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RESEARCH PAPER

Construction and evaluation of a transformant library of *Lasiodiplodia theobromae* generated through Restriction Enzyme-Mediated Integration

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Summary. Grapevine dieback, caused by *Lasiodiplodia theobromae*, is an important trunk disease worldwide. Transformants of *L. theobromae* were generated in an attempt to identify potential pathogenicity-related genes. *Lasiodiplodia theobromae* strain JZB 0300251, a highly virulent isolate, was selected for the genetic transformation. Based on optimised conditions, the Restriction Enzyme-Mediated Integration (REMI) methodology was established in *L. theobromae* using pUCATPH (a plasmid carrying a hygromycin B marker). A total of 6,036 transformants were generated with four restriction enzymes, respectively and the transformant library was evaluated based on 200 randomly selected transformants. Mutants that exhibited various degrees of virulence and different growth rates were obtained. The study provides basic results that will lead to increased understanding of the role of the pathogenicity-related genes involved in the infection process of *L. theobromae*.

Key words: mutants, grapevine Botryosphaeria dieback, pathogenicity variant.

Introduction

Table and wine grapevines are grown in most provinces of China, and in 2010 the total area of grapevines was approx. 530,000 ha (C.Q. Duan, China Agricultural Research System, personal communication). Grape powdery and downy mildews have been considered as the most destructive diseases, but grapevine Botryosphaeria dieback (formerly Botryosphaeria canker) has recently become more prevalent in China (Yan *et al.*, 2012). Species in the Botryosphaeriaceae, which are well-known as opportunistic pathogens to a wide range of hosts, cause serious dieback in many woody plants (Maas and Uecker, 1984; Rumbos, 1987; Michailides, 1991; Phillips, 2002; van Niekerk *et al.*, 2004; Amponsah *et al.*, 2011), and 21 species, including, *Botryospaeria dothidea*, *Diplodia mutila*, *D. porosum*, *D. seriata*, *Dothiorella sarmentorum*, *Lasiodiplodia crassispora, L. theobromae, Neofusicoccum australe*, *N. luteum*, *N. mediterraneum*, *N. parvum*, *N. ribis*, *N. viticlavatum*, *N. vitifusiforme* and *Spencermartinsia viticola*, have been associated with grapevine dieback (Ma *et al.*, 2001; Siebert, 2001; Taylor *et al.*, 2005; Úrbez-Torres *et al.*, 2007a,b; Úrbez-Torres and Gubler, 2009). Botryosphaeria dieback has been reported in almost all of the main grape growing countries, including Africa, Australia, Chile, China, Egypt, France, Hungary, Italy, Lebanon, Portugal, Spain, and the USA (El-Goorani and El Meleigi, 1972; Lehoczky, 1974; Leavitt and Munnecke, 1987; Rovesti and Montermini, 1987; Castillo-Pando *et al.*, 2001; Larignon and Dubos, 2001; Phillips, 2002; Auger *et al.*, 2004; van Niekerk *et al.*, 2004; Luque *et al.*, 2005; Choueiri *et al.*, 2006; Li *et al.*, 2010).

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Yan *et al*. (2011) reported that *L. theobromae* (Pat.) Griffon & Maubl. is one of the pathogens associated with grapevine Botryosphaeria dieback in China, and a subsequent pathogenicity test showed that the fungus was the most virulent of four species that were identified from diseased samples collected from China (unpublished data). The role of *L. theobromae* as the cause of grapevine dieback has been studied over the past few years (Úrbez-Torres, 2011; Yan *et al*., 2011), but the infection mechanism and pathogenicity-related genes of the fungus have not yet been elucidated.

