Cloning of the sterol C-14 reductase gene of the tomato pathogenic fungus *Septoria lycopersici* and its complementation of the *erg-3* mutation of *Neurospora crassa*

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Abstract

We have cloned the *erg-3* gene, which encodes the ergosterol biosynthetic enzyme sterol C-14 reductase, from the tomato pathogenic fungus *Septoria lycopersici*. Its nucleotide sequence, reported here, encodes a 512-amino-acid polypeptide with 54% sequence identity to sterol C-14 reductase of *Neurospora crassa*. The *Septoria* gene complemented the pisatin-sensitive, tomatine-resistant and female-sterile phenotypes of a *Neurospora erg-3* mutant.

[Aparna K., Sandrock R.W. and Kasbekar D. P. 1998 Cloning of the sterol C-14 reductase gene of the tomato pathogenic fungus *Septoria lycopersici* and its complementation of the *erg-3* mutation of *Neurospora crassa*. *J. Genet.* **77**, 71–75]

Introduction

In *Neurospora crassa* the *erg-1* and *erg-3* genes encode the ergosterol biosynthetic enzymes sterol Δ8-7 isomerase and sterol C-14 reductase respectively. Mutations in these genes affect the sensitivity of this fungus to the tomato phytoanticipin α-tomatine and to the pea phytoalexin pisatin (Sengupta *et al.* 1995; D.P. Kasbekar, unpublished results). Whereas the wild type is tomatine sensitive and pisatin resistant, the *erg-1* and *erg-3* mutants (including null mutants) are tomatine resistant and pisatin sensitive. Tomatine probably serves as a chemical defence against microbial infection in tomato (Arneson and Durbin 1968a). This idea is supported by the fact that fungal pathogens of tomato possess enzymes that can degrade tomatine into less toxic metabolites (Arneson and Durbin 1967, 1968b; Schlosser 1975; Verhoeff and Liem 1975; Ford *et al.* 1977; Pegg and Woodward 1986; Sandrock *et al.* 1995). Tomato pathogens might have also evolved additional mechanisms of tomatine tolerance that do not depend on its detoxification but nondegradative mechanisms of tomatine tolerance have not yet been reported in any fungal species. In view of the tomatine-resistant phenotype of the *Neurospora erg* mutants it was conceivable that tomato pathogens might have coopted their *erg* gene homologues for tomatine tolerance mechanisms. For instance, their transcription might be downregulated by high concentrations of tomatine so as to increase the pathogen's tolerance to tomatine. As a first step to investigate such possibilities we undertook to isolate the *erg-3* homologue from *Septoria lycopersici*, a fungal pathogen of tomato.

Functional sterol C-14 reductase genes have thus far been isolated only from *Saccharomyces cerevisiae* and *Neurospora crassa* (Lorenz and Parks 1992; Marcireau *et al.* 1992; Papavinasasundaram and Kasbekar 1993, 1994; Lai *et al.* 1994). A homologous gene was isolated from *Schizosaccharomyces pombe* (Smith 1995), but the function of its product has not been tested. We demonstrate here that the *Septoria* sterol C-14 reductase gene can complement the *Neurospora erg-3* mutation.

Methods

Strains and growth conditions: The *N. crassa* wild-type strain 74-OR23-1 *mat-A* (FGSC #987) and the mutant *erg-3 mat-a*

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Keywords. *Septoria lycopersici*; *Neurospora crassa*; sterol C-14 reductase; *erg-3*; pisatin; tomatine.

(FGSC #2725) were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas Medical Center, Kansas City, KS 66103, USA. From a cross between these two strains we recovered an *erg-3 mat-A* recombinant.

Crossing and maintenance of the *Neurospora* strains were essentially as described by Davis and De Serres (1970). Resistance to tomatine was tested by examining growth from conidia streaked onto 1.5% agar plates containing Vogel's N medium plus FGS (0.05% fructose, 0.05% glucose and 2% sorbose) and supplemented with 90 μg/ml α-tomatine (Sigma) from a stock solution (25 mg/ml) in DMF. After incubation overnight at 30°C colonial growth can be observed from the *erg-3* but not the wild-type conidia (Sengupta *et al.* 1995). Resistance to pisatin was scored by streaking conidia onto Vogel's FGS medium supplemented with 50 μg/ml pisatin from a stock solution (55 mg/ml) in DMSO. Pisatin was extracted from germinated pea seeds by the procedure of Sweigard and VanEtten (1987). More recently we have found that the commercially available isoflavone biochanin A (10 μg/ml made from a stock solution of 20 mg/ml in DMSO) can be used instead of pisatin (A. Prakash and D.P. Kasbekar, unpublished results).

