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A Highly Efficient Bioassay System for Screening *Ralstonia solanacearum* Mutants with Altered Virulence

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ABSTRACT: *Ralstonia solanacearum*, the causing agent of crop bacterial wilt, is a devastating bacterium with an unusually wide host range. To effectively integrate durable resistance to *R. solanacearum* into crops, comprehensive and global information on pathogenesis mechanisms employed by this pathogen is important. In this study, we report the development of an efficient *Arabidopsis*-based bioassay system useful for screening of large number of bacterium mutants with altered virulence. The effectiveness and uniqueness of this system was further demonstrated by the isolation of *R. solanacearum* mutants exhibiting differential pathogenesis and *in vitro* characteristics, and by the identification of mutants with the transposon inserted in known and novel loci. This highly efficient bioassay system, together with the accompanying *in planta* and *in vitro* bioassay systems described here, would facilitate comprehensive study on *R. solanacearum* pathogenesis mechanisms.

KEY WORDS: *Ralstonia solanacearum*, mutant, screening, *Arabidopsis*.

INTRODUCTION

Bacterial wilt is a very complex and deadly soil-borne vascular disease of many agronomically important crop species and occurs mainly in tropic, sub-tropic and warm temperature zones (Jaunet and Wang, 1999; Wang et al., 2000). However, this disease has extended to more temperate areas (Kim et al., 2003). Because conventional control strategies for this disease are not effective, production loss due to this disease can be 100%. The causing bacterium of bacterial wilt, *Ralstonia solanacearum*, is a gram-negative, aerobic rod bacterium belonging to β-proteobacteria. This bacterium has an unusual wide host range over 200 species and can survive in soil for a long period of time (Hayward, 1991). *R. solanacearum* could be classified into phylotypes, biovars, races and many strains based on biochemical, molecular, and metabolic characteristics (Denny, 2006; Castillo and Greenberg, 2007). The strains in race 1 are found to be highly diverse both in their genotypes and aggressiveness (Jaunet and Wang, 1999). Due to the diversity of this bacterium, variations in stability of host plant resistance over different field locations exist (Lopes et al., 1994; Hanson et al., 1998).

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In the natural infection process, *R. solanacearum* invades plants at the sites of emergence of secondary roots or at root tips and propagates intercellularly (Vasse et al., 1995). After the bacterium enters into the xylem system, the infection becomes systemically, with further bacterial multiplication and the production of large amounts of extracellular polysaccharides, leading to complete wilting and the death of infected plants (Buddenhagen and Kelman, 1964). The bacterium then returns to the soil, where it can be associated with plant debris and weed rhizosphere and survive under humid conditions over a long time. It has been suggested that *R. solanacearum* may develop a viable-but-nonculturable state, which could be involved in long-term survival of the bacterium and in plant infection (Grey and Steck, 2001). To successfully establish a pathogenic interaction with host plants, *R. solanacearum* has to win many "battles" throughout its journey from the soil up into the stem of host plants. A large battery of specialized gene products is required to accomplish tasks at different steps of the infection process (Schell, 2000; Genin and Boucher, 2004). However, our knowledge on the complex and apparently highly coordinated pathogenesis of this bacterium is far from sufficient.

With the availability of genome sequences of four distinct *R. solanacearum* strains, new pathogenicity determinants putatively involved in various aspects of bacterial pathogenesis have been identified by bioinformatics approaches (Salanoubat et al., 2002; Gabriel et al., 2006; Boucher et al., unpublished

data). However, the actual functions and roles of these genes in *R. solanacearum* pathogenesis often remain to be determined. Although bioinformatic tools have become a useful approach for genome-wide identification of candidate pathogenicity genes, certain genes involved in multiple facets of pathogenesis may not necessarily be identifiable *in silico*. This is particularly true in the case of genes that encode hypothetical proteins, the function of which remains to be established. Additionally, it is well-known that certain genes could play multiple roles in different biological functions. Consequently, large scale bioassay-based screening for pathogenesis-related genes remains essential for the identification of new pathogenicity genes.