In the past two decades, after genetic transformation systems was introduced, molecular-level studies on phytopathogenic fungi have been performed, and many other technologies, such as gene knockout and complementary, have been developed for the confirmation of the function of genes that play key roles in infection pathways. The Restriction Enzyme-Mediated Integration (REMI) technique was first applied to *Saccharomyces cerevisiae* (Schiestl and Petes, 1991), and has been subsequently used in many phytopathogenic fungi, such as *Alternaria alternata*, *Cochliobolus heterostrophus*, *Colletotrichum gloeosporioides*, *Coprinus cinereus*, *Lentinus edodes*, and *Magnaporthe oryzae* (Lu *et al*., 1994; Shi *et al*., 1995; Akamatsu *et al*., 1997; Granado *et al*., 1997; Yakoby *et al*., 2000). The principal conditions and vectors of the REMI process have also been optimised to obtain high-efficiency transformations in different microbes, and have been commonly used in recent years (Liu *et al*., 2008). REMI allows the introduction of random tagged mutations into the host genome, and also allows the generation of mutants with specific phenotypes and mutations in single loci, from which specific functional genes can be isolated (Todd *et al*., 1999; Choi *et al*., 2008).

The present study reports the successful application of REMI in *L. theobromae* to produce a series of mutants with different phenotypes. These can be used for functional gene cloning and to analyse the functions of the genes involved in the infection processes.

Materials and methods

Fungal strain, grapevine cultivar, and transformant plasmid

Lasiodiplodia theobromae strain JZB 0300251 was used as the wild-type strain for transformation. This strain was first isolated from a grapevine in Changsha, Hunan Province, and was maintained in the laboratory of the Institute of Plant and Environment Protection at the Beijing Academy of Agriculture and Forestry Sciences (BAAFS). Strain JZB 0300251 grows rapidly on potato dextrose agar (PDA) at 28°C. The grapevine cultivar used for pathogenicity tests was Black Summer, and was provided by Prof. HY Xu (Institute of Forestry and Pomology Science, BAAFS). The transformation vector pUCATPH, which carries hygromycin B (*Hyg*) and ampicillin (*amp*) resistance genes as its selective markers, was provided by Y.L. Peng (China Agricultural University).

Hygromycin B resistance determination

The natural resistance of wild-type JZB 0300251 toward the antibiotic hygromycin B was evaluated through cultivation on PDA medium containing different concentrations of hygromycin B (20, 30, 40, 50, 60, 70, 80, 90, and 100 μg mL^{-1}) at 28°C for 2 d.

Protoplast preparation

Protoplasts were prepared as described be Shi *et al*., (1995) with minor modifications. Mycelium cultured in liquid complete medium (CM, 0.3% casein acids hydrolysate, 0.3% casein enzymatic hydrolysate, 0.6% yeast extract, 1% sucrose) was used for the protoplast preparation. The mycelium was washed twice with 0.7 M NaCl and then digested with 2 mL of an enzyme solution (20 mg mL^{-1} Driselase, Sigma Company, and 20 mg mL^{-1} Snailase, Bei Jing Hao Cheng Zhi Yuan Biotech Co., Ltd.) per 1 μg of mycelia, at 28°C for 4 h. Protoplasts were harvested by filtration through three layers of filter paper and washed twice with STC buffer (10 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, and 1.2 M sorbitol). The protoplasts were then suspended in STC buffer, and the final concentration was adjusted to 1×10^8 protoplasts mL^{-1}.

Transformation procedure

Transformation of *L. theobromae* was performed using the REMI method as described by Shi *et al*., (1995) with some modifications. Fresh protoplasts (150 μL, 1×10^8 protoplasts/ mL^{-1}) were gently mixed with 2 μg of the linearised plasmid pUCATPH in a 50-mL sterile distillation tube. The restriction enzyme was then added, and the STC buffer was amended to a final volume of 300 μL. The mixture

was kept on ice for 20 min, then 2 mL of 60% PEG solution [60% PEG3350, 10 mM Tris-HCl (pH 7.5), and 50 mM $CaCl_2$] was added, and the mixture was kept on ice for a further 20 min. Subsequently, 15 mL of STC buffer was added, and the mixture was mixed and then centrifuged at 2,000 g for 15 min at 4°C. The pelleted protoplasts were suspended in 2 mL of liquid regeneration medium (LRM, 0.1% yeast extract, 0.1% casein enzymatic hydrolysate, and 1 M sucrose) and incubated at 28°C for 8–12 h. The incubated mixture was mixed evenly with pre-heated (45°C) solid regeneration medium (LRM plus 1.6% agar) and then dispersed into 90 mm diam. Petri dishes. After the mixture solidified, each transformation plate was covered with 15 mL of 0.8% water agar medium (containing 70 μg mL^{-1} hygromycin B) and incubated for 2–3 d at 28°C.

