Isolation of a benomyl-resistant allele of the β -tubulin gene from *Septoria nodorum* and its use as a dominant selectable marker

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We have developed a homologous transformation system for the wheat-pathogenic fungus *Septoria nodorum* based on a benomyl-(MBC-) resistant allele of the β -tubulin gene. The β -tubulin gene was isolated by heterologous hybridization from a cosmid library prepared from an MBC-resistant mutant. Cosmids carrying the gene conferred MBC resistance when introduced into a sensitive strain, demonstrating that resistance to MBC fungicides in *S. nodorum* may be determined by the β -tubulin gene. This MBC resistant allele of the β -tubulin gene (*tubA*^R) was subcloned into pUC18 and used as a dominant selectable marker for transformation of wild-type sensitive strains. Transformants arose at frequencies of approximately 5 per μ g of DNA, were integrative in nature and were mitotically stable. Some transformants showed a marked reduction in vigour, both in the presence and absence of MBC; this is thought to arise from overproduction of β -tubulin. The *S. nodorum tubA*^R gene also conferred MBC resistance on the related species *Leptosphaeria maculans*, a pathogen of *Brassica*, following its introduction by cotransformation. Probing digested *S. nodorum* DNA with *tubA*^R at low stringency revealed only a single β -tubulin gene. We anticipate that *tubA*^R will prove a useful tool for the investigation of the pathogenicity of *S. nodorum* and other fungi.

Introduction

Recombinant DNA technology is increasingly being used to investigate the mechanisms of fungal pathogenesis to plants (Yoder *et al.*, 1986; Leong & Holden, 1989). We are attempting to clone by complementation genes involved in pathogenesis from *Septoria nodorum* (teleomorph *Leptosphaeria nodorum*), which is a major pathogen of wheat (King *et al.*, 1983). This approach requires a gene transfer system for analysis of the phenotypic effects of cloned sequences following their reintroduction into *S. nodorum* cells. We succeeded in transforming *S. nodorum* with a vector, pAN7-1, which carries the *Escherichia coli* hygromycin B phosphotransferase gene under the control of expression sequences from *Aspergillus nidulans* (Cooley *et al.*, 1988). However, the frequency of transformation with this vector, 5–25 transformants per μ g DNA, is too low to permit screening of a gene library by complementation. To

determine whether the use of a selectable marker originating from *S. nodorum* would increase the efficiency of transformation in this species, we undertook to develop a homologous transformation system to replace the heterologous system based on the *E. coli* hygromycin B resistance gene.

With genetically undeveloped fungi, such as *S. nodorum*, it is often necessary to transform wild-type recipients and hence any system must be based on a dominant selectable gene. Antibiotic resistance genes have frequently been used in this way (Yoder *et al.*, 1986; Punt *et al.*, 1987; Rambosek & Leach, 1987) but their bacterial origin excluded them from our purpose. Fungicide resistance is an obvious analogy that could be exploited in a homologous system. However, the molecular basis of the resistance to many fungicides is poorly understood and candidate selectable genes cannot be readily identified and cloned. An exception is resistance to the benzimidazole (MBC) group of fungicides which in *A. nidulans*, *Neurospora crassa* and *Saccharomyces cerevisiae* arises through mutations in the β -tubulin gene (Sheir-Neiss *et al.*, 1978; Orbach *et al.*, 1986; Thomas *et al.*, 1985). A cloned benomyl-resistance gene from *N.*

Abbreviations: MBC, methyl benzimidazole-2-yl carbamate (carben-dazim), the active ingredient of benomyl; HmB, hygromycin B; superscript R, resistant.

crassa (*tub-2*) has been used in a highly efficient homologous transformation system for that species (Orbach *et al.*, 1986; Vollmer & Yanofsky, 1986). Wild-type *S. nodorum* is sensitive to MBC fungicides and can be transformed to resistance by the *Neurospora tub-2* gene (Cooley *et al.*, 1990), suggesting that MBC resistance might form the basis of a homologous transformation system. Mutants resistant to MBC can be readily isolated in *S. nodorum* (Horsten & Fehrmann, 1980) but the mechanism of resistance in this species has not been established. On the assumption that resistance arises through modification of the β -tubulin, we set out to clone the β -tubulin gene from an MBC-resistant mutant of *S. nodorum* and to use it as a dominant selectable marker for transformation of this species and other phytopathogenic fungi.