To effectively control bacterial wilt in a comprehensive fashion, extensive and global information on the mechanisms and genes involved in plant-*R. solanacearum* interactions would be highly desirable and necessary. The era of genomics in biology has brought in high-throughput methods for studies and gathering a large amount of information on plant-microbe interactions. These include large-scale of mutant screening, DNA microarray, proteomics and metabolomics technologies. However, due to the complexity of bacterial wilt, well established, efficient systematic bioassay systems will be very helpful and essential for large-scale plant-*R. solanacearum* interaction studies. Here, we report the development and use of an efficient Arabidopsis-based bioassay system suitable for high-throughput screening of *R. solanacearum* mutants.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Media used for this study included: TTC medium (1% peptone, 0.1% casein hydrolysate, 0.5% glucose, 1.5% Bacto agar, 0.005% TTC), TTC-SDS medium (TTC medium supplemented with 0.01% SDS), 523 medium (0.03% magnesium sulfate heptahydrate, 0.2% potassium phosphate, 0.4% yeast extract, 0.8% casein hydrolysate, 1% sucrose, and/or 1.5% Bacto agar), and minimal medium (0.68% sodium phosphate dibasic, 0.3% potassium phosphate monobasic, 0.05% sodium chloride, 0.1% ammonium chloride, 0.024% magnesium sulfate, 0.001% calcium chloride, 2.5% glucose, and 1.5% Bacto agar).

The wild-type strains used in this study are two local strains, Pss190 and Rd15, isolated from tomato and radish in Taiwan *R. solanacearum* mutants were generated using EZ::TN^{TM} <KAN-2> Tnp Transposome^{TM} kit (Epicentre) following the manufacture's instruction. Aliquots of the electroporated cells were plated onto TTC medium plates containing kanamycin (100 μg/ml). Following 2 days incubation at 28°C, all colonies were picked, cultured in 523 medium for 2 days and stored in 30% glycerol at -80°C.

Mutant screening on *Arabidopsis*

Seeds of *Arabidopsis thaliana* ecotype Col0 were surface-sterilized with 70% ethanol for 1 min, treated with 10% sodium hypochlorite for 20 minutes, washed five times with sterile water, then stored at 4°C for vernalization. Before sowing the seeds on the Murashige and Skoog medium (MS, Gibco, USA) supplemented with 1% sucrose and 0.4% phytagel (pH 5.7), 5 ml of bacterial suspension with an optical density of 0.4 or 0.8 at 600nm was poured onto each plate, left for 5 minutes and the left-over bacterial suspension was poured off from the plate. The vernalized seeds were then spread out on the plates and incubated under 16 h photoperiod at 28°C. Disease progress was monitored every week over two weeks.

Pathogenesis assays on tomato plants

Three tomato (*Solanum esculentum*) cultivars with various degrees of susceptibility to most *R. solanacearum* strains were used for pathogenicity tests: L390 (highly susceptible), L180 (medium resistant), H7996 (highly resistant). Three-week old plants grown in soil were inoculated by soil drench without root severing, as described by Jaunet and Wang (1999). The concentration of bacterial inoculum was 10^8 CFU/ml. For each mutant strain, six plants were inoculated in each soil drench experiment and the experiment was repeated at least two times, giving a total of twelve test plants. Inoculated plants were kept in a greenhouse with natural light and mean temperature at 28°C. Percentage of plants showing wilting symptom was recorded after 21 days.

Hypersensitive response assays on tobacco plants

Bacterial suspensions at a concentration of 10^8 CFU/ml were infiltrated into *Nicotiana tabacum* cultivar W38. The bacteria were infiltrated on the same leaves. The test was carried out on two leaves for each trial and at least two independent trials were performed. Inoculated plants were kept in a greenhouse with natural light and mean temperature at room temperature. Hypersensitive response was recorded two days after inoculation.

In vitro* growth of *R. solanacearum

For analysis of *in vitro* growth of *R. solanacearum* in various media, the bacterial stocks were streaked out from -80°C onto the TTC plate containing kanamycin, incubated at 28°C for 2 days and single colonies were grown in 523 medium broth at 28°C for 2 days. The bacterial suspensions were then diluted to give an optical density of 0.3 at 600 nm (approximately 10^8 CFU/ml) followed by 10-fold serial dilutions up to 10^5 fold. Ten microliters of each diluted bacterial samples were spotted onto the indicated medium plates and bacterial growth was recorded two or three days after incubation at 28°C.