Transformant selection and storage

When a single colony of a transformant appeared, the colony was transferred to a new CM medium plate containing 50 μg mL^{-1} hygromycin B and incubated at 28°C for further subculture confirmation. The positive transformants were transferred to fresh PDA plates covered with several pieces of sterilised and dried filter paper. When the paper was covered by mycelia, the paper was transferred to a paper bag and dried. The bag was then transferred to a box sealed with tape and maintained at -20°C in a refrigerator.

Transformant detection by PCR

The fungal mycelium cultured in CM liquid medium at 28°C was used for DNA extraction. The genomic DNA of randomly selected transformants and wild-type strain JZB 0300251 was extracted using the CTAB method (Graham *et al.*, 1994). The presence of *Hyg* in the transformants was determined by PCR, using the specific primers *Hyg*-up (5′-GATGTAGGAGGGCGTGGATA-3′) and *Hyg*-down (5′-TCAAGACCAATGCGGAGCA-3′). The PCR reaction consisted of 5–10 ng of template, 0.3 μL of Taq, 50 μM *Hyg*-up primer, 50 μM *Hyg*-down primer, and a dNTP and buffer mixture. The PCR reaction was performed in a total volume of 25 μL, which was obtained with double distilled H_2O. All of the components were obtained from Takara, Dalian China. The PCR program was performed under the following conditions: 94°C for 3 min and then 35 amplification cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. DNA of the strain JZB 0300251 was used as the negative control.

Genomic Southern blot analysis of transformants

Approximately 15 μg of the genomic DNA digested with the restriction enzyme *Xho*I (Takara) was separated through 0.8% agarose gel electrophoresis and blotted onto a Hybond N^+ membrane (Amersham) according to the manufacturer's instructions. The coding region of *Hyg* was labelled as the probe for the confirmation of the transformants. The DNA probe was generated by PCR amplification using hygromycin B gene prime pairs (*Hyg*-up, 5′-GATGTAGGAGGGCGTGGATA-3′, and *Hyg*-down, 5′-TCAAGACCAATGCGGAGCA-3′), using the pUCATPH plasmid as the template. DNA labelling, hybridisation, and signal detection were performed using a DIG High Prime DNA labelling and detection starter kit I (Roche) according to the manufacturer's instructions.

Phenotypic evaluation of transformants

Eighty randomly selected REMI transformants were cultured on PDA for 48 h at 28°C. Mycelium plugs of the REMI transformants and strain JZB 0300251 were cut with a 4 mm diam. cork borer, then transferred to PDA plates and incubated for 24 h, when colony diameters were measured. The experiment was conducted three times. The data were analysed for significant differences using the DPS statistical analysis system (Version 13.5).

Green shoots (8–10 mm diam., 30 cm length) were used for the pathogenicity test. All of the leaves and tendrils were removed from each shoot, and wounds were made on the shoots with a sterile 4 mm diam. metal borer. A 4 mm diam. inoculum plug from a 2-d-old colony growing on PDA was then placed into each hole, and each hole was then covered with parafilm (BEMIS). Control shoots were filled with non-colonised plugs of PDA. Each inoculated shoot was inserted into apot filled with sterile soil. The pots were placed in a greenhouse and maintained under controlled temperature (28±2°C) for 4 d. The lesion length was then measured on each shoot. The experiment was repeated twice. The data were analysed for significant differences using DPS (Version 13.5).