Methods

Strains and vectors. The wheat-adapted strain of *Septoria nodorum* BS171 (Osborn *et al.*, 1986) was used as the wild-type throughout. The *Leptosphaeria maculans* strain used (345A4) was obtained from Dr K. J. Doughty (Institute of Arable Crops Research–Rothamsted Experimental Station, UK). *Escherichia coli* strain DH1 was used as the host for all plasmids, except pUC18 derivatives containing subcloned *S. nodorum* DNA fragments, where JM83 was used. Strain JA221 was used as the recipient in transfections of the cosmid library.

The plasmid pAN7-1 was constructed by Punt *et al.* (1987) and contains the *E. coli* hygromycin B (HmB) resistance gene under the control of *Aspergillus nidulans* expression sequences. Cosmid pAN7-2 is a derivative of pAN7-1 (P. J. Punt, personal communication) containing a unique *Bgl*II site and a λ -*cos* site. Plasmid pB5 was used as the source of the *A. nidulans* β -tubulin gene *benA* (May *et al.*, 1985).

Media and culture conditions. Standard methods for the storage, growth and sporulation of *S. nodorum* and the composition of minimal medium (MM) and sporulation medium (CzV8CS) are described elsewhere (Newton & Caten, 1988). The medium used in *S. nodorum* transformations is described by Cooley *et al.* (1988). Oxoid potato dextrose agar (PDA) was used as the basal medium for growth of *L. maculans* and spores were produced by growing colonies on CzV8CS medium under the same conditions as used for sporulation of *S. nodorum*. *L. maculans* was also grown in CzV8CS medium. MBC was added to media as carbendazim from a 1 mg ml⁻¹ stock solution in methanol.

Isolation of MBC-resistant mutants. MBC-resistant (MBC^R) mutants of *S. nodorum* were isolated by overlaying BS171 spore suspensions, which had been UV-irradiated to give a survival rate of 3–10%, on MM plates containing 2.0 µg MBC ml⁻¹. Approximately 2 × 10⁶ viable spores were inoculated on to each plate and these were incubated at 25 °C in the dark.

Pathogenicity assays. Pathogenicity was tested in detached wheat leaf assays as described by Cooley *et al.* (1988). To test MBC resistance during growth on plant tissue, the detached wheat leaves were placed on water-agar containing benlate (Du Pont) at a range of concentrations up to 40 µg ml⁻¹. Benlate contains 50% active ingredient of MBC.

Preparation and transformation of protoplasts. Protoplasts of *S. nodorum* and *L. maculans* were prepared and transformed as described

by Cooley *et al.* (1988). *L. maculans* protoplasts were regenerated on PDA medium buffered with 1 M-sucrose. Direct selection for MBC resistance was applied by overlaying protoplasts on to plates containing MBC at a final concentration of 2.5 µg ml⁻¹.

DNA isolation and molecular analyses. DNA was isolated from *S. nodorum* as described by Cooley *et al.* (1988). ³²P-labelled DNA probes were produced by hexanucleotide random primer labelling (Feinberg & Vogelstein, 1983, 1984). All other DNA manipulations, including restriction digests, ligations, *in vitro* packaging, colony hybridization and Southern blotting, were as described by Maniatis *et al.* (1982) or in accordance with the manufacturers' recommendations.

Construction of an *S. nodorum* genomic library and isolation of β -tubulin-specific cosmid clones. High-molecular-mass DNA from MBC^R mutant BSm300 was partially digested with *Sau*3A and size-fractionated on salt gradients (1.25–5 M-NaCl) in order to recover fragments approximately 30 kb in size. These were ligated into *Bgl*II-linearized pAN7-2 pre-treated with alkaline phosphatase: 0.1 µg insert DNA was ligated with 0.4 µg vector DNA in the presence of 20 mM-Tris/HCl, pH 7.6, 10 mM-MgCl₂, 1 mM-ATP, 1 mM-DTT, and 0.1 unit of T4 DNA ligase in a final volume of 15 µl for 2 h at room temperature. The products were packaged, transfected into *E. coli* JA221 and the library plated on to nitrocellulose filters overlaying L-agar containing 150 µg ampicillin ml⁻¹. Replica filters, each containing approximately 3000 colonies, were hybridized to ³²P-labelled 5.4 kb *Pst*I fragment of pB5, containing the *A. nidulans benA* gene (May *et al.*, 1985). Hybridization was carried out under conditions of medium stringency at 42 °C in a buffer containing 37.5% (v/v) formamide, 5 × SSC, 1 × Denhardt's solution, 100 µg salmon sperm DNA ml⁻¹, 10% (w/v) dextran sulphate, 20 mM-orthophosphate, pH 6.5.

