Chlorimuronethyl Resistance Selectable Marker Unsuited for the Transformation of Rice Blast Fungus (Magnaporthe Grisea)

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Chlorimuronethyl Resistance Selectable Marker Unsuited for the transformation of rice blast fungus (*Magnaporthe grisea*)

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Abstract: Chlorimuronethyl resistance gene is increasingly used as a selectable marker for transformation, especially fungal transformation. *Magnaporthe grisea* is an important model organism for investigating fungal pathogenicity, and *Agrobacterium tumefaciens*-mediated transformation (ATMT) is used for functional mutagenesis of the fungus. However, our results showed that rice blast strains collected from infectious rice fields have highly conserved resistance to chlorimuronethyl, even comparable to transformants which carrying chlorimuronethyl resistance genes as selectable marker in laboratory conditions. PCR results showed that all tested field strains presented the amplified products of the same size as the selectable marker amplified from plasmid carrying chlorimuronethyl gene. Sequence analysis of PCR products amplified from field strains confirmed that field strains harbored the highly identity homolog of chlorimuronethyl resistance gene. Blast search in GenBank suggested that the fragment is presenting in reference genome sequence of 70-15, but it is not a wide-spread gene in other organisms, excepted for *Herpetosiphon aurantiacus*. Although the origin and reason of the conserved chlorimuronethyl resistance gene in field isolates of blast fungus is unclear, the ecological function of the gene is noteworthy.

Key words: *Magnaporthe grisea*, chlorimuronethyl resistance gene, selectable marker, fungi transformation

Introduction

Fungi play important roles in many human, plant, and animal activities, including biotechnological processes, phytopathological and biomedical research. They are also excellent models for molecular and genetic studies (Casas-Flores *et al*., 2004). Molecular studies of fungal biology have been greatly advanced by *Agrobacterium tumefaciens*-mediated transfromation (ATMT) techniques. Transformation via non-homologous integration of plasmid DNA carrying a selectable marker has been widely used for fungal transformation (Chang *et al*., 2006). For comprehensive and in-depth study of the interaction between pathogen and host, a series of vectors bearing more available selectable markers (e.g. more antibiotic resistant genes) must be constantly developed to meet ATMT.

Magnaporthe grisea has been extensively utilized as a model of fungal pathogen for understanding the molecular basis of host plant-fungus interaction, due to its genetic and molecular tractability (Dean, 1997; Talbot, 1995), as well as the economical importance of the disease it caused. ATMT have been widely used to investigate the infection process and the genes involved in the complex interaction between *M. grisea* and rice. To date, many selectable markers have been used for fungal transformation, such as hygromycin, bialaphos, zeocin and chlorimuronethyl resistance genes. Based on current reports, chlorimuronethyl has not yet been reported to be used for *M. grisea* transformation, but other selective markers have been used for the purpose. Chlorimuronethyl belonged to the sulfonylureas series herbicide. The chlorimuronethyl resistance gene has been engineered to be modified and eliminate sites for the most common restriction enzymes, and chlorimuronethyl selectable markers have been used to construct a series of vectors for fungal transformation (Sweigard *et al*., 1997).

Sulfonylureas, especially chlorimuronethyl, which was the active ingredient of the herbicide Classic^{reg}, inhibits acetolactate synthase (Sweigard *et al*., 1997). The sulfonylurea resistant allele of *M. grisea ILV*1 has been subcloned as a 2.8 kb fragment and modified by the elimination of eight enzyme sites (Sweigard *et al*., 1997). The chlorimuronethyl resistance gene that was cloned from allele of the *Magnaporthe grisea ILV*1 encoded acetolactate synthase involved isoleucine and valine synthesis (Sweigard *et al.*, 1997). ILV1 was a homologue of MGG_07224 (threonine dehydratase) from *Magnaporthe grisea* 70-15 (Hoffmann and Valencia, 2004). Chlorimuronethyl selectable markers was used for *Neurospora crassa* (Li *et al*., 2005), *Cercospora nicotianae* (Chen *et al*., 2007) and other fungal transformations. Although *M. grisea* transformation has many available selectable markers, in some special cases, other selectable markers such as the marker bearing the chlorimuronethyl resistance gene has been developed to cater for special requirements. This begs the question whether this selectable marker could be successfully used for *M. grisea* transformation. In the present study, the chlorimuronethyl resistance gene has been studied as a potentially available marker for *M. grisea* transformation.