Results

Hygromycin susceptibility

The hygromycin sensitivity of strain JZB 0300251 was evaluated by its growth on PDA with different concentrations of hygromycin B (up to 100 μg mL^{-1}). Colony growth of the strain was completely inhibited at a hygromycin B concentration of 70 μg mL^{-1} at 28°C (Figure 1). Therefore, a hygromycin B concentration of 70 μg mL^{-1} was used for selection of transformants.

Confirmation of REMI transformants

The detection of hygromycin B resistance in nine randomly selected REMI transformants was confirmed by PCR amplification. The hygromycin gene was detected in the genomic DNA of all nine transformants and the positive control plasmid pUCATPH, but not in strain JZB 0300251 (Figure 2).

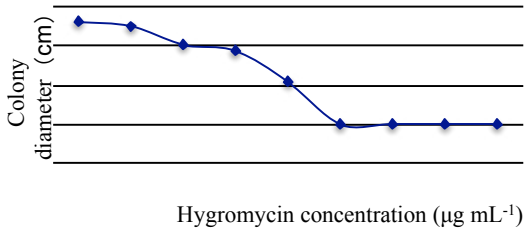

Figure 1. Mean diameter of *Lasiodiplodia theobromae* colonies incubated for 2 d on PDA containing different concentrations of hygromycin B.

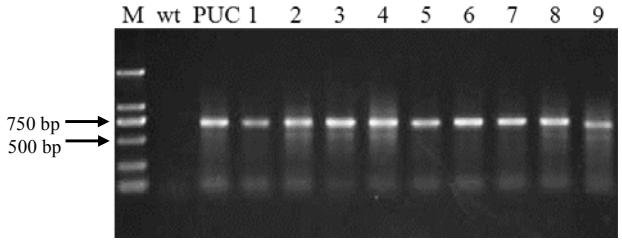

Figure 2. Identification of *Lasiodiplodia theobromae* transformants through PCR using specific primers for hygromycin B. Legend: M, 2,000-bp marker (Takara); Wt, wild-type JZB 0300251 strain; PUC, pUCATPH plasmid; 1-9, randomly selected transformants.

REMI transformant library

To investigate the stability of the transformants, they were successively cultured on PDA medium for five rounds and then transferred to selection PDA plates containing 70 μg mL^{-1} hygromycin B. All of the randomly selected transformants retained their resistance to hygromycin B, which indicates that the transformants containing the extra vector were stable. In addition, many transformants exhibited different phenotypic characteristics compared to strain JZB 0300251 (Figure 3). Some mutants grew more or less rapidly than the wild type strain, and some mutants exhibited different colours or mycelial density compared with the wild type strain.

An REMI library of *L. theobromae* containing 6,036 transformants was constructed, and the detailed characteristics of the transformation are shown in Table 1. Almost all the transformants were generated