Results and Discussion

Isolation of MBC^R mutants of *S. nodorum*

Following incubation of the MBC^R mutant selection plates, strongly growing colonies developed amongst a weak background growth at a rate of 5 × 10⁻⁶. After hyphal transfer on to fresh plates approximately one-third of these initially selected colonies were able to grow at 20 µg MBC ml⁻¹, which is ten times the concentration needed to completely inhibit growth of *S. nodorum* BS171. Similar levels of resistance occur in benomyl-resistant mutants of *N. crassa* (Borck & Braymer, 1974) and *A. nidulans* (van Tuyl, 1977).

Six of these MBC^R mutants were tested for pathogenicity in detached wheat leaf assays in the presence and absence of benlate. Without benlate all six mutants produced lesions at least the size of those produced by BS171. Furthermore, all six also produced sizeable lesions on leaves placed on water-agar containing 20 µg benlate ml⁻¹, whilst BS171 gave a much reduced lesion under these conditions. One mutant, BSm300, still produced large lesions on water-agar containing 40 µg benlate ml⁻¹. Thus, these mutants express MBC resistance both *in vivo* and *in planta* and show no loss of pathogenicity.

Isolation and characterization of β -tubulin-specific cosmid clones

Thirteen cosmid clones from the BSm300 genomic library produced positive signals on five pairs of duplicate filters when probed with the *A. nidulans benA* gene. These were picked and purified, and cosmid DNA from each of these β -tubulin-specific clones was isolated, digested with *Bam*HI and probed with the *A. nidulans benA* gene in Southern hybridizations. Ten of these 13 clones produced positive signals corresponding to fragments of 3.0 and 4.1 kb, suggesting that the region which contains homology to the *benA* probe possesses an internal *Bam*HI site (data not shown). The other three clones each gave single positive signals, corresponding to fragments of 3.0, 4.1 and 5.5 kb, and probably represent incomplete clones of the β -tubulin-specific region cloned in the other 10 clones. These results suggest that *S. nodorum* has a single β -tubulin gene, or at least only one gene with enough homology to the *A. nidulans benA* gene to allow its isolation under the conditions used.

Ten of the β -tubulin-specific cosmid clones, including the three presumed incomplete clones, were transformed into *S. nodorum* BS171 by selecting for hygromycin resistance. HmB^R transformants arose at relatively low frequencies, between 0.4 and 1.2 per μ g DNA, compared to the frequency obtained using pAN7-1 (5–25 per μ g DNA; Cooley *et al.*, 1988). These HmB^R transformants were transferred to MM plates containing HmB or MBC. As expected, all grew on plates containing enough HmB to completely inhibit growth of BS171 (150 μ g HmB ml⁻¹). Furthermore, transformants from seven of the 10 cosmid clones tested grew on plates containing 2.5 μ g MBC ml⁻¹, whilst BS171 and transformants of the other three clones were completely inhibited at this concentration. The seven cosmid clones that conferred MBC resistance all contained *benA*-specific 3.0 and 4.1 kb *Bam*HI fragments, whilst the three that did not lacked one or more of these fragments. These results demonstrate that in *S. nodorum*, as in a number of other fungi, resistance to benzimidazole fungicides may be determined by the β -tubulin gene (Sheir-Neiss *et al.*, 1978; Orbach *et al.*, 1986; Thomas *et al.*, 1985; Hiraoka *et al.*, 1984). That the three cosmid clones which gave single positive hybridization signals were unable to confer MBC resistance is consistent with the idea that these are incomplete clones of the same gene contained in the other 10 clones.

