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NOTE

Construction and Characterisation of an Antifungal Recombinant *Bacillus thuringiensis* with an Expanded Host Spectrum[§]

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A novel antifungal *Bacillus thuringiensis* strain 19–22, ssp. *kurstaki* (H3a3b3c), was characterised. This strain included *cry1Aa*, *cry1Ab*, *cry1Ac*, and *cry1D*, which have high insecticidal activities against lepidopteran larvae other than *Spodoptera exigua*. To expand the host spectrum, a *cry1E* gene whose product is active against *S. exigua* was introduced into the isolate. The transformant successfully expressed the Cry1E protein without any loss of its original antifungal activities. These results indicate that this recombinant strain exhibits dual activities and may be used as an integrated control agent to control plant diseases and insect pests.

Keywords: *Bacillus thuringiensis*, antifungal activity, *cry1E*, insecticidal activity

Although *B. thuringiensis* has been used as a biopesticide since the 1960s, most studies have focused on its insecticidal δ-endotoxins and strains used in the biopesticide formulations, which are selected based mainly on their entomocidal activities. However, *B. thuringiensis* can also act as a potential biocontrol agent against different fungal and bacterial species because it expresses many antimicrobial molecules, including zwittermicin A (Stabb *et al.*, 1994; Cherif *et al.*, 2003), chitinases (Arora *et al.*, 2003), bacteriocins and quorum-sensing – sensing-quenching N-acyl homoserine lactone-lactonases (Dong *et al.*, 2002). The *B. thuringiensis* strain CMB26, which produces the lipopeptide fengycin, was found to affect the cell surface of the phytopathogenic fungus *Collectotrichum gloeosporioides* (Kim *et al.*, 2004). Cherif *et al.* (2003) reported that *B. thuringiensis* ssp. *entomocidus* HD9 produced a novel bacteriocin, entomocin 9, which showed antifungal activity against *Aspergillus nidulans* and *Fusarium graminis* in dual culture tests. Additionally, some reports have focused on *B. thuringiensis* exochitinase activity against phytopathogenic fungi (de la Vega *et al.*, 2006) or zwittermicin A activity against alfalfa damping-off caused by *Phytophthora medicaginis* (Stabb *et al.*, 1994).

Spodoptera exigua (Lepidoptera: Noctuidae) is a worldwide pest that attacks many plant species, including important crop plants, such as cotton, cabbage, maize, tomatoes, soybeans and cauliflower (Dingha *et al.*, 2004). *B. thuringiensis* subsp. *kenyae* (Visser *et al.*, 1990; Masson *et al.*, 1992), ssp. *darmstadiensis* (Chambers *et al.*, 1991), ssp. *aizawai* (Sanchis *et al.*, 1989) and ssp. *galleriae* (Kalman *et al.*, 1995), all of which harbour *cry1C* or *cry1E* genes, have been showed significant insecticidal activity against *S. exigua* and other *Spodoptera* species. Previously, a strain of *B. thuringiensis* subsp. *kenyae*, STB-3, that expressed only the *cry1E* crystal gene and exhibited insecticidal activity against *S. exigua* was isolated and characterised (Chang *et al.*, 1998).

In this study, we characterised a new antifungal *B. thuringiensis* strain, 19–22, and introduced a *cry1E* gene (cloned from *B. thuringiensis* subsp. *kenyae*, STB-3) into the isolate to generate a recombinant *B. thuringiensis* strain that is able to control plant fungal diseases and insect pests simultaneously.

The *B. thuringiensis* strain 19–22 isolate, which has antifungal activity against several phytopathogens, was obtained from soil samples collected in Korea as described by Ohba and Aizawa (1978). The isolate *B. thuringiensis* 19–22 was determined to belong to ssp. *kurstaki* using the H antiserum agglutination test (data not shown). For *cry*-gene typing in the strain 19–22, plasmid DNA was extracted using an alkaline lysis method (Reyes-Ramirez and Ibarra, 2008) including lysozyme treatment. PCR tests were performed using primer sets specific to 20 major *cry*/*cyt* genes (1Aa, 1Ab, 1Ac, 1B, 1C, 1D, 1E, 1F, 1G, 2A, 3A, 3B, 3C, 4A, 4B, 7A, 9A, 10A, 11A and cyt1A) as described by Lee *et al.* (2001). Nucleotide sequencing of the amplified PCR products showed that *B. thuringiensis* 19–22 harbours the *cry1Aa*, *cry1Ab*, *cry1Ac*, and *cry1D* genes (Fig. 1A).