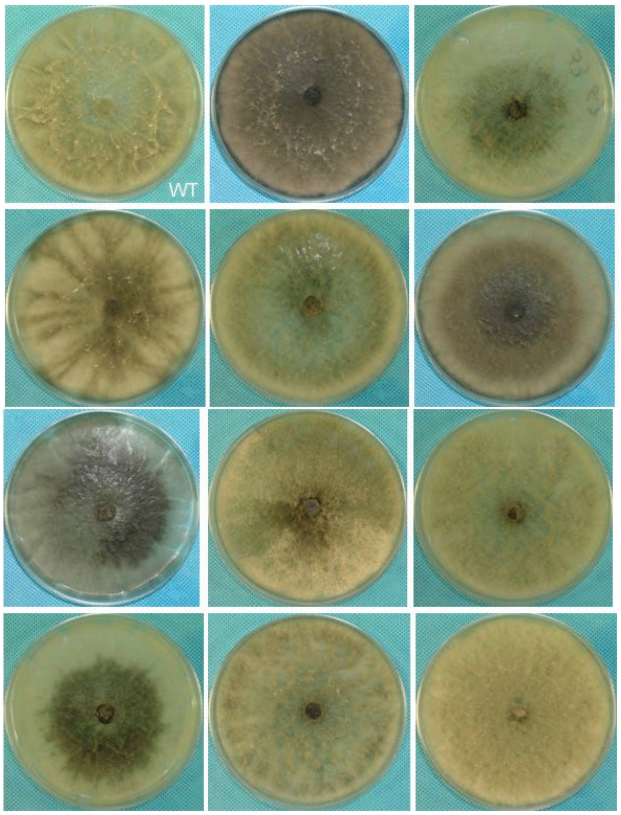

Figure 3. Different phenotypes of REMI transformants of *Lasiodiplodia theobromae*. Legend: WT, wild-type JZB 0300251 strain; the other images are of transformants generated by REMI.

Table 1. Number of *Lasiodiplodia theobromae* transformants obtained with different enzyme quantities by REMI.

Restriction enzyme	Enzyme quantity (U/μg*)					Transformants number
	0	15	30	45	60	
*Hin*d	-	108	367	3039	498	4012
K*pn*	-	40	73	427	607	1147
S*ac*	-	57	3	27	72	159
S*ma*l	-	3	62	28	53	146
No enzyme[b]	542	-	-	-	-	542
Blank[c]	30	-	-	-	-	30
Total	542	208	505	3521	1230	6036

[a] The quantities (Units) of enzymes added per μg of vector during the REMI procedure.
[b] Transformation obtained in the absence of an enzyme during the REMI procedure.
[c] Transformation obtained without an enzyme and a vector during the REMI procedure.

with *Hin*dIII (45 U μg^{-1}). Analysis of the genomic Southern blot showed additional single locus insertion according to this ratio (data not shown), and the Southern blot of three randomly selected pathogenicity mutants also exhibited single locus insertion (Figure 4).

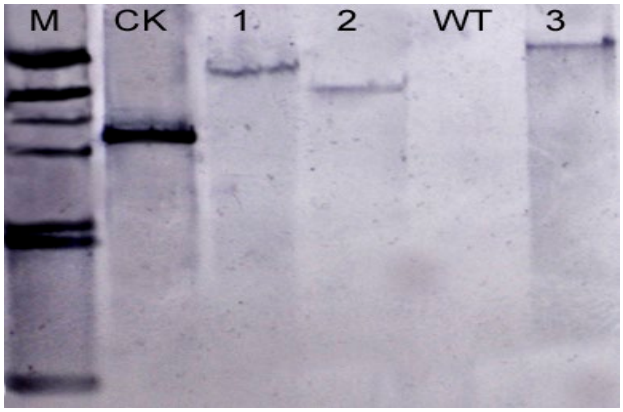

Figure 4. Genomic Southern blot analysis of virulent *Lasiodiplodia theobromae* mutants with a probe (parts of hygromycin B). Legend: M, marker, λDNA digested with *Hin*dIII; CK, positive control, vector; 1, 2, and 3, mutants that exhibited a stronger virulence compared with the wild-type strain; WT, wild-type JZB 0300251 strain.

Growth rate assay

Eighty transformants derived from strain JZB 0300251 were incubated on PDA for 24 h, and the colony diameters were then measured. This showed significant differences in growth rate between most of the transformants and strain JZB 0300251, as shown in Figure 5. When the colony diameters of strain JZB 0300251 reached almost 6 cm, the colony diameters of some of the transformants, such as 169, 164, 215, 233 and 174, were less than 3 cm, whereas some of the transformants, such as 197, 39, 125, and 112, had almost filled the Petri dishes (colony diam. 9 cm). The different integration sites in the genome of the transformants may have resulted in the disruption of specific genes and thus produced the different growth rate phenotypes.

