Expression of a *Bacillus thuringiensis cry1B* synthetic gene protects Mediterranean rice against the striped stem borer

Received: 7 February 2000 / Revision received: 18 May 2000 / Accepted: 29 May 2000

**Abstract** We investigated the expression in transgenic rice of a synthetic gene encoding the toxic part of the *Bacillus thuringiensis* Cry1Ba endotoxin, which was shown to exhibit a tenfold lower lethal concentration 50 (LC50) than Cry1Ac in a Striped Stem Borer (SSB) diet incorporation assay. The 1,950-bp synthetic cry1B gene, possessing an overall GC content of 58%, was cloned under the control of the maize ubiquitin promoter first intron and first exon regions. The resulting vector, designated as pUbi-cry1B, was transferred to two commercial Mediterranean cultivars of rice. Ariete and Senia, using microprojectile acceleration-mediated transformation. Thirty-two and 47 T0 events were generated in cvs. Ariete and Senia, respectively. Southern blot and immunoblot analyses allowed the identification of 7 Senia and 1 Ariete events harbouring both an intact gene cassette and expressing Cry1B at a level ranging from 0.01% to 0.4% of the total soluble proteins. Three Senia and 1 Ariete events were found to be protected against second instar SSB larvae in whole plant feeding assays, exhibiting 90–100% mortality 7 days after infestation. Spatial and temporal variation in transgene expression was further examined in resistant event 64 of cv. Ariete. Stable accumulation of Cry1B, representing 0.4% of the total soluble proteins, was observed over the T2 to T4 generations in leaf tissue 20, 40, 70 and 90 days after germination in both young and old leaves and in internodes. Ariete event 64 was found to be fully protected from attacks of third and fourth instar SSB larvae over subsequent generations.

**Key words** *Bacillus thuringiensis* · cry1B · Insect resistance · *Oryza sativa* L. · Synthetic gene · Transgenic plants

**Introduction**

The Striped Stem Borer (SSB) (*Chilo suppressalis* Walker) of Asian origin is one of the major constraints affecting rice production in southern European countries, causing yield losses reaching up to 15–20% in Spain and France. This problem has been addressed by means of chemical treatments with pyrethroid insecticides and sprays with *Bacillus thuringiensis* (Bt) insecticidal formulations. However, the former has proven to be environmentally damaging and the latter of limited efficiency due to their poor persistence and the biological localization of the pest, which develops in the culm. Conventional breeding for SSB tolerance has also proven to be difficult due to the polygenic control of the trait (Khush and Brar 1991), and no gene for host resistance has been mapped (Bennett et al. 1997). An alternative is the deployment of engineered rice cultivars adapted to local growth conditions and consumer requirements that harbour one or several endotoxin genes from the soil bacterium *Bt*, which encodes insecticidal proteins active against the SSB. The production of transgenic plants harbouring synthetic cry1A genes driven by constitutive promoters and exhibiting full protection against insect attacks [SSB, yellow stem borer (*YSB*, *Scirpophaga incertulas* and leaffolder-*Cnaphalocrocis medinalis*) has been extensively reported in *japonica*(cry1Ab: Fujimoto et al. 1993, Cheng et al. 1998; cry1Ac: Cheng et al. 1998) and *indica*(cry1Ab: Wünn et al. 1996, Datta et al. 1998; cry1Ac: Nayak et al. 1997) rice]. These studies were aimed at obtaining a high level of expression of the insecticidal genes in the transgenic rice plant. The
delivery of a high dose of toxins combined with the presence of refuges in the Bt crops is considered by many entomologists as the most promising strategy to prevent or delay resistance build up in the target pest population (Frutos et al. 1999).

As mentioned above, the expression of genes whose efficacy have been assessed in rice has so far been limited to cry1Ab and cry1Ac, which might not encode the toxins displaying the highest activity against the SSB. A diet incorporation assay involving seven Bt toxins has shown that the toxin exhibiting the highest toxicity towards SSB larvae is Cry1Aa, followed by Cry1B and Cry1Ac (Fiuza et al. 1996). As in transgenic plants insect mortality results from both a high dose delivery and specific toxicity of the toxin on the target pest, there is a need to engineer novel synthetic Bt genes encoding the most active toxins that have been shown to cause high mortality in SSB diet incorporation assays. This is particularly true for the SSB, which appears to be far less sensitive to Bt toxins than YSB and leafhopper (M. Cohen, personal communication). Very recently, the expression of a cry2A synthetic gene in transgenic indica rice has proven efficient in fully controlling YSB and leafhopper (Bano-Maqbool et al. 1998). However, this toxin has also been reported to be the least effective among those exhibiting toxicity to SSB (Fiuza 1996; Aguda et al. 1997).