The expression vector, pHT1K-1E, encoding the insecticidal *cry1E* gene of *B. thuringiensis* under the control of its en-

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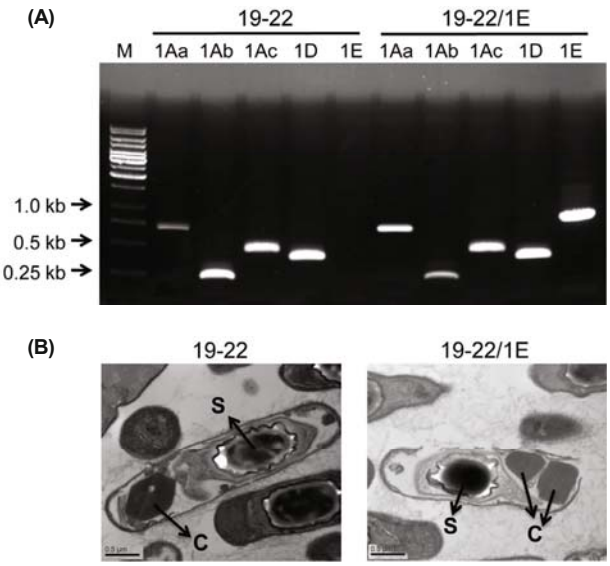

Fig. 1. ***cry*** **Gene profiles (A) and transmission electron microscopy (B) of the wild-type *B. thuringiensis* strain 19–22 and the recombinant strain 19–22/1E**. C and S indicate the parasporal crystal and spore, respectively (40,000×).

dogenous promoter was transformed into the wild-type *B. thuringiensis* 19–22 isolate using a slightly modified protocol (25 μF, 2 kV, 400 Ω) commonly used for the transformation of *B. thuringiensis* (Lereclus *et al*., 1989) in 0.2 cm electrode gap electroporation cuvettes (Bio-Rad, USA). Transformants were selected on nutrient agar plates containing erythromycin (25 μg/ml). The *cry* gene profiles of these transformants showed that most of them had lost one or more resident plasmids (Supplementary data Table S1). As described by Yue *et al*. (2005), the transformants usually demonstrated a decrease in the expression of endogenous crystal protein, possibly due to the loss of some native plasmids from the parent strain in the transformation process. Finally, one

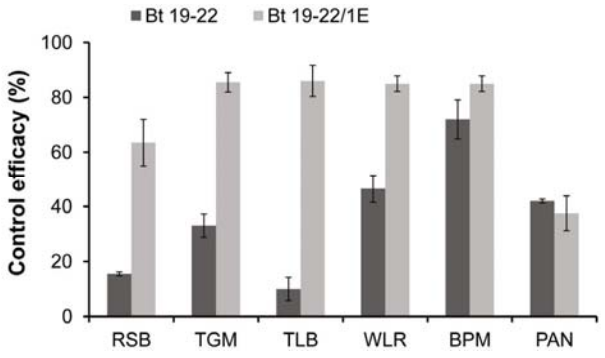

Fig. 2. ***In vivo*** **antifungal activity of *B. thuringiensis* 19–22/1E against six fungal phytopathogens.** RSB, rice sheath blight; TGM, tomato grey mould; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barley powder mildew; PAN, red pepper anthracnose; Bt 19–22, wild-type *B. thuringiensis* 19–22; Bt 19–22/1E, recombinant *B. thuringiensis* 19–22/1E.

transformant that maintained all of its resident *cry* genes was selected and designated as *B. thuringiensis* 19–22/1E. PCR was used to detect the *cry* gene pattern and the test showed that, in addition to the newly introduced *cry1E* gene, the *cry1Aa*, *cry1Ab*, *cry1Ac*, and *cry1D* genes from the parent strain were also present in *B. thuringiensis* 19–22/1E (Fig. 1A).

As shown in Fig. 1B, the wild-type *B. thuringiensis* 19–22 formed a single cube-shaped parasporal crystal in one sporangium, and the crystal protein appeared as a major band of approximately 130 kDa in SDS-PAGE analysis (Supplementary data Fig. S1). The expression of the introduced Cry1E protein in the recombinant strain 19–22/1E was also detected by 12% SDS-PAGE, and an increased amount of the 130 kDa protein was detected (Supplementary data Fig. S1). To further confirm the production of Cry1E, thin sections of sporulated cells were also examined by transmission electron microscopy (Fig. 1B). Two separated parasporal crystals were observed in one sporangium, in contrast the wild-type strain, indicating that the expression of the Cry1E protein caused an increase in the total amount of Cry1-type protein expressed in the recombinant strain 19–22/1E.

