A genetically unique strain of the Fusarium wilt pathogen was first recognized in wilted and dead Upland cotton seedlings in Australia in 1993. Since that time the disease spread rapidly despite stringent containment practices. The Australian biotype isolates of *Fusarium oxysporum* f. sp. *vasinfectum* (Fov) produced copious amount of fusaric acid in potato dextrose cultures and their virulence was directly correlated to amounts of fusaric acid produced. Though fusaric acid has long been implicated in the pathogenesis of Fusarium wilt for a number other plant species including tomato, watermelon, and flax, direct evidence for the role of fusaric acid in the virulence is still lacking as the biosynthetic enzymes and genes for fusaric acid have not been identified and cloned. We set out to identify and clone the polyketide synthase (PKS) gene involved in the fusaric acid biosynthesis through differential display. We identified a partial clone whose expression matched the expected profile of the fusaric acid production in the fungus. Using homology based strategy, we cloned a gene cluster containing the PKS gene as well as an amino acid kinase gene and the corresponding cDNAs. Targeted gene disruption of either of these genes in the Australian biotype Fov isolate resulted in complete blockage of fusaric acid production. These fusaric acid knockout mutants showed much weaker pathogenicity than their wild type progenitor toward tomato seedling in a seedling germination bioassay on agar plates. These results indicate that fusaric acid plays an important role in the pathogenicity of the Australian biotype isolates.

**Introduction**

Fusarium wilt of cotton is caused by *Fusarium oxysporum* Sclecht f. sp. *vasinfectum* (Atk.) Sny. and Hans (Fov). Fov occurs in most countries where cotton is grown (Bell, 1999; Hillocks, 1992). Yield losses can be significant on farms where soil conditions, nematode populations, and indigenous populations of the pathogen favor the disease (DeVay et al., 1997; Hillocks and Kibani, 2002). A genetically unique strain of the Fusarium wilt pathogen was first recognized in wilted and dead Upland cotton seedlings in Australia in 1993 (Davis et al., 1996; 2006; Bentley et al., 2000). Since that time the disease spread rapidly despite stringent containment practices. On some Australian farms where it was first discovered, losses greater than 90% have forced cotton production to be abandoned. In contrast to U.S. race A isolates, which are often associated with root-knot nematodes and thrives in light textured sandy acid soils, the Australian biotypes attack cotton seedlings without root-knot nematode in heavy alkaline clay soils. They also produce prodigious quantities of fusaric acid when grown on Czapek media. Fusaric acid is a potent phytotoxin especially to cotton. For the Australian biotype isolates, their virulence was directly correlated to amounts of fusaric acid produced in potato dextrose broth cultures (Bell et al., 2003). Fusaric acid has also long been implicated in the pathogenesis of Fusarium wilt for a number other plant species including tomato (Gaumann, 1957), watermelon (Nishimura, 1958), and flax (Trione, 1960). However, controversies exist regarding the role of fusaric acid in the pathogenesis (Kuo and Scheffer, 1964). Direct evidence for the role of fusaric acid in the virulence is still lacking as the biosynthetic enzymes and genes have not been identified and cloned to date.

Fusaric acid was discovered and characterized as early as 1937 from *Fusarium heterosporum* Nees (Yabuta et al.). Subsequently it was detected in a wide range of *Fusarium* species (Bacon et al., 1996; Amalfitano et al., 2002). Elucidation of the biosynthetic origin of fusaric acid using $^{13}$C-, $^{14}$C-, $^{15}$N-labelled precursors indicated that fusaric acid is derived via a triketide and oxaloacetate, suggesting involvement of a polyketide synthase (Hill et al., 1966; Desaty et al., 1968). Here we describe the identification and cloning of a polyketide synthase gene and an aspartate kinase like gene are required for the biosynthesis of fusaric acid in *Fusarium oxysporum* f. sp. *vasinfectum*.
kinase like gene required in the biosynthesis of fusaric acid. Using targeted gene disruption mutants of these genes, we also demonstrated that fusaric acid production was required for the full virulence of the Australian biotype Fov isolates.

**Materials and Methods**

DNA and RNA were extracted from mycelium of an Australian Fov isolate grown in shake cultures of Czapek media for 6 days. RNA was also extracted from mycelium grown in the presence of Zn which suppressed fusaric acid production. RT-PCR was used to amplify the ketoacyl synthase (KS) domain of PKS genes using consensus sequences of the fungal KS domain. The amplified fragments were cloned into pUC18 and sequenced. For sequencing the full clone of the genes, primers were designed based on homologous sequences available from public databases.

Gene replacement cassettes containing the hygromycin resistance gene were constructed using the double-joint PCR method (Yu 2004). The wild type Australian isolate were transformed with the construct by the polyethylene glycol (PEG) mediated method. Hygromycin resistant transformants were purified by two rounds of a monoconidial isolation method before their DNA was extracted. Isolates were grown on Czapek media with or without Zn for 15 days and fusaric acid concentrations were determined using HPLC.