The cry1Ba gene, which was isolated from Bt strain HD2 by Brizzard and Whiteley (1988), encodes an endotoxin whose protoxin and toxic regions share only 55.5% and 34.8% amino acid homology with Cry1Ac, respectively. The sequence from nucleotides 1–1944 encoding the active toxin plus 29 amino acids at the N-terminal end of the wild-type cry1Ba gene was fully modified to optimize its expression in monocotyledonous crops, as reported previously (Bohorova et al. 1999). The GC content of the resulting 1944-bp modified gene is 58%, while that of the native cry1B is 39.3%.

The objective of the study reported here was to attain full protection of Mediterranean rice against SSB through the constitutive expression of this novel, monocot codon-optimized cry1B synthetic gene. We first reinvestigated the toxicity of Cry1B relative to that of Cry1Aa and Cry1Ac in a diet incorporation assay of a newly field-collected SSB population that will serve for bioassay material. The maize ubiquitin promoter region (Christensen and Quail 1995) was then fused to the cry1B coding sequence and the resulting construct transferred to two Mediterranean rice cultivars that are susceptible to SSB attack. We further examined the level of protection afforded by expression of the cry1B gene.

### Materials and methods

#### Chilo suppressalis rearing and diet incorporation assays

A field-collected Chilo suppressalis population was reared on an artificial diet as previously described (Bordat et al. 1977). Lethal concentration (LC50) bioassays were performed by spreading 100 μl of various concentrations of Cry1Aa, Cry1B or Cry1Ac Bt toxin solutions in PBS buffer onto a 5-mm-deep layer of artificial medium contained in 1-cm² individual square wells. Excess liquid was allowed to dry under a laminar flow hood. For each toxin dose, forty 10-day-old L2 larvae were individually placed on the artificial media with a small paintbrush. Percentages of mortality were recorded 7 days after release. Data were analyzed using a specific software (WindL, 1998) according to the Probit method (Finney 1971), corrected for the estimation of natural mortality (Dempster et al. 1977; Hasselblad et al. 1980).

#### Plant material

Transformation was carried out using two japonica (isozyme group VI according to Glasmann 1987) rice (Oryza sativa L.) varieties, Ariete and Senia. Ariete and Senia are two top commercial cultivars in France and Spain and are highly susceptible to SSB (Chilo suppressalis) attacks. Mature seed embryos were used to induce callus cultures, which subsequently served as material for particle bombardment according to Chen et al. (1998b).

#### Transformation vectors

The synthetic cry1B gene was cloned under the control of the promoter and the entire 3′ untranslated region of the maize polyubiquitin gene Ubi-1 (promoter-exon1-intron1) and the nos 3′ polyadenylation sequence into the BamHI site of plasmid pAH25 (Christensen and Quail 1995) deleted for the uidA coding sequence. A HindIII fragment containing the Ubi-cry1B-nos gene cassette was inserted into the HindIII restriction site of pBKS (Stratagene), resulting in the 7.19-kbp pUbi-cry1B plasmid. The 5.1-kbp plasmid pILTAB227 consisting of the CaMV35S promoter with a duplicated enhancer sequence (e35S) controlling the hygromycin phosphotransferase bhp gene and followed by the nos 3′ terminator (kindly supplied by Dr. C. Fauquet, ILTAB, La Jolla, Calif.) was used as the selectable construct in the microprojectile bombardment experiment. Schematic representation of the pUbi-cry1B plasmid is provided in Fig. 1. All plasmids were prepared according to Birnboim and Doly (1979), purified over a CsCl gradient and resuspended in water at a concentration of 1 μg/μl.

![Diagram of pUbi-cry1B plasmid](image-url)