Determination of the disrupted loci

Bacterial genomic DNA was isolated using MasterPure™ DNA Purification kit (Epicentre) and subjected to direct sequencing using primers designed based on the kanamycin resistance gene contained in Tn5 (Hoffman and Jendrisak, 1999). The sequences of the primers were: 5'-ACCTACAACAAAGCTCTCATCAACC-3' and 5'-GCAATGTAACATCAGAGATTTTGAG-3'. To determine the identity of the disrupted loci, the identified flanking sequences were then compared to the GeneBank databases using BLASTN.

RESULTS

The screening system

To identify genes involved in *R. solanacearum* pathogenesis, an effective and efficient bioassay system established on *Arabidopsis* germinating seeds was developed and used for screening of *R. solanacearum* mutants with altered pathogenicity and virulence. Using the defined condition, the response of *Arabidopsis* germinating seeds can be clearly observed 10 to 14 days post inoculation (Fig. 1). In this assay, the parental strains Pss190 and Rd15 were able to kill 100% of the young seedlings within two weeks, while germinating seeds inoculated with sterile water grew normally. The bacteria did not multiply visibly on MS medium until the death of *Arabidopsis* seedlings. This was followed by the rapid appearance of significant bacterial multiplication surrounding the dead tissues. This system is simple, efficient, time-economic and thus useful for screening of large number of mutants. In addition, this system allows the isolation of mutants that are unable to harm very young seedlings, implying the disrupted genes could play a key role in bacterial pathogenesis.

Screening for *R. solanacearum* mutants with altered virulence

Using the screening system, 480 *R. solanacearum* Tn5-mutants in strain Pss190 background and 96 mutants in strain Rd15 background were randomly screened for altered virulence. After confirmation by performing three additional assays, five Pss190 mutants and one Rd15 mutant reproducibly failed to kill *Arabidopsis* germinating seeds were identified (Fig. 1). The survival rate of germinating seeds inoculated with the mutants was higher than 90% after two weeks, while no plant survived inoculation with the wild-type strain. To further evaluate the virulence of the identified mutants on *Arabidopsis* germinating seeds, we further tested their pathogenesis using higher bacterial dose for inoculation. The results showed that, the virulence of four mutants (73, 124, 125, 198) remained severely reduced even at a high bacterial inoculum concentration, while two mutants (20-63 and 59) exhibited increased virulence when higher bacterial dose was used for inoculation (Table 1).

Pathogenesis of *R. solanacearum* mutants on tomato and tobacco plants

To further characterize the identified mutants, we evaluated their pathogenesis on Solanaceous plants. As shown in Table 1, all mutants consistently exhibited various degrees of reduction in their virulence on L390, a tomato cultivar highly susceptible to most *R. solanacearum* strains. Consistently, these mutants conferred even more significantly reduced virulence on tomato cultivars L180 (medium resistant) and H7996 (highly resistant). However, when inoculated into tobacco leaves, these mutants reproducibly caused hypersensitive response not differently from the wild-type strains did (Fig. 2, data not shown).

***In vitro* characteristics of *R. solanacearum* mutants**

In order to determine whether the reduced virulence of the mutants was due to growth defects, several media were used to monitor their growth and EPS production (Table 2). In addition to rich medium (TTC) and minimal medium (MM), as the pathogenesis-defective mutants were originally selected on *Arabidopsis* germinating seeds grown on Murashige and Skoog (MS) agar medium, the growth of all mutants was also evaluated on MS medium. Moreover, in order to test whether these mutants could be defected in cell envelope or membrane integrity (Titarenko et al., 1997), their growth on