Pathogenicity assay

Fifty randomly selected transformants were used for the pathogenicity assay. No lesions were observed in the non-wounded excised green shoots. Strain JZB 0300251 and the transformants all produced dark-brown lesions in the wounded excised green shoots (Figure 6). The virulence of some of the transformants was obviously reduced, whereas some of the transformants exhibited increased virulence compared with the wild type strain. Most of the REMI transformants exhibited significant differences compared with strain JZB 0300251 strain ($P<0.05$).

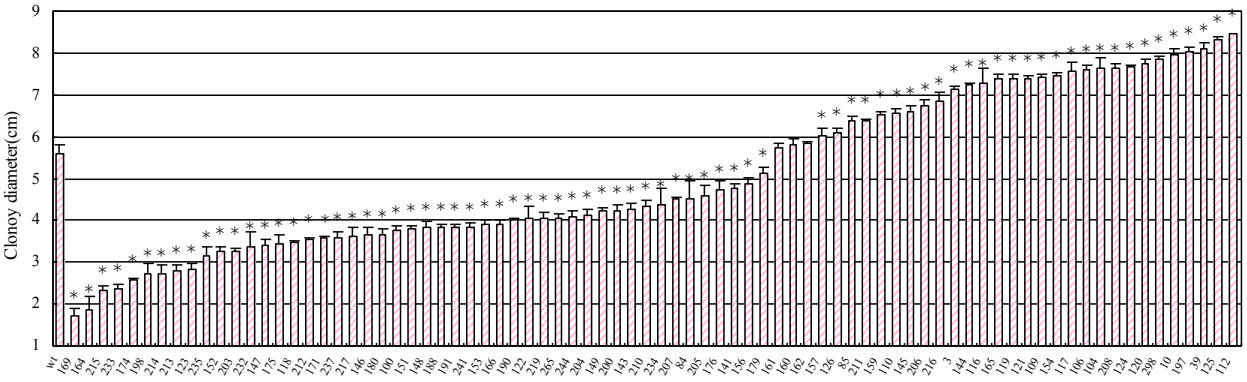

Figure 5. Mean colony diameters of randomly selected REMI *Lasiodiplodia theobromae* transformants. Legend: wt, JZB 0300251 strain; * indicates significant differences (P<0.05) between JZB0300251 and a specific transformant, as determined through Duncan's method. Standard deviations of the means are indicated.

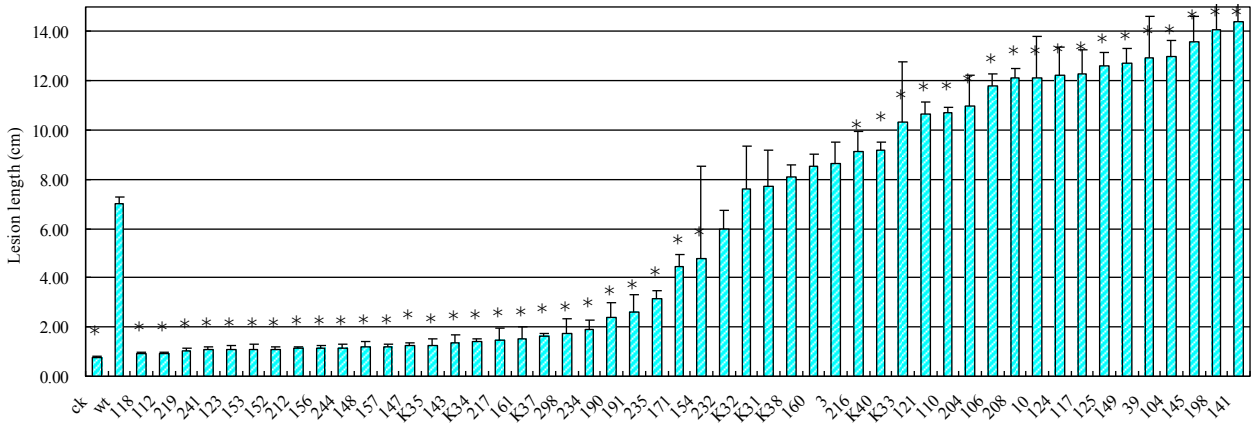

Figure 6. Mean lesion lengths resulting from inoculations of randomly selected *Lasiodiplodia theobromae* REMI transformants, in a pathogenicity test. Legend: wt: JZB 0300251 strain; ck: non-colonised plugs of PDA; * indicates significant differences (P<0.05) between the JZB 0300251 strain and a specific transformant, as determined through Duncan's method. Standard deviations of the means are indicated.