Materials and methods

Fungal strain and cultural conditions

Thirty field isolates and two transformants of *M. grisea* were used in this study. Field field isolates were collected from different regions of Yunnan Province and the two transformants were obtained through Y98-16 transformation. pBIMgNIP04 was constructed plasmid bearing the chlorimuronethyl resistance gene. Fungal cultures were grown on oatmeal agar (OMA; 40 g of oatmeal for 1 L) at 25℃ under continuous fluorescent light to promote conidiation (Lee and Lee, 1998). Conidia were harvested from 7- to 10-day old cultures using sterilized water.

Transformation

The *A. tumefaciens* strain GV3101, containing pBIMgNIP04, was grown at 28℃ for 48 h in a minimal medium (MM; Hooykaas *et al.*, 1979) supplemented with kanamycin (50 μg/ml). Bacterial cells in a 2 ml aliquot of this culture were harvested and washed with an induction medium (IM; Bundock *et al*., 1995). The cells were resuspended with 5 ml of IM containing 200 μM acetosyringone (AS). The cells were grown for an additional 4-6 h before mixing with an equal volume of conidial suspension of *M. grisea* field strain Y98-16. This mix (200 μl) was plated on filter paper on a co-cultivation medium, adding 200 μM AS. Following co-cultivation at 25°C for 72 h, the filter paper was cut into strips using scissors, and they were reversely plated on PDA medium plates containing chlorimuronethyl (purchased from Japan, CAS: 9082-32-4, No.036-16671) as the selection agent for fungal transformants. Concentrations of chlorimuronethyl were 100, 200 and 300 μg/ml. In order to eliminate the remaining *A. tumefaciens* cells, the cefotaxime (200 μg/ml) and spectinomycin (200 μg/ml) were added in the PDA medium. After 3-day old selective cultivation, individual transformants were transferred into OMA medium containing chlorimuronethyl (300 μg/ml) and incubated until conidiation. Conidia of the individual transformants were picked and transferred to OMA.

DNA extraction and PCR determination

Based on chlorimuronethyl resistance gene nucleotide sequence, PCR primer pairs were designed by software Primer Designer. The primer sequences followed as 5'-GCAAGGAGTGGTTCGACCAGATCAA-3' and 5'-GTCAGAGCATCACCGACATCGTCAG-3', and the PCR product size was 562 bp. DNA was extracted from fungal mycelia grown in potato dextrose broth for 4 days at 28℃ at 120 rpm. The DNA extraction method followed Chadha and Gopalakrishna (2005). PCR reaction was performed on an Eppendorf PCR machine. Each tube contained a 25 μl reaction mixture, including *Taq* polymerase (TAKARA Biotechnology (Dalian) Co. Ltd). Thermal cycling conditions consisted of 2.5 min at 95℃ following by 35 cycles of 30 s at 94℃ and 30 s at 62℃, 1 min at 72℃, and one final cycle of 10 min at 72℃.

Results

PCR results showed that all tested strains, including the two transformants, were expected to appear at ~500 bp band (Fig. 1). To confirm whether the PCR products were identical, the PCR product from wild type strain Y98-16 and one transformant MgNIP04-1 bearing the chlorimuronethyl resistance gene were cloned into pGEM-T vector and sequenced. The sequencing results were aligned with 70-15 genome sequences and partial coding sequence of chlorimuronethyl resistance gene using BioEdit (Fig. 2). Based on alignment results, a section of DNA sequences, which located in supercontig 6.18, ranging from

1272316 to 1275124 of 70-15 strains, were homologous with the chlorimuronethyl resistance gene and PCR product from Y98-16. For all aligned sequences, almost all of bases were identical.

Chlorimuronethyl resistance gene sequence was blasted in NCBI, the result showed that the gene appeared higher identity with *M. grisea* acetolactate synthase gene and partial coding sequence of MGG06868 (E-value: 0.0), while appeared lower identity with *Herpetosiphon aurantiacus* ATCC 23779 (E-value: 4e-05), but it is absent in other organisms presented database, suggested that it is suitable marker for transformation of other fungi and organisms. To understand the genomic environment of the gene, sequences located upstream and downstream of the gene was carried out. There was no any transponson around the gene. G+C% content of *M. grisea* homologue to chlorimuronethyl gene, -1818 bp of 5' and 3' terminal of the homologue was analyzed using Seqool, respectively. The result showed that GC% content of the homologue was 52.96%, -1818bp of 5' terminal was 55.23%, and -1818bp of 3' terminal was 47.47%.

