-163.

Suzuki, K., Imai, Y., Yamashita, I. and Fukui, S. (1983), In vivo ligation of linear DNA molecule to circular forms in the yeast *Saccharomyces cerevisiae*. *J. Bacteriol*., **155**, 747-754.

Tanaka, A., Shiotani, H., Yamamoto, M. and Tsuge, T. (1999), Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-toxin in the Japanese pear pathotype of *Alternaria alternata. MPMI*, **12**, 691-702.

Thon, M. R., Nuckles, E. M. and Vaillancourt, L. J. (2000), Restriction enzyme-mediated Integration used to produce pathogenicity mutants of *Colletotrichum graminicola. MPMI*, **13**, 1356-1365.

Warwar, V., Oved, S. and Dickman, M. B. (2000), Antisense expression of the calmodulin gene from *Colletotrichum trifolii* impairs prepenetration development. *FEMS Microbiol. Lett*., **191**, 213-219.

Yelton, M. M., Hamer, J. E. and Timberlake, W. E. (1984), Transformation of *Aspergillus nidulans* by using a *trp*C gene. *Proc. Natl. Acad. Sci. U S A*, **81**, 1470-1474.

Received: April 24, 2006;
Revised: September 19, 2006;
Accepted: July 19, 2007.