The level of MBC resistance of the cosmid clone transformants was intermediate between that of the wild-type recipient (BS171) and the MBC^R mutant (BSm300) from which the β -tubulin gene was obtained. Similar results have been seen in benomyl-resistant transformants of *A. nidulans* and *N. crassa*, where

Fig. 1. Restriction map of the 5.8 kb *Hind*III fragment contained in pTUBA4. B, *Bam*HI; H, *Hind*III; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; Sm, *Sma*I; Ss, *Sst*I; X, *Xho*I.

resistant alleles of the *benA* and *tub-2* genes were used, respectively (G. S. May & M. J. Orbach, personal communications). It is likely that the transformed resistant allele shows incomplete dominance with respect to the sensitive recipient allele, so that both resistant and sensitive β -tubulin polypeptides are produced and incorporated into the microtubules of transformants, producing intermediate levels of resistance.

Subcloning the *S. nodorum* β -tubulin gene and its use as a dominant selectable marker

The *S. nodorum* β -tubulin gene was subcloned into pUC18 as a 5.8 kb *Hind*III fragment (Fig. 1) to produce plasmid pTUBA4. This plasmid was tested for its ability to transform BS171, selecting directly for MBC resistance. After 14 d incubation at 25 °C, strongly growing MBC^R colonies appeared at frequencies of 3–6 per μ g pTUBA4 DNA, but were absent from minus-DNA control plates (Fig. 2a). After transfer on to fresh plates all putative transformants tested grew at 2.5 μ g MBC ml⁻¹ whilst BS171 was completely inhibited at this concentration (Fig. 2b). These putative transformants varied in growth vigour on MBC-containing media, suggesting different levels of MBC resistance. However, some showed reduced growth and abnormal colony morphology in the absence of MBC, making the relative resistance levels difficult to assess. The reduced growth in the absence of fungicide is likely to be due to the inhibitory effects of overproduction of β -tubulin similar to the overexpression of either *benA* or *tubC* in *A. nidulans* (Waring *et al.*, 1989). The heterogeneity of phenotype in the absence of MBC was not apparent amongst the cosmid clone transformants.

In addition to the large MBC^R colonies, small colonies developed on both the pTUBA4 transformation plates and the minus-DNA control plates. They appeared at a higher frequency on the transformation plates (Fig. 2a). The small colonies on the minus-DNA control plates may have been due to leaky background growth, perhaps from particularly large protoplast aggregates. Those on the transformation plates were probably a mixture of the above plus abortive transformants, similar to those

Fig. 2. The selection (a) and retesting (b) of pTUBA4 transformants. (a) Aliquots of BS171 protoplasts were treated with pTUBA4 (left) or used as minus-DNA controls (right) and plated on buffered MM containing $2.5 \mu\text{g MBC ml}^{-1}$. Large MBC^{R} colonies arose only on plates inoculated with DNA-treated protoplasts. (b) Six putative pTUBA4 transformants and strains BS171 and BSm300 (lower right and left inocula on each plate, respectively) were retested on MM (left) and MM containing $2.5 \mu\text{g MBC ml}^{-1}$ (right). All six putative transformants grew on MBC, despite some also showing altered growth in its absence.

observed when using pAN7-1 to produce HmB^{R} transformants (Cooley *et al.*, 1988). None of these small colonies from either pTUBA4-treated or minus-DNA plates were able to grow following hyphal transfer to fresh plates containing $2.5 \mu\text{g MBC ml}^{-1}$.

Molecular analysis of putative MBC^{R} transformants

DNA from two HmB^{R} colonies, from each of three cosmid clones which conferred MBC resistance, was probed with ^{32}P -labelled whole pAN7-2 DNA in Southern hybridizations. All six $\text{HmB}^{\text{R}}/\text{MBC}^{\text{R}}$ strains gave positive hybridization signals, confirming that they had been transformed with the pAN7-2 cosmid clones (data not shown). DNA from BS171 gave no such signal.

DNA from BS171 and three putative MBC^{R} pTUBA4 transformants (T1, T2, T3) was probed with ^{32}P -labelled pUC18 DNA in Southern hybridizations (Fig. 3). Uncut DNA gave a single high-molecular-mass positive signal, which was absent from the BS171 track. This both confirms that these are pTUBA4 transformants and demonstrates the integrative nature of transformation.