Based on the concentration of chlorimuronethyl introduced by Sweigard (1998), which was 100 μg/ml, it was necessary to verify whether the wild type strains were resistant to chlorimuronethyl under different concentrations. The transformant Y98-16 and CY2 were screened on PDA medium plates containing chlorimuronethyl (100, 200 and 300 μg/ml, respectively) as the selection agent. Results showed that all test strains, including Y98-16 and CY2, showed resistance to chlorimuronethyl in the tested concentrations (100, 200 and 300 μg/ml)(Fig 3).

Discussion

Fungal genetic transformation has greatly accelerated the analysis of gene function. Fungal transformation methods include protoplast, biolistic and agrobacterium tumefaciens-mediated transformations (ATMT). And *Agrobacterium tumefaciens*-mediated transformation (ATMT) is used for functional mutagenesis of the fungus(Jeon and Lee et al, 2007). No matter which methods are used, there must be antibiotic resistance genes as selectable markers for successfully selecting incorporated genes for a desired trait during transformation. For transformants to be successfully screened, the plasmid containing an antibiotic resistance gene must be constructed. There are four types of selectable marker genes, including antibiotic resistant (e.g. neomycin and kanamycin), herbicide tolerant (e.g. bialaphos and chlorimuronethyl), metabolic/auxotrophic and screenable marker genes (www.nuffieldfoundation.org/bioethics/publication/modifiedcrops/rep0007969.html). Of these selectable markers, herbicide tolerant marker was originally used in screening transgenic plants, but now this marker was also used for fungal transformation.

Since green evolution, quantities of herbicides have been used to control grasses. Some grasses had developed tolerance to these chemical components during long-term competition

with chemicals. While chlorimuronethyl as for a kind of herbicide was applied in fields in early 1980s and it widely applied in rice fields, soybean fields, maize fields, wheat crop fields, rape fields, lawn and other weeds in non-cultivated land for a long time. In addition, increasing transgenic organisms carrying herbicide-resistance genes such as chlorimuronethyl and bialaphos were released into the fields, and possibility of gene flow from these transgenic organisms to other organisms, especially under herbicide stress is present. If a specific DNA sequence of a strain had higher or lower G+C content than its genome mean G+C content, or up- and down-stream sequence, which indicated that this specific DNA sequence was obtained from exogenous bacterium or plasmid of other species (Li et al, 2008). The GC content of *M. grisea* homologue to chlorimuronethyl gene was analyzed, the result showed that GC content of the homologue and its 5' terminal were higher than *M. grisea* genome (GC% of content was 51.57%), and its 3'terminal was lower than 51.57%. which offered a speculation that *M. grisea* homologue to chlorimuronethyl gene possibly was obtained from exogenous bacterium or plasmid of other species. It was necessary to verify the speculation through experimental method. So, the selectable marker carrying chlorimuronethyl gene was unsuitable for *M. grisea* genetic transformation.

If transgenic plants or fungi carrying Chlorimuronethyl were released into fields or markets, it would inevitably threaten human and/or animal health. Therefore, it is necessary to develop suitable and safe selectable markers in the future. Crop developers have been seeking more useful markers for selecting transgenic plants, animal or fungi and these methods have been adopted in the selection process (Dale and Ow, 1991; Ebinuma, et al., 1997). In conclusion, some markers carrying antibiotic genes such as chlorimuronethyl, were neither suitable for *M. grisea* transformation nor other fungi or plant transformation from the long-term perspective of global food and environmental safety.

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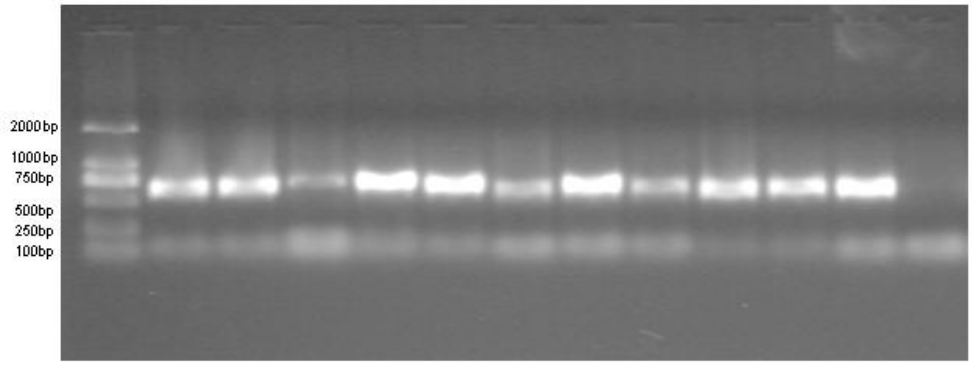

