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Construction of *Beauveria bassiana* T-DNA insertion mutant collections and identification of thermosensitive and osmosensitive mutants

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Abstract: [Objective] This study was to identify genes involved in thermotolerance and osmotolerance of *Beauveria bassiana*. [Methods] A collection of T-DNA random insertion mutagenesis was constructed and thermosensitive and osmosensitive mutants were screened. The DNA fragments flanking to inserted T-DNA sequence in the mutants were isolated by using Y-shaped adaptor dependent extension (YADE) method. [Results] Five mutants impaired in thermotolerance or osmotolerance were identified and characterized. Mutant 212 and 2550 could not grow at 32℃, while mutants 139, 2737 and 2812 showed depressed growth under osmotic stress. Conidial viabilities of 212 was decreased by 23%, 52% and 21% after stressed by ultraviolet, heat shock, and H_2O_2 oxidation, respectively, compared to the wild-type strain. Virulence of mutant 212 and 2737 against aphids *Myzus persicae* were reduced by 12% and 40%, respectively. Sequencing analysis indicated that two function-unknown genes were interrupted by insertion of T-DNA in mutant 212 and 2737. In other three mutants, the insertions are located in the non-encoding regions. [Conclusion] The results demonstrate the successful use of T-DNA insertion mutagenesis in identification of genes involved in thermotolerance and osmotolerance of *B. bassiana*.

Keywords: *Beauveria bassiana*; insertion mutagenesis; thermotolerance; osmotolerance
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1 INTRODUCTION

Entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, are considered as eco-friendly and promising biological control agents for a variety of agricultural pests. However, the slow rate of kill in controlling pests and low tolerance to adverse condition in the field limit their widespread application. Adverse environmental factors, such as high temperature and osmostress, inhibit the conidial germination, hyphal growth, invasion speed and virulence[1-3], and determine the shelf-life of conidia[4]. The inherent defect is still the restrictive factor for the development of fungal-based insecticides. A fungal formulation with greater resistance to environment would be of more potential for practical application[5]. The resistance of insect-pathogenic fungi to adverse condition has attracted great attention[5-8]. However, the mechanism of adaptation to high temperature and osmostress in insecticidal fungi has been poorly understood.

T-DNA insertion mutagenesis represents a promising approach to the molecular isolation of genes with essential functions in filamentous fungi[9-12]. To identify genes involved in thermotolerance and osmotolerance of *B. bassiana*, a widely used insect disease fungus, the T-DNA insertion collections of the fungus had been constructed by *Agrobaterium tumefaciens*-mediated transformation. From the collections, five mutants

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sensitive to thermal and osmotic stresses had been identified. The fragments flanking to T-DNA were also cloned. Sequence analysis revealed that two insertion events respectively occurred in encoding regions of two different function-unknown genes in mutant 212 and 2737.

2 MATERIAL AND METHODS

2.1 Bacterial and fungal strains

B. bassiana Bb0062 single spore isolate was previously described[13]. *Escherichia coli* DH5α was employed for routine DNA manipulations. *A. tumefaciens* AGL1 was used for transformation of *B. bassiana*.

2.2 Construction of pPK2PBT and transformation of *B. bassiana*

To construct vector pK2PBT (Fig. 1), hygromycin phosphotransferase (HPH) cassette in pPK2 vector (kindly provided by Dr. Covert) was replaced by element of phosphinothricin acetlytransferase (BAR) excised from the vector pPBT by *Eco*RI and *Xba* Ⅰ. T-DNA insertion mutant collections of *B. bassiana* were constructed using pK2PBT by *A. tumefaciens*-mediated transformation[13].

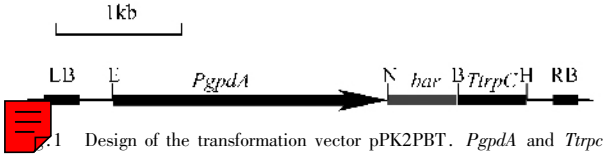

Fig.1 Design of the transformation vector pPK2PBT. *PgpdA* and *Ttrpc* are the *Aspergillus nidulans gpd* promoter and *trpc* terminator, respectively. *Bar* is the herbicide resistance gene from *Streptomyces hygroscopicus*. LB: left border, RB: right border, B: *Bam*HⅠ, E: *Eco*RⅠ, H: *Hin*dⅢ, N: *Not*Ⅰ.