PCR: In view of the significant amino acid sequence conservation (40% identity) between the sterol C-14 reductases of *Neurospora* and yeast (Papavinasasundaram and Kasbekar 1994), a PCR-based approach was chosen to isolate the *Septoria* homologue. The PCR was done with *Septoria* genomic DNA as template and degenerate oligonucleotide primers made to the conserved amino acid residues. The forward primer (5′ATIGGIMGIGARYTIAAYCCIMG3′), encodes the amino acids IGRELNPR which are residues 210–217 of the *Neurospora* enzyme and 199–206 of the yeast enzyme. The reverse primer (5′CCARTCICCIARRTARTTIATRTG3′) is complementary to the nucleotide sequence encoding the amino acids HINY(L/F)GDW which are residues 388–395 and 368–375 of the *Neurospora* and yeast sterol reductases respectively (Lorenz and Parks 1992, Papavinasasundaram and Kasbekar 1994). The PCR conditions were a 5-minute 'hot start' at 94°C followed by 30 cycles each of a 1-minute denaturation at 94°C, 1-minute annealing at 45°C, and 2.5-minute extension at 72°C.

Transformation of Neurospora spheroplasts: Spheroplasts of the *Neurospora erg-3 mat-a* strain were prepared and transformed as described previously (Papavinasasundaram and Kasbekar 1993). Plasmid pMP6 DNA was used in cotransformation experiments. This plasmid contains the *E. coli* gene *hph* for resistance to hygromycin B and allows for the selection of transformants on medium containing hygromycin (200 μg/ml, made from a stock solution of 100 mg/ml in water). Cotransformations were done with ~ 500 ng of cosmid DNA together with ~ 400 ng of plasmid pMP6. Routinely the majority (>50%) of the hygromycin-resistant transformants also carried the unselected cotransformed DNA.

Plasmid constructions and sequencing: *E. coli* strain DH5α and the plasmid vector pBluescript KS were used for all plasmid manipulations. Plasmid DNA was isolated by the boiling lysis method (Maniatis *et al.* 1982). Subclones for sequencing were obtained by exonuclease-III-generated deletions of the plasmid insert using the Erase-a-base system (Promega) and also by sequencing PCR products obtained with primers based on the sequence from the deletion clones. Sequencing was either done manually by the chain-termination method (Sanger *et al.* 1977) or automated with the ABI Prism 377 DNA sequencer. M13 forward and reverse and T_7 promoter primers were used for manual sequencing. The Dye terminator/Dye primer cycle sequencing kit (Perkin Elmer) was used for the automated sequencing. Sequence data were aligned using PCGENE software.

Results and discussion

Cloning of the Septoria sterol C-14 reductase gene

We performed a PCR with *Septoria* genomic DNA as template and the degenerate oligonucleotide primers based on amino acid residues conserved between the *Neurospora* and yeast sterol C-14 reductases (see the Methods section). The resulting amplified DNA ran as a single major band when electrophoresed through a 2% agarose gel and was comparable in size (~ 550 bp) with the product of a control PCR with *Neurospora* genomic DNA (557 bp). The band was isolated from the gel and the DNA was cloned into the pMosBlue vector (Amersham) and its partial nucleotide sequence determined. The sequence revealed an ORF with ~50% amino acid sequence identity with the corresponding amino acid sequence of *Neurospora* sterol C-14 reductase, suggesting that the amplified DNA represented the *Septoria* sterol C-14 reductase gene. An aliquot of the gel-excised DNA was used to probe two *S. lycopersici* genomic cosmid libraries. Six positive cosmid clones were identified in these screens.

When *Neurospora erg-3 mat-a* mutant protoplasts were transformed with one of the cosmid clones (cosmid 2-1) complementation of the pisatin-sensitive and tomatine-resistant mutant phenotype was observed. This indicated that cosmid 2-1 indeed carried a sterol C-14 reductase gene and demonstrated that this gene could be expressed in *Neurospora*. Complementation of the tomatine-resistance phenotype suggested that the *Septoria* sterol C-14 reductase gene lacked *cis* elements for autonomous downregulation by tomatine (figure 1).