DNA digested with *Xba*I, an enzyme with a unique site in pTUBA4, gave different patterns of hybridization

Fig. 3. Southern hybridization analysis of pTUBA4 transformants. DNA from strain BS171 (lanes 2 and 6) and three pTUBA4 transformants (T1, lanes 3 and 7; T2, lanes 4 and 8; T3, lanes 5 and 9) was probed with ^{32}P -labelled pUC18. DNA in lanes 2-5 was undigested, whilst that in lanes 6-9 was digested with *Xba*I. Lane 1 contained λ -HindIII size standards. Lanes 2-9 were each loaded with $0.2 \mu\text{g}$ of DNA.

between transformants. T2 and T3 (Fig. 3, lanes 8 and 9) produced major positive signals corresponding to a fragment the same size as linearized pTUBA4 (8.4 kb), a pattern indicative of multiple integration events at one or more sites. A number of less intense signals were also seen in these two lanes, representing the border regions of these multiple integration events and/or the products of single integration events. The large number of these bands indicates that integration had taken place at a number of sites. Thus, integration does not only take place through homologous recombination at the site of the endogenous wild-type *tubA* allele. T1 (Fig. 3, lane 7) gave only a single hybridization signal. Single integration events normally produce two such signals. However, the pattern observed would be consistent with this type of event if the site of integration on the vector were close to the *Xba*I site, producing a fragment bearing little or no homology to the pUC18 probe. These integration events are similar to those seen when selecting for the HmB resistance carried by pAN7-1, except that no single integration events have been detected in the latter (Cooley *et al.*, 1988).

The results in Fig. 3 demonstrate that different transformants vary in the number of copies of pTUBA4 integrated. The reduced growth rate in the absence of MBC and the levels of MBC resistance shown by pTUBA4 transformants did not correlate simply with variation in copy number. For example, two of the three transformants shown in Fig. 3, T1 and T2, had similar phenotypes, with reduced growth in the absence of MBC but no further inhibition in its presence, despite having different copy numbers of integrated pTUBA4. Also, T3 was uninhibited in either the presence or absence of MBC despite having approximately the same copy number of integrated pTUBA4 as T2. Overproduction of β -tubulin may be responsible for the inhibition of growth in the absence of fungicide in T1 and T2; however, this does not simply result from an increased copy number of the β -tubulin gene in these transformants.

Mitotic stability of MBC^R transformants

The stability of primary transformants was tested to determine whether or not they arose as heterokaryons. An origin in this way is possible since PEG 4000 stimulates protoplast fusion. We found segregation of the MBC^R phenotype amongst single-spore progenies derived from individual primary transformant colonies in four out of nine cosmid clone transformants and two out of six pTUBA4 transformants. This single-spore analysis was repeated for one resistant single-spore colony from each original transformant. This time no segregation was seen. These results are similar to those obtained with pAN7-1 transformants (Cooley *et al.*, 1988) and indicate

Fig. 4. Transformation of *L. maculans* to MBC resistance. *L. maculans* strain 345A4 was used in pAN7-1 transformation and pAN7-1/pTUBA4 cotransformation experiments. Three HmB^R pAN7-1 transformants (top row), strain 345A4 (bottom row) and five HmB^R transformants from a pAN7-1/pTUBA4 cotransformation experiment (middle rows) were each plated on three types of media: (a) PDA, (b) PDA containing 150 μ g HmB ml⁻¹ and (c) PDA containing 2.5 μ g MBC ml⁻¹. Four out of the five HmB^R transformants from the pAN7-1/pTUBA4 experiment had been cotransformed to MBC resistance.

that, although primary transformant colonies may be heterokaryons, purified MBC^R transformants are stable through mitotic divisions.