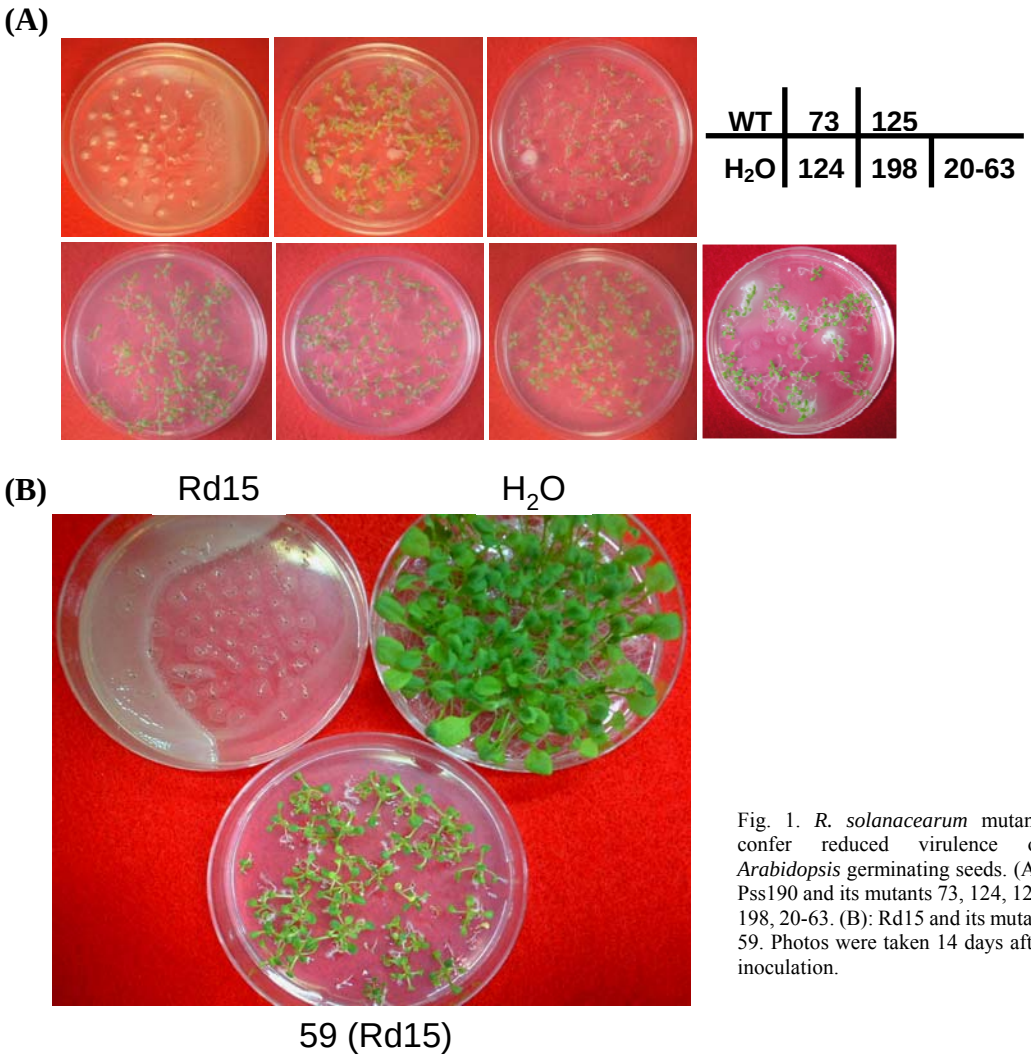

Fig. 1. *R. solanacearum* mutants confer reduced virulence on *Arabidopsis* germinating seeds. (A): Pss190 and its mutants 73, 124, 125, 198, 20-63. (B): Rd15 and its mutant 59. Photos were taken 14 days after inoculation.