Neurospora protoplasts can take up linear DNA fragments and integrate them into chromosomes by nonhomologous recombination. We therefore transformed protoplasts of the *erg-3 mat-a* strain with different restriction digests of cosmid 2-1 and examined the transformants for complementation. Digestion by enzymes whose recognition sites are contained within the *erg-3* gene should destroy the complementing ability but digestion by enzymes for which

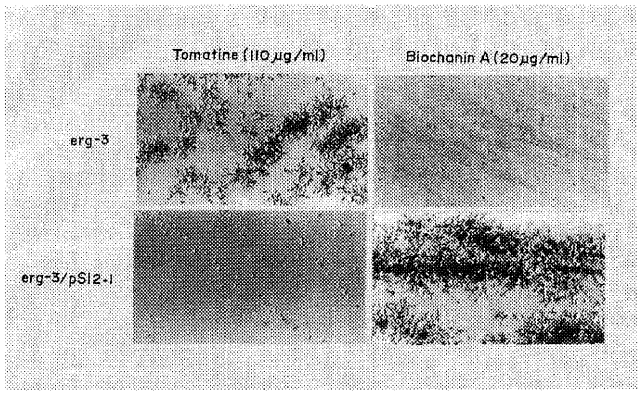

Figure 1. Complementation of the *erg-3* mutant phenotype of *N. crassa* by the *S. lycopersici*-derived *erg-3* transgene. Vogel's FGS plates supplemented with either tomatine (left panel) or biochanin A (right panel) were streaked with conidia of either the *erg-3* mutant strain (top) or of a strain carrying the *erg-3* mutation and the pS12-1-derived Septoria *erg-3* transgene (bottom). The mutant is tomatine resistant and biochanin A sensitive whereas the transgenic strain is tomatine sensitive and biochanin A resistant.

the cleavage sites are not present within the gene should not do so. In this way the complementing ability of cosmid 2-1 could be localized to a 3.2-kb *Pst*I fragment. This fragment was cloned into the pBluescript KS plasmid vector and the resulting plasmid was designated pS12-1.

Characterization of Septoria erg-3

We determined 2.2 kb of nucleotide sequence of the insert of pS12-1 and found on ORF for a protein of 512 amino acid residues (relative molecular mass 57,578 Da) (figure 2a). This ORF is interrupted by an intron of 57 bp whose splicing regulatory sequences are similar to those reported for *Neurospora* genes (Bruchez *et al.* 1993b). Throughout its length the predicted protein shows similarity (54% identity) to *Neurospora* sterol C-14 reductase (figure 2b). The intron is located at the same position relative to the amino acid sequence as the intron in the *Neurospora* gene. No significant gaps were found in the alignment of the *Neurospora* and *Septoria* sequences. This argued against a hypothesis that the nucleotides for residues 406–437 of the *Neurospora* sterol C-14 reductase might, in fact, represent an additional intron (Papavinasasundaram and Kasbekar 1994).

We found *cis* elements used for transcription and translation by *Neurospora*, and presumably also by *Septoria*, immediately upstream of the ORF (figure 2a). The sequence TCATCAGC was found, which matches the consensus TCATCANC for transcription initiation in *Neurospora* (Bruchez *et al.* 1993b). An equivalent sequence is lacking in the *Neurospora* sterol reductase gene. The predicted methionine start codon is embedded in the sequence GACA ATG GC which has a reasonably good match with the consensus for initiation of translation of *Neurospora* mRNA (CA(A/C)(A/C/G)ATGGC) (Bruchez *et al.* 1993a). The upstream region does not reveal any putative CAAT or TATA boxes, but this is true for many fungal genes, including the β_2-tomatinase gene of *Septoria* (Sandrock *et al.* 1995). The sequence AATAT is found 13 bases downstream of the stop codon and might represent the polyadenylation signal sequence. On the basis of the sequence conservation and ability to complement a *Neurospora erg-3* mutation we designate this gene *Septoria erg-3*.