Pathogenicity assays of the gene disrupted mutants versus wild-type Australian Fov isolates were conducted using germinating tomato seeds on Czapek agar in Petrie dishes. We plated 200 ml of 10⁶ conidia per ml on each plate and 5 tomato seeds, and incubated for 13 days in a diurnal cycle of 12 hr light at 25o C and 12 hr dark at 22o C.

**Results and Discussion**

**Identification and cloning of a polyketide synthase gene and an aspartate kinase like gene involved in the biosynthesis of fusaric acid**

A pair of degenerate primers corresponding to the conserved regions of the KS domain of the PKS genes was used to amplify the KS domain of the PKS genes from mRNA extracted from an Australian biotype Fov isolate grown under fusaric acid producing conditions. The amplified fragments were cloned and sequenced. A BLAST search of the insert sequences identified 6 individual PKS genes. Based on the sequences of the six cloned fragments of individual PKS genes, we designed corresponding gene specific primers. Gene expression analysis was carried out with these gene specific primers. Among six cloned PKS gene fragments, only one had a matching expression profile with the fusaric acid production profile. The corresponding full clone sequence was obtained using a homology based strategy. A gene cluster containing the corresponding PKS (fua1) gene and an amino acid kinase like gene (fua2) was obtained (Fig. 1). cDNA sequencing revealed that the cloned PKS (fua1) gene coded for a 2410 amino acid protein (Fig. 2). It contained KS, AT, and ACP domains, as well as three reducing domains, KR, DH, and ER; this is consistent with the biosynthesis of the reduced side chain of fusaric acid via a reducing PKS. The presence of an amino acid kinase like gene next to the cloned PKS gene is consistent with derivation of fusaric acid from one unit of aspartate.

![Fig. 1 Fusaric acid biosynthetic gene cluster.](image-url)
Generation of fusaric acid biosynthetic gene knock-out mutants by targeted gene disruption

We used the double-joint PCR method to construct the gene replacement cassettes containing the hygromycin resistance gene. For the PKS gene disruption, the gene replacement cassette was constructed by flanking Hyg B resistant gene with the wilt type gene sequences flanking the AT and DH domain of the PKS gene (Fig. 3). Double cross over during the transformation produced a Hyg B resistant mutant with the AT and DH replaced, causing the disruption of the PKS gene. Using a similar approach, we constructed the gene replacement cassette for the amino acid kinase gene. This time, the whole gene was replaced by the Hyg B resistance gene (Fig. 4). Generation of gene replacement mutants were carried out with an Australian biotype isolate using a PEG-mediated transformation method.

We were able to generate one PKS and one amino acid kinase gene disruption mutant which were confirmed by PCR analysis. These mutants failed to produce any detectable amounts of fusaric acid, confirming the cloned genes indeed code for fusaric acid biosynthetic genes. The relative conidia counts and the growth rates of the mutants are not significantly different from that of their wild type progenitor. Furthermore, the colony morphology of the mutants on several different media is also very similar to that of their wild type progenitor. These results indicate that the gene disruption did knock out the production of fusaric acid, but did not significantly change the isolate’s overall physiology.

Pathogenicity test with germinating tomato seeds

In the presence of PKS gene knock out mutants, more than 4 out of 5 seeds germinated and grew healthy. The roots were long and not discolored, and the cotyledonary leaves are fully developed (Fig. 5). In contrast, in the presence of wild-type Australian Fov isolates only two out of 5 seeds in each plate germinated and the cotyledonary leaves of the germinating seedlings are severely stunned. The roots are scarce and dark brown in color. These results support our hypothesis and indicate that fusaric acid is a major contributor to virulence of the Australian Fov biotype isolates.
Summary

A number of PKS gene fragments were identified using a pair of degenerate primers targeted at the conserved sequences of the fungal KS domain. The PKS gene involved in the biosynthesis of fusaric acid was identified by comparing expression levels of the cloned genes under fusaric acid inducing conditions vs. suppressing conditions. A gene cluster containing a PKS gene and an amino acid kinase like gene and the corresponding cDNAs were cloned and sequenced. The cloned PKS gene coded for a 2410 amino acid protein and contained KS, AT, and ACP domains, as well as three reducing domains, KR, DH, and ER which is consistent with the biosynthesis of the reduced side chain of fusaric acid via a reducing PKS. The presence of an amino acid kinase like gene next to the cloned PKS gene is consistent with derivation of fusaric acid from one unit of aspartate. Targeted gene disruption of either of these genes in the Australian biotype Fov isolate resulted in complete blockage of fusaric acid production indicating that the cloned genes are indeed involved in the biosynthesis of fusaric acid. These fusaric acid knockout mutants showed much weaker pathogenicity than their wild type progenitor toward tomato seedling in the seedling germination bioassay on agar plates. These results indicate that fusaric acid is an important contributor to the pathogenicity of the Australian biotype Fov isolates.

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References