Transformation of a heterologous species to MBC resistance

Plasmid pTUBA4 was transferred into *L. maculans* strain 345A4 by cotransformation with pAN7-1. Protoplasts were treated with an equimolar mixture of both plasmids and transformants selected for HmB resistance. HmB^R transformants arose at a frequency of approximately 1 per μ g pAN7-1 DNA used, somewhat lower than previous reports of transformation frequency for this species (Farman & Oliver, 1988). Five potential cotransformant colonies were inoculated by hyphal transfer on to PDA plates, PDA plates containing 150 μ g HmB ml⁻¹ and PDA plates containing 2.5 μ g MBC ml⁻¹ (Fig. 4). The recipient strain 345A4 and a number of HmB^R transformants arising from treatment with pAN7-1 alone were also inoculated on these plates. All but the recipient grew on the HmB plates. The recipient, all the transformants from the pAN7-1 transformation and one of the transformants from the pAN7-1/pTUBA4 transformation were completely inhibited by MBC. However,

the other four of the latter transformants grew in the presence of MBC, indicating that they had been cotransformed with pAN7-1 and pTUBA4 and showing that high-frequency cotransformation takes place in *L. maculans* as well as in *S. nodorum* and other fungal species (Kelly & Hynes, 1985; Punt *et al.*, 1987; Wernars *et al.*, 1987; Cooley *et al.*, 1990).

These cotransformation experiments in *L. maculans* show that the *Septoria tubA*^R gene confers MBC resistance in a heterologous species and suggest that pTUBA4 could be used to transform other Ascomycete and Deuteromycete species by direct selection. In addition to the cotransformation with pTUBA4 reported here, *L. maculans* has also been transformed with this vector by selection for MBC resistance (M. L. Farman, personal communication). The *N. crassa* and *A. niger* tubulin genes have also been shown to confer MBC resistance following transformation into several Ascomycetes and Deuteromycetes (Dickman, 1988; Henson *et al.*, 1988; Rambosek & Leach, 1987; Cooley *et al.*, 1990; Bernier *et al.*, 1989), suggesting that β -tubulin genes are sufficiently conserved within and between these taxa for their products to copolymerize and function in heterologous backgrounds.

S. nodorum has a single β -tubulin gene

Digested genomic DNA from *S. nodorum* BS171 was probed in Southern hybridizations at low stringency [at 42 °C in 25% (v/v) formamide, 5 × SSC, 1 × Denhardt's solution, 100 µg salmon sperm DNA ml⁻¹, 10% (w/v) dextran sulphate, 20 mM-orthophosphate, pH 6.5] with the 2.0 kb *Pst*I fragment of pTUBA4 which shows homology with *benA* and which spans the *Bam*HI site of *tubA*^R (Fig. 1). With either *Pst*I- or *Hind*III-digested DNA, a single positive hybridization signal was observed corresponding to fragments of 2.0 and 5.8 kb, respectively (Fig. 5). These are the sizes of fragments predicted by the restriction map of the *Septoria* insert of pTUBA4, suggesting that *S. nodorum* has a single β -tubulin gene, or at least only one gene with sufficient homology to hybridize to a *tubA*-specific sequence. *S. nodorum* is like *N. crassa* in this respect (Orbach *et al.*, 1986) and unlike *A. nidulans*, which possesses two β -tubulin genes (May *et al.*, 1987). These data also demonstrate that the β -tubulin gene was not rearranged during its cloning.

Conclusion

Our aim of developing a homologous transformation system for *S. nodorum* based on selection for an MBC-resistant β -tubulin gene was realized. However, the transformation frequency obtained using the *tubA*^R gene

Fig. 5. Southern hybridization analysis of the *S. nodorum* β -tubulin gene. DNA (0.4 µg) from strain BS171 was digested with *Pst*I (lane 2) and *Hind*III (lane 3) and probed with the ³²P-labelled 2.0 kb *Pst*I fragment of pTUBA4. Lane 1 contained λ -*Hind*III standards.

as a homologous dominant selectable marker was disappointing as it did not show any improvement on the frequency obtained using the heterologous hygromycin resistance encoded by pAN7-1 (Cooley *et al.*, 1988). Panaccione *et al.* (1988) reported that comparable frequencies were obtained when transforming *Colletotrichum graminicola* with homologous and heterologous benomyl-resistance genes. These results suggest that with a highly conserved gene like β -tubulin homology is not an important factor determining transformation frequency within Ascomycetes and Deuteromycetes. This is consistent with the high frequency of non-homologous integration observed even with vectors carrying extensive regions of homology (Fincham, 1989). Despite this failure to improve the efficiency of transformation of *S. nodorum*, the MBC-resistance gene cloned may be generally useful as a dominant selectable marker for transformation of related phytopathogenic fungi, particularly those naturally resistant to hygromycin.

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