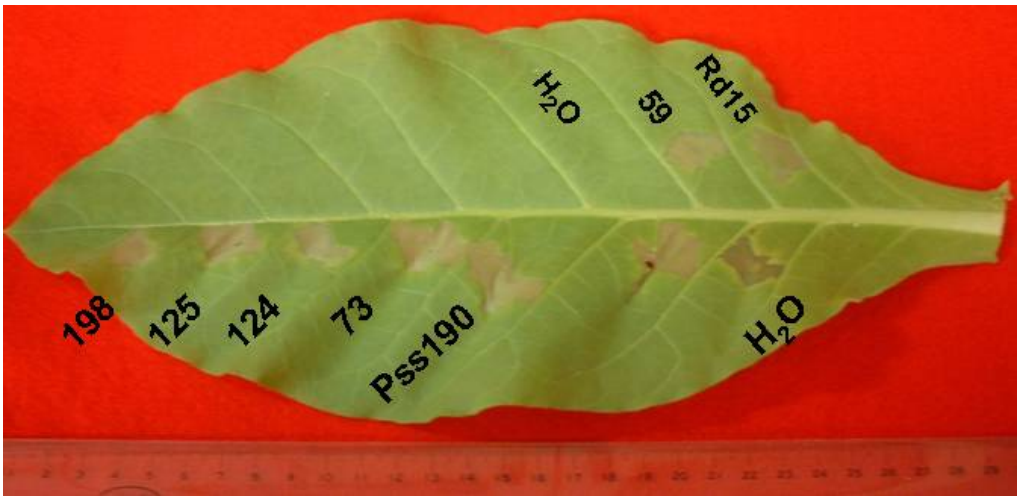

Fig. 2. *R. solanacearum* mutants are capable of triggering normal hypersensitive response on tobacco leaf. The bacteria were infiltrated on the same leaves. The test was carried out on two leaves for each trial and at least two independent trials were performed. The response of inoculated leaves was recorded 2 days after inoculation. Consistent results were observed and representative results are shown.

Table 1. Virulence of *R. solanacearum* mutants on *Arabidopsis* germinating seeds and three-week old tomato plants.

Strains	Arabidopsis[a] (death %)		Tomato[b] (wilting %)		
	OD 0.4	OD 0.8	L390	L180	H7996
Pss190					
wild-type	100	100	100	83	67
73	0	0	83	n.d.	n.d.
124	0	0	0	0	0
125	10	10	16	0	0
198	0	0	0	0	0
20-63	0	36	33	n.d.	n.d.
Rd15					
wild-type	100	100	100	67	16
59	0	67	50	0	0

[a] Percentage of ecotype Col0 germinating seeds not survived 14 days after inoculation with the bacteria at an OD_{600} equals to 0.4 or 0.8.

[b] Percentage of plants showing wilting symptom 21 days after inoculation with bacteria at an OD_{600} equals to 0.3. Three tomato cultivars with various degrees of susceptibility to most *R. solanacearum* strains were used for the tests: L390 (highly susceptible), L180 (medium resistant), H7996 (highly resistant). n.d., not determined.

Table 2. Selected *in vitro* characteristics and Tn insertion site of *R. solanacearum* mutants.

Strains	Multiplication in media[a]			Colony fluidity[b]	Tn insertion site
	TTC	TTC-SDS	MM/MS		
Pss190					
wild-type				+	
73	=	↓	=	+	n.d[c]
124	=	=	×	+	*serC*
125	=	=	×	+	RSc1326
198	=	=	×	+	n.d.
20-63	=	↓	=	+	*rfaF*
Rd15					
wild-type				+	
59	=	↓	×	+	*clpA*

[a] The bacterial suspensions were then diluted to give an optical density of 0.3 at 600nm (approximately 10^8 CFU/ml) followed by 10-fold serial dilutions to give bacterial samples of 10^5, 10^4, 10^3 and 10^2 CFU/ml. Ten microliters of each diluted bacterial samples were spotted onto the indicated medium plates and bacterial growth was recorded two days after an incubation at 28°C. Wild-type strains grew well on all media at all dilutions. =, not significantly different from the wild-type strain; ↓, colonies show at 10^5, 10^4 and 10^3 dilution; ×, no growth at all dilutions.

[b] +, not significantly different from the wild-type strain.

[c] n.d., not determined.

TTC agar medium containing SDS (TTC-SDS) was also assessed. The results showed that all of the mutants multiplied normally on TTC. The mutants 73 and 20-63 grew normally on MM and MS media, whilst the other mutants failed to grow on these media after two-day incubation at 28°C. In addition, colonies of all mutants were fluidal, suggesting the EPS production was not significantly affected as judged from colony fluidity. Furthermore, except for the mutants 124, 125 and 198, the mutants 73, 20-63 and 59 exhibited reduced growth in the presence of SDS, indicating possible disorders in components of cell membrane and envelope, transporters or extracellular protecting materials.