2.3 Screening and identification of mutants

Czapek's medium containing 0.8 mol/L NaCl was used to screen osmosensitive mutants at 26℃. And thermosensitive mutants were identified on modified Czapek's medium with 1 g/L tryptone at 32℃. The approach was as follows: the conidia of each T-DNA insertion transformants were suspended in solution (0.05% Tween-80, V/V). Each 3 μL of mixture containing about 3000 conidia was inoculated on the centre of the medium in a 60 mm diameter Petri dish and incubated for 7 days. Compared with wild-type strain Bb0062, mutants characterized with reduced growth were selected.

2.4 Analysis of preliminary characterization of mutants

Conidia yield was examined by the method of Fang et al[13] after incubation for 4 weeks at 26℃ with a 15 : 9 h light : dark cycle.

In order to evaluate thermotolerance of aerial conidia, 500 μL conidial suspension (1×10^6 conidia) was heat-shocked in 50℃ water-bath for 5 min and cooled on ice for 3 min. A 100 μL suspension was plated on SDY medium in 90 mm diameter Petri dish. To test aerial conidial tolerance to ultraviolet radiation, a 100 μL mixture containing 10^5 conidia was equally plated on SDY medium in 90 mm diameter Petri dish and allowed to dry under clean bench. Then the conidia plated on medium were respectively exposed to 20 W ultraviolet light for 3 min and maintained in dark for 30 min. To detect resistance of conidia to oxidative stress, 100 μL suspension containing 10^5 conidia was equally mixed with 100 mmol/L H_2O_2 for 2 min, respectively. At the indicated time point, conidia were harvested by centrifugation at 12000 rpm for 3 min and plated onto SDY medium. Conidial germination of all treatments was detected after incubation at 26℃ for 24 h.

The bioassay was performed as previously described[14] to determine the virulence of the mutants.

2.5 T-DNA flanking sequence isolation

Linear and exponential PCR primers designed according to T-DNA sequence, L1 (5′-TGTCGTGCC-AGCTGCATTAA-3′) and R1 (5′-TTTCGCCAGCTGG-CGTAATA-3′), L2 (5′-GCAATTCGGCGTTAATT-CAG-3′) and R2 (5′-GAGCTTGGATCAGATTGTCG-3′), were used to amplify T-DNA flanking sequence. It was carried out by the method of Xiao et al[15].

3 RESULTS

3.1 Identification of mutants sensitive to thermal and osmotic stresses

To obtain mutants sensitive to high temperature and osmostress, over 3000 T-DNA insertion transformants were screened. Five mutants, named 212, 2550, 139, 2737 and 2812 were identified from the collections (Fig. 2). Compared with wild type strain, 212 and 2550 showed more sensitive to high temperature and the growth

of the colonies were restricted grow at 32℃. Under osmostress (Czapek's medium containing 0.8 mol/L NaCl), 139, 2737, 2812, and 212 exhibited decreased growth in comparision with wild type strain. The growth of 212 was also slower than that of the wild type strain even at the optimum temperature (26℃).

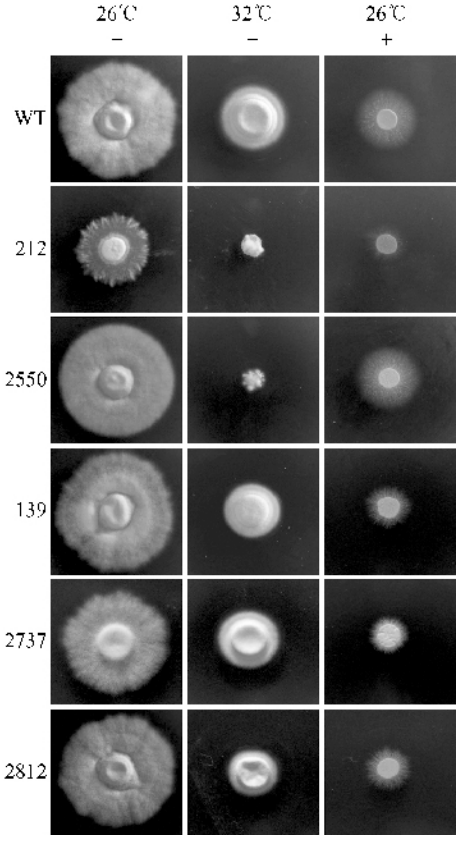

Fig.2 The growth of mutants was depressed under stress conditions. Left images were morphology of each strain grown on Czapek medium at 26℃, middle and right images were colonies grown on Czapek medium supplied with 0.1% tryptone or 0.8 mol/L NaCl (+) at indicated temperature.