The primary transformants obtained following transformation of spheroplasts of the *N. crassa erg-3 mat-a* strain with the cosmid 2-1 or the plasmid pS12-1 initially showed complementation of the *erg-3* mutant phenotype but they reverted to the pisatin-sensitive and tomatine-resistant mutant phenotype within a couple of vegetative transfers. *Neurospora* spheroplasts are frequently multinucleate and transformation with exogenous DNA often results in heterokaryons in which only one nucleus contains the transforming DNA (Pandit and Russo 1992; Grotelueschen and Metzenberg 1995; Miao *et al.* 1995). It is possible that the instability of the complementation phenotype reflected rapid loss of transformed nuclei in cultures derived from the heterokaryotic transformants. An alternative possibility is that the reversion might be due to quelling, a transgene-induced gene silencing process, which has been best studied in the *al-1* locus (Cogoni *et al.* 1996). These two possibilities are not mutually exclusive.

To rescue the *Septoria erg-3* gene in a homokaryotic background we crossed two independently obtained transformants with an *erg-3 mat-A* strain. Ordinarily a cross between *erg-3 mat-a* and *erg-3 mat-A* strains is infertile because the *erg-3* mutation confers a female-sterile phenotype (Perkins *et al.* 1982), but complementation by the *Septoria* transgene now allowed the transformed parent to serve as the female. Both crosses were poorly fertile and displayed the 'barren' phenotype resembling that reported for crosses involving segmental aneuploid strains. The poor fertility of the crosses might reflect the fact that the transformed parents had already reverted to a large extent. Nevertheless we obtained a few perithecia and viable ascospores from both crosses and identified segregants in which the transgenic nucleus had been rescued in a homokaryotic state.

Conclusions

We have cloned the sterol C-14 reductase gene of *Septoria lycopersici* and determined that the encoded protein has 54% amino acid sequence identity with its *Neurospora* homologue. The *Septoria* gene could complement the pisatin-sensitive, tomatine-resistant and female-sterile phenotypes of a *Neurospora erg-3* mutant. The experimental approach described here has also been used in our laboratory to clone the sterol C-14 reductase genes of *Nectria haematococca* MP VI and *Ascobolus immersus* (D.P. Kasbekar and H.D. VanEtten, unpublished results; D.P. Kasbekar and G. Faugeron, unpublished results).