Identification of the disrupted loci in *R. solanacearum* mutants

Southern hybridization revealed a single transposon insertion in each of these mutants (data not shown). To identify genes possibly disrupted by transposon insertions, the genomic regions flanking the transposon insertion sites of selected mutants were further determined (Table 2). Sequence analysis revealed that, the mutant 124 had the transposon inserted at RSc0903 (*serC*), which is predicted to encode a probable phosphoserine aminotransferase protein. In mutant 125, the transposon inserted in RSc1326, a locus homologous to genes encoding probable aspartate aminotransferase proteins. Because the two mutants disrupted in RSc0903 or RSc1326 failed to grow normally on MM medium, these two loci are apparently involved in amino acid biosynthesis. The mutant 20-63 contained a transposon insertion at RSc0565 (*rfaF*), which may encode a probable ADP-heptose-lipopolysaccharide heptosyltransferase II protein, an enzyme involving in production of lipopolysaccharides. *R. solanacearum* mutant disrupted in this gene has been shown to be sensitive to antimicrobial peptides and avirulent (Titarenko et al., 1997). In mutant 59, the

transposon was found to insert at RSc2464, a locus homologous to genes encoding ClpA, an ATP-dependent protease (ATP-binding specificity subunit) protein.

DISCUSSION

In this study, we developed and used an efficient *Arabidopsis* bioassay system for systematic screening of *R. solanacearum* mutants with altered pathogenicity and virulence, aiming to identify genes involved in *R. solanacearum* pathogenesis. This system is simple, efficient and time-economic, nicely meeting the requirements of methods used for screening of large mutant populations. It is high-throughput as compared with the regular root drench inoculation method. Particularly, this system allows the isolation of mutants unable to damage very young seedlings, implying the genes identified by this system could play a key role in bacterial pathogenesis. Most importantly, because studies on bacterial wilt are mostly carried out on mature crop plants, this efficient bioassay system would certainly provide a new aspect for studying the interactions between *R. solanacearum* and different host plants. The effectiveness of this screening system was further demonstrated by the successful identification of mutants with the transposon inserted in known and novel loci (see below).

Although germinating seeds apparently are more vulnerable to pathogen attack, and we may thus miss pathogenicity genes important for *R. solanacearum* pathogenesis on older plants, our results demonstrated that this method is highly efficient by several facts. First, genes known essential for *R. solanacearum* pathogenesis, RSc0565 (*rfaF*) (Titarenko et al., 1997), was identified using this seed system. Secondly, our preliminary data suggested that the impairment of pathogenicity of all of the mutants identified using this system reflected their pathogenesis defects on older *Arabidopsis* plants. Thirdly, mutants selected by the seed system displayed reduced virulence at various levels on tomato plants (Table 1). Fourthly, novel mutants defective in components of cell membrane and envelope or lipopolysaccharides, including mutants 73, 20-63 and 59, were identified mutants, suggesting the uniqueness of this screening system. Although a few mutants identified using this system are deficient in metabolite biosynthesis, and thus accounted for their pathogenesis defects within plants (Table 2), pre-selecting out auxotrophs during mutant library propagation before subjected to bioassay-based screening would enable this system to be efficiently used for future screening of additional mutants.

In conclusion, from this study, an efficient bioassay system has been established. The effectiveness and uniqueness of this system have been demonstrated by employing it for high-throughput screening of *R. solanacearum* mutants conferring reduced virulence. This highly efficient bioassay system, together with the accompanying *in planta* and *in vitro* bioassay systems described in current study, can readily facilitate the work on comprehensive and globally dissecting the pathogenesis mechanisms employed by *R. solanacearum*. We currently continue to employ this useful system to screen pools of *R. solanacearum* mutants, aiming to identify genes playing key roles in multifaceted pathogenesis mechanisms.

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青枯病菌病原性改變突變株之高效篩選系統

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摘　　要

由青枯病菌(*Ralstonia solanacearum*)所引起之細菌性萎凋病會導致眾多重要作物的嚴重損失，為整體性地考量有效且持久的病害防治措施，全面性瞭解青枯病菌的致病機制當然十分重要。本研究中，我們研發以阿拉伯芥為基礎的生物測試平台，可快速且有效大量篩選病原性改變之病菌菌株。我們已成功地運用此系統篩選出致病力及多種特性各異的青枯病菌突變株，並更發現這其中包含了在已知或新穎的病原性相關基因被破壞的突變株，進一步驗證並支持這個系統的有效性與獨特性。藉由此高效率之生物檢定平台，並配合本研究所使用的多種宿主植物內及宿主體外分析系統，將極利於進行深入且全面性的青枯病菌致病機制研究。

關鍵詞：青枯病菌、突變株、篩選、阿拉伯芥。

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