Fig.1 PCR product of chorimuronethyl resistance gene in genomic DNA of rice blast fungus, *M. grisea*
Lane1: DL2000 marker, lane2-9: partial rice blast strains from different fields from Yunnan, lane10: wild type strain Y98-16 genomic DNA as template; lane11: wild type strain CY2 genomic DNA as template; lane12: genetic transformant carrying chlorimuronethyl resistance gene as selection marker; lane13: negative control.

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                                10        20        30        40        50        60        70        80        90
                                |         |         |         |         |         |         |         |         |
Chlorimuronethylgene            CGATCGCAAGGAGTGGTTCGACCAGATCAAGGAGTGGAAGGAGAAGTGGCCTCTGTCACATTATGAGAGGGCCGAGCGTAGTGGTCTCATC
M.grisea70-15                   .........................................................................................
Transformant                    .........................................................................................
Y98-16                          .........................................................................................

                               110       120       130       140       150       160       170       180       190
                                |         |         |         |         |         |         |         |         |
Chlorimuronethylgene            ACTCTGATCGAGGAGCTGAGCAACCTGACTGCTGACCGCAAGGACATGACCTACATCACAACCGGTGTTGGCCAGCACCAAATGTGGACAG
M.grisea70-15                   .........................................................................................
Transformant                    .........................................................................................
Y98-16                          .........................................................................................

                               210       220       230       240       250       260       270       280       290
                                |         |         |         |         |         |         |         |         |
Chlorimuronethylgene            TCAGGTGGAGGCACCCACGGTCCATGATCACCTCTGGCGGTTTGGGAACCATGGGATATGGTCTGCCGGCAGCGATTGGCGCCAAGGTTGC
M.grisea70-15                   .............................................T...........................................
Transformant                    .............................................T...........................................
Y98-16                          .............................................T...........................................

                               310       320       330       340       350       360       370       380       390
                                |         |         |         |         |         |         |         |         |
Chlorimuronethylgene            TGCTTTGGTCATTGACATCGACGGCGACGCATCGTTCAACATGACTCTGACAGAGCTTTCGACGGCGGCACAGTTCAACATTGGCGTCAAG
M.grisea70-15                   ..................T.....T.....................................................................
Transformant                    ..................T.....T.....................................................................
Y98-16                          ..................T.....T.....................................................................

                               410       420       430       440       450       460       470       480       490
                                |         |         |         |         |         |         |         |         |
Chlorimuronethylgene            TTGAACAACGAGGAGCAGGGAATGGTGACCCAATGGCAGAACTTGTTCTACGAGGACCGCTACTCACATACACACCAGCGCAACCCAGACT
M.grisea70-15                   .........................................................................................
Transformant                    .........................................................................................
Y98-16                          .........................................................................................

                               510       520       530       540       550       560
                                |         |         |         |         |         |
Chlorimuronethylgene            TCGCCGATGCAATGGACGTTCAACATCGCCGTGTTTCGAAGCCTGACGATGTCGGTGATGCTCTGAC
M.grisea70-15                   ..........................................................T..............
Transformant                    ............................................................................
Y98-16                          ............................................................................
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Fig.2 Sequence alignment of chlorimuronethyl resistant gene with *M. grisea* 70-15, transformant carrying

chlorimuronethyl resistance gene and wild type strain of Y98-16. The aligned sequences of transformant and Y98-16 were cloned and sequenced from PCR products that using primer pairs of chlorimuronethyl resistant gene to amplify transformant DNA and Y98-16 DNA, respectively.

Fig.3 Chlorimuronethyl resistance test of wild type strains of CY2 and Y98-16 and two transformants of T1 and T2. CK (control): no chlorimuronethyl; 100 μg/ml mean concentration of chlorimuronethyl was 100 μg/ml; 200 μg/ml mean concentration of chlorimuronethyl was 200 μg/ml; 300 μg/ml mean concentration of chlorimuronethyl was 300 μg/ml.