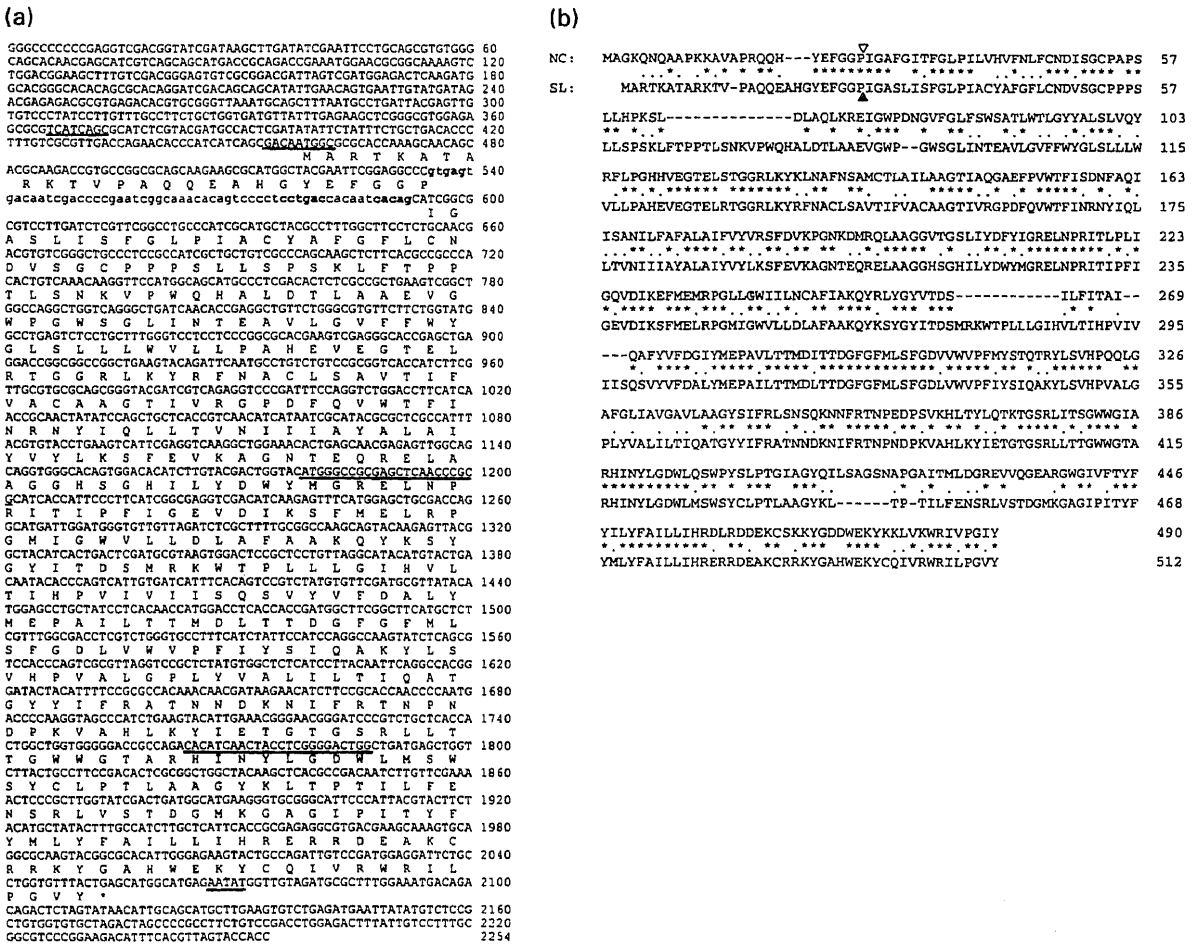

Figure 2. (a) Sequence of the *S. lycopersici erg-3* gene (EMBL nucleotide sequence database accession number Y14389). The deduced amino acid sequence starting with the first methionine is displayed in one-letter code below the middle bases of the codons. The stop codon (TGA) is indicated by an asterisk (*). The intron (nucleotides 535–592) is indicated by lower-case letters; the donor, acceptor and lariat sequences are in bold letters. Starting from the 5′ end the following sequences have been underlined: the putative transcription initiation signal, the putative translation initiation signal, sequence recognized by the forward primer, sequence recognized by the reverse primer, and the putative polyadenylation signal. (b) Alignment of the predicted amino acid sequences of the sterol C-14 reductases of *N. crassa* (NC) and *S. lycopersici* (SL). Asterisks indicate conserved amino acid residues, dots indicate similar residues, and dashes indicate gaps introduced to maximize the alignment. The arrowheads indicate intron locations.

Acknowledgements

We thank Bhavani Prasanna for technical assistance, Narender for media and glassware, and Nagesh for the primers. The cosmid clone 2-1 was isolated by D.P.K. and R.W.S. in the laboratory of Hans VanEtten, University of Arizona. D.P.K.'s visit to VanEtten's laboratory was funded by a Rockefeller Foundation Biotechnology Career Fellowship. This research was supported in part by a grant to D.P.K. from the Department of Science and Technology, Government of India.

References

Arneson P. A. and Durbin R. D. 1967 Hydrolysis of tomatine by *Septoria lycopersici*: A detoxification mechanism. *Phytopathology* **57**, 1358–1360.

Arneson P. A. and Durbin R. D. 1968a The sensitivity of fungi to α-tomatine. *Phytopathology* **58**, 536–537.

Arneson P. A. and Durbin R. D. 1968b Studies on the mode of action of tomatine as a fungitoxic agent. *Plant Physiol.* **43**, 683–686.

Bruchez J. J. P., Eberle J. and Russo V. E. A. 1993a Regulatory sequences involved in the translation of *Neurospora crassa* mRNA: Kozak sequences and stop codons. *Fungal Genet. Newsl.* **40**, 85–88.

Bruchez J. J. P., Eberle J. and Russo V. E. A. 1993b Regulatory sequences in the transcription of *Neurospora crassa* genes: CAAT box, TATA box, introns, poly(A) tail formation sequences. *Fungal Genet. Newsl.* **40**, 89–96.

Cogoni C., Irelan J. T., Schumacher M., Schmidhauser T. J., Selker E. U. and Macino G. 1996 Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA : DNA interaction or DNA methylation. *EMBO J.* **15**, 3153–3163.