Discussion

The opportunistic species of Botryosphaeriaceae that cause plant diseases have received increasing attention, and are important grapevine pathogens. Species and genera of the Botryosphaeriaceae are now clearly defined (Phillips *et al.*, 2013), and are known to infect many woody plants to induce dieback or canker symptoms, determined with updated techniques and methods (von Arx and Muller, 1954; Barr, 1987; Crous *et al.*, 2006; Úrbez-Torres 2011). *Lasiodiplodia theobromae* is widespread in the tropics and subtropics (Punithalingam, 1980), and exhibits optimal growth at higher temperatures than most of the other species in the Botryosphaeriaceae. *Lasiodiplodia theobromae* has demonstrated different virulence in different countries. For example the pathogen is considered as the most virulent specie in Mexico, through *in vivo* pathogenicity tests performed in Western Australia on cuttings and on green shoots and rooted cuttings in Mexico, while in South Africa, this specie showed weak pathogenicity on green shoots *in vivo* but high virulent on mature canes *in vitro* (van Niekerk *et al.*, 2004; Taylor *et al.*, 2005; Úrbez-Torres and Gubler, 2009). In China, *L. theobromae* causing grapevine dieback is mainly confined to the tropical and subtropical regions, i.e., in the Guangxi,

Hunan, and Zhejiang provinces (Yan *et al*., 2013). We have found that isolates of this species are more virulent than any of the other species on Black Summer and 20 other grape cultivars grown in China (data not shown). For this reason we chose one isolate of *L. theobromae* for the study reported here.

Grapevine dieback caused by *L. theobromae* is becoming increasingly common in many grape-growing countries (van Niekerk *et al*., 2004; Úrbez-Torres *et al*., 2006; Pitt *et al*., 2010). Almost all of the reports related to *L. theobromae* have focused on its biological and morphological characterisation, whereas infection mechanism and specific functional genes of the pathogen have not yet been studied. The REMI method has previously been used to generate 2,184 transformants that were screened for toxin production in *Eutypa lata*, which is the first grapevine trunk pathogen to be studied using REMI transformation (Jordan *et al*., 2010). In the present study, an REMI system for *L. theobromae* resulted in the construction of a transformant library containing more than 6,000 independent transformants. This research will provide material for specific phenotype gene cloning. Using plasmid rescue or other techniques, we can easily obtain key genes controlling specific phenotypes and then analyse their roles in infection processes. This will provide increased understanding of infection development and mechanisms for this organism.

The Southern blot analysis of the randomly selected transformants showed that the single copy insertion rate by *Hin*dIII was approximately 60%, which can be used for the analysis of specific genes through molecular manipulation procedures. During the pathogenicity test, we also found that some of the mutants had lost their pathogenicity, and these can be used for cloning specific genes in the future. The pathogenicity variation ratio of the isolate used in this study was greater than that obtained for other fungi, such as *Magnaporthe oryzae* and *Fusarium oxysporum* (Sweigard *et al*., 1998; Inoue *et al*., 2001). We have confirmed the effect on the isolate through the REMI method in the absence of plasmid and restriction enzyme, and the results showed that virulence of the transformants was not changed. This indicates that the REMI procedure alone did not induce variation. Another interesting result was that some mutants obtained through REMI showed increased virulence. In other studies, such as with *Magnaporthe oryzae* and *Fusarium oxysporum* (Sweigard *et al*., 1998; Inoue *et al*., 2001), very few mutants showed increased virulence. We hypothesise that some inhibitor(s) controlling the virulence of the isolate may have been disrupted.

Acknowledgements

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