In order to examine *in vivo* antifungal activity, *B. thuringiensis* 19–22 and the recombinant strain 19–22/1E were assayed against six phytopathogens: rice sheath blight (RSB, *R. solani*), tomato grey mould (TGM, *B. cinerea*), tomato late blight (TLB, *P. infestans*), wheat leaf rust (WLR, *P. recondita*), barley powdery mildew (BPM, *E. graminis*) and red pepper anthracnose (PAN, *C. gloeosporioides*). Tomato (*Lycopersicon esculentum* Mill., cv. Seokwang), barley (*Hordeum sativum Jessen*, cv., Dongburi) and wheat (*Triticum aestivum* L., cv. Chokwang) plants were grown in a greenhouse at 25±5°C for 1–3 weeks. Dilutions of each *B. thuringiensis* strains grown in TSB medium for 72 h at 30°C were sprayed on the plants, and the sprayed plants were inoculated with fungal spores after 24 h. At 3–7 days post-inoculation, disease symptoms were rated. The tests were repeated three times independently in a growth chamber, and the mean value of six estimates for each treatment was used to calculate the percentage of disease control. The percentage of disease control was determined using the equation previously reported (Choi *et al*., 2007). As shown in Fig. 2, the antifungal activities of the wild-type *B. thuringiensis* 19–22 strain were maintained in the recombinant strain 19–22/1E.

The *cry1E* gene encodes a crystal protein highly toxic to *Spodoptera* species including *S. exigua*, *S. exempta*, and *S. littoralis*. The insecticidal activity of the recombinant strain was determined against *S. exigua* larvae as described previously (Chang *et al*., 1998). Bioassays were conducted at 25°C in 60–70% humidity with a 16L/8D cycle. Sporulated cultures of *B. thuringiensis* were serially diluted in 0.01% (v/v) Triton X-100, and 100 μl aliquots of each dilution were ap-

Table 1. **Insecticidal activity of the *B. thuringiensis* 19-22 transformant against second-instar *S. exigua* larvae**

Strain	LC_{50} ($\times 10^5$ CFU/cm^2)	FL_{95}[a] ($\times 10^5$ CFU/cm^2)
B. thuringiensis 19-22	ND[b]	ND
B. thuringiensis 19-22/1E	1.51	0.11–4.46

[a] 95% fiducial limits
[b] not determined (only 47.6% mortality even at 1×10^7 CFU/cm^2)

plied to the surface of vegetables inoculated with 30 second-instar *S. exigua* larvae. Larval mortality was recorded every 24 h for 5 days. All tests were performed with spore-parasporal inclusion suspensions and repeated three times independently. The median lethal concentration (LC_{50}) was calculated by the Probit method using SoftTOX version 1.1 (Soft LabWare, USA). The LC_{50} of *B. thuringiensis* 19–22/1E was lower than that of the wild-type strain (Table 1), clearly confirming that the expression of *cry1E* in the recombinant 19–22/1E strain caused enhanced toxicity against *S. exigua*. This also demonstrated that the recombinant *B. thuringiensis* strain expressed the insecticidal Cry1E protein stably and functionally.

The study of *B. thuringiensis* as a biological control agent has been focused on the use of Cry proteins (Clark *et al.*, 2005), and a few studies on its antifungal activity have been reported recently. In this study, *B. thuringiensis* 19–22 was isolated from a Korean soil sample, and its biochemical and molecular biological characteristics were analysed. *B. thuringiensis* 19–22 which belonged to ssp. *kurstaki*, was selected for its toxicity against barley powdery mildew. Then, the strain was subjected to a transformation procedure designed specifically to introduce an exogenous *cry1E* gene into its genome to broaden its target spectrum and stably maintain the foreign gene. The instability and incompatibility of introduced plasmids in *B. thuringiensis* often cause a loss or decrease of toxicity in recombinant strains. Other reports also confirmed synergistic interactions among various *B. thuringiensis* toxins (Poncet *et al.*, 1995). In many subspecies of *B. thuringiensis,* crystal protein genes are encoded by one or more large plasmids; in a few subspecies, these genes are encoded by the chromosome. When shuttle vectors containing *B. thuringiensis* replicons or exogenous plasmids harbouring crystal protein genes are transferred into *B. thuringiensis*, the resident plasmids that carry important crystal protein genes are often displaced (Baum *et al.*, 1990; Gamel and Piot, 1992). Thus, a variety of integrated vectors have been used to introduce and insert cloned *cry* genes by homologous recombination into resident plasmids (Lereclus *et al.*, 1992; Adams *et al.*, 1994) or the *B. thuringiensis* chromosome (Kalman *et al.*, 1995) to allow the stable expression of the introduced genes and simultaneous maintenance of the resident plasmids. To increase the efficacy and broaden the host spectrum of *B. thuringiensis*, *cry* genes that have a greater toxicity against the same target or are toxic to other insect orders have been directly transformed into *B. thuringiensis* strains (Zhu, 2006; Wang *et al.*, 2008). We avoided the loss of synergism between toxins by using a mild electroporation method, and identified and assayed a transformant strain (*B. thuringiensis* 19–22/1E) that had maintained all of its native *cry* genes. The recombinant strain *B. thuringiensis* 19–22/1E showed higher mortality against *S. exigua* than the original isolate, indicating that the foreign *cry1E* gene was expressed well within the transformant cell. The results of this study demonstrate that a recombinant *B. thuringiensis* strain is able to control harmful insect pests as well as plant fungal diseases in one crop and may be used as a potential biocontrol agent in the future.

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