3.2 Preliminary characterization of mutants

All the five mutants decreased in conidiation on SDY medium (Table 1). The conidial yield of 212 is only 8% of that of wild type strain, while that of other four mutants decreased by 58% (mutant 139), 62% (mutant 2550), 13% (mutant 2737) and 33% (mutant 2812), respectively.

To further investigate resistance of each mutant to adversity, the viability of aerial conidia, stressed respectively by ultraviolet radiation, oxidation and heat-shock, was measured. The conidia viabilities of mutant 212 were respectively reduced by 23%, 52% and 31% compared with that of wild type strain. Further details are shown in Figure 3.

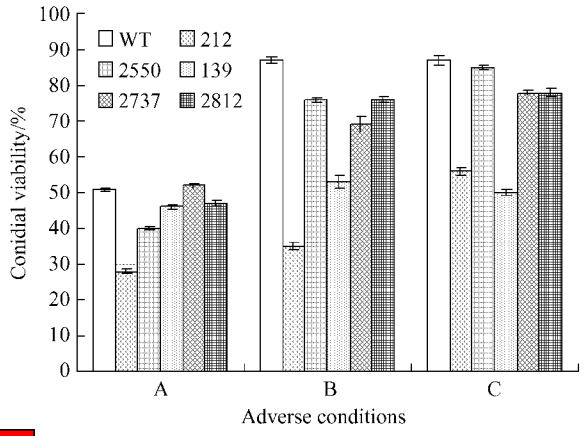

Fig.3 Conidial resistance to adverse factors. A, B and C are the conidia viabilities after stressed by ultraviolet radiation for 3 min, 50 mmol/L H_2O_2 for 5 min, and heat shock at 50℃ for 5 min, respectively.

3.3 The virulence of mutants against aphids *M. persicae*

To determine the virulence of the mutants, bioassay was carried out using aphid adults (*M. persicae*) as target pest. Accumulative mortalities caused by mutant 212 and 2737 decrease by 12% and 40%. LT_{50} (time needed for the pathogen to kill 50% of aphids) of 212 and 2550 are significantly increased over that of wild type strain (Table 1). This suggested that the mutagenesis of mutant 212, 2550 and 2737 were also involved in virulence to insects.

Table 1 Conidial yield on SDY medium and virulence against aphids (*M. persicae*)

Strain	Conidial yield/(conidia/mm^2)	Accumulative mortality/%	LT_{50} (days) (95% confidence limits)
Wild type	$5.50 \pm 0.13 \times 10^8$	69.81 ± 4.03	5.84 (5.69 ~ 5.99)
212	$0.43 \pm 0.21 \times 10^8$	57.94 ± 1.57	7.01 (6.83 ~ 1.19)
2550	$2.09 \pm 0.04 \times 10^8$	63.73 ± 0.68	6.89 (6.71 ~ 7.07)
139	$2.37 \pm 0.11 \times 10^8$	67.06 ± 4.04	6.22 (6.05 ~ 6.39)
2737	$4.78 \pm 0.60 \times 10^8$	29.34 ± 2.96	Nc*
2812	$3.66 \pm 0.34 \times 10^8$	67.59 ± 1.55	6.2 (6.04 ~ 6.36)

*. LT_{50} (days) could not be calculated as less than 50% of test insects died at this concentration. Experiments were carried out in three independent experiments.

3.4 Analysis of sequences flanking to T-DNA in mutants

To identify genes disrupted by insertion T-DNA in the mutants, sequences flanking to the T-DNA insertion sites were isolated using YADE method (Table 2). The junction fragments were analyzed by BLASTX searches. In mutant 212 and 2737, T-DNA insertion interrupted two encoding regions of two function-unknown genes,

the putative proteins of which were similar to FG00406 and FG04721, respectively. In mutant 139, 2550 and 2812, the insertions are located in the non-encoding regions.