Davis R. H. and de Serres J. F. 1970 Genetic and microbiological research techniques for *Neurospora crassa*. *Meth. Enzymol.* **A17**, 79–143.

Ford J. E., McCance D. J. and Drysdale R. B. 1977 The detoxification of α-tomatine by *Fusarium oxysporum* f. sp. *lycopersici*. *Phytochemistry* **16**, 545–546.

Grotelueschen J. and Metzenberg R. L. 1995 Some property of the nucleus determines the competence of *Neurospora crassa* for transformation. *Genetics* **139**, 1545–1551.

Lai M. H., Bard M., Pierson C. A., Alexander J. F., Goebl M., Carter G. T. and Kirsch D. R. 1994 The identification of a gene family in the *Saccharomyces cerevisiae* ergosterol biosynthesis pathway. *Gene* **140**, 41–49.

Lorenz R. T. and Parks L. W. 1992 Cloning, sequencing and disruption of the gene encoding sterol C-14 reductase in *Saccharomyces cerevisiae*. *DNA Cell Biol.* **11**, 685–692.

Maniatis T., Fritsch E. F. and Sambrook J. 1982 *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Marcireau C., Guyonnet D. and Karst F. 1992 Construction and growth properties of a yeast strain defective in sterol 14-reductase. *Curr. Genet.* **22**, 267–272.

Miao V. P. W., Rountree M. R. and Selker E. U. 1995 Ectopic integration of transforming DNA is rare among *Neurospora* transformants selected for gene replacement. *Genetics* **139**, 1533–1544.

Pandit N. N. and Russo V. E. A. 1992 Reversible inactivation of a foreign gene, *hph*, during the asexual cycle in *Neurospora crassa* transformants. *Mol. Gen. Genet.* **234**, 412–422.

Papavinasasundaram K. G. and Kasbekar D. P. 1993 Pisatin resistance in *Dictyostelium discoideum* and *Neurospora crassa*: comparison of mutant phenotypes. *J. Gen. Microbiol.* **139**, 3035–3041.

Papavinasasundaram K. G. and Kasbekar D. P. 1994 The *Neurospora crassa erg-3* gene encodes a protein with sequence homology to both yeast sterol C-14 reductase and chicken lamin B receptor. *J. Genet.* **73**, 33-41.

Pegg G. F. and Woodward S. 1986 Synthesis and metabolism of α-tomatine in tomato isolines in relation to resistance to *Verticillium albo-atrum*. *Physiol. Plant Pathol.* **28**, 187–201.

Perkins D. D., Radford A., Newmeyer D. and Bjorkman M. 1982 Chromosomal loci of *Neurospora crassa*. *Microbiol. Rev.* **46**, 426–570.

Sandrock R. W., DellaPenna D. and VanEtten H. D. 1995 Purification and characterization of β_2-tomatinase, an enzyme involved in the degradation of α-tomatine, and isolation of gene encoding β_2-tomatinase from *Septoria lycopersici*. *Mol. Plant-Microb. Interact.* **8**, 960–970.

Sanger F., Nicklen S. and Coulson A. R. 1977 DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Schlosser E. 1975 Role of saponins in antifungal resistance IV. Tomatine-dependent development of species of *Alternaria* on tomato fruits. *Acta Phytopathol.* **10**, 77–87.

Sengupta S., Bhavani Prasanna T. and Kasbekar D. P. 1995 Sterol 14,15 reductase (*erg-3*) mutants switch the phenotype of *Neurospora crassa* from sensitivity to the tomato saponin α-tomatine to sensitivity to the pea phytoalexin pisatin. *Fungal Genet. Newsl.* **42**, 71–72.

Smith S. 1995 Cloning and sequence analysis of an *ERG24* homolog from *Schizosaccharomyces pombe*. *Gene* **155**, 139–140.

Sweigard J. and VanEtten H. D. 1987 Reduction in pisatin sensitivity of *Aphanomyces euteiches* by polar lipid extracts. *Phytopathology* **77**, 771–775.

Verhoeff K. and Liem J. I. 1975 Toxicity of tomatine to *Botrytis cinerea* in relation to latency. *Phytopathol. Z.* **82**, 333–338.

Received 27 May 1998; in revised form 19 September 1998