Table 2 Analysis of T-DNA borders and mutant flanking sequences

Mutant	T-DNA borders and junction sequences (5′→3′)†	Homologue
212	...*GTTCTCCACAACTCG*TGTGGTGTAAACAAA...	FG00406; Score = 77.4; Expect = 9e-18
2550	...*ATGCTCGTACACAGA*TATTGTGGTGTAAAC...	No‡
139	...*AGGTTCGTCTATTCT*TATTGTGGTGTAAAC...	No‡
2737	...*CCGAACAAAAAAAAA*TTGTGGTGTAAACA...	No‡
	···CTATCAGTGTTTGAC*CACGACGCTATGGAT*...	FG04721; Score = 125; Expect = 8e-28
2812	...CTATCAGTGTTTGACG*CATTGCATGTATGA*...	No‡

†. Underlined and italic sequences are T-DNA borders and its junction fragments, respectively.
‡. No homologue of the mutated gene was found by the BLASTX algorithm.

4 DISCUSSION

In filamentous fungi, T-DNA insertion mutagenesis was a simple and convenient method for identification of genes with essential functions. The borders of insertion T-DNA are conserved and flanking sequences can be cloned by tail-PCR. Recently, it has been used as an effective tool for insertion mutagenesis of filamentous fungi[9-12]. In this study, T-DNA insertion mutant collections of *B. bassiana* had been constructed for identification of mutants impaired in thermotolerance and osmotolerance. We identified five mutants displaying an array of phenotypes in relation to resistance to adverse condition, conidial yield, and virulence. Furthermore, the fragments flanking to T-DNA in mutants were isolated. Sequence analysis revealed that 2 insertion events occurred in encoding regions of two different function-unknown genes. Wang et al. identified the *M. anisopliae Mos*1 gene. The antisense repression of *Mos1* increased sensitivity to osmotic and oxidative stresses and reduced virulence[3]. Here, 4 mutants also showed impaired in thermotolerance and ultraviolate tolerance except for the increased sensitivity to osmotic and oxidative stresses. But the fragments flanking to T-DNA sequence in these mutants have no homology with *Mos*1.

The disrupted-gene in mutant 212 encoded a putative protein which contained an acid phosphatase domain. It might be involved in the use of phosphorus and dephosphorylation of some substrates in *B. bassiana*. In mutant 2737, the disrupted gene was similar to a putative transcriptional regulatory protein (XP-001383922) gene in *Pichia stipitis*. It may serve as a regulator in the process of the fungal adaptation to osmostress. T-DNA inserted in un-encoding regions in other three mutants. The expression of some genes might be influenced by the T-DNA insertions, which could be response for the various changes of the mutants.

This study demonstrates the use of T-DNA insertion mutagenesis in isolation of gene involved in thermotolerance and osmotolerance of *B. bassiana*. Fungal resistance to adverse condition is a complex process. In order to understand the details of the mechanism, it is essential to identify more resistance-related genes of the fungus.

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球孢白僵菌 T-DNA 突变体库的构建及高温和高渗敏感型突变体的筛选

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摘要：【目的】筛选对高温和高渗等逆境胁迫敏感的球孢白僵菌 T-DNA 随机插入突变体，并克隆相关基因，为研究杀虫真菌适应逆境的分子机理奠定基础。【方法】利用逆境胁迫的方法从球孢白僵菌的 T-DNA 随机插入突变库中筛选对高温和高渗敏感的突变体，进而利用 YADE (Y-shaped adaptor dependent extension)技术克隆相关基因。【结果】筛选得到 5 个对高温和高渗敏感的突变体。其中 2 个(212 和 2550)对高温敏感，在 32℃下生长完全受抑；其它 3 个突变体对高渗胁迫敏感。突变体 212 的分生孢子对逆境胁迫的抗性也显著降低。与野生菌株相比，分生孢子经紫外线、高温和氧胁迫后，其存活率分别下降了 23%、52%和 21%。突变体 212 和 2737 对桃蚜的致死率(接种浓度为 1×10^7 个孢子/mL)分别下降了 12%和 40%。T-DNA 侧翼序列分析显示，突变体 212 和 2737 的 2 个功能未知基因的编码框被 T-DNA 随机插入阻断，而其它 3 个突变体的 T-DNA 插入位点位于非编码区域。【结论】本研究结果表明，利用 T-DNA 随机插入突变筛选高温与高渗等逆境相关突变体，进而克隆相关基因是一种行之有效的方法。

关键词：球孢白僵菌；插入突变；高温；高渗

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