View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Rothamsted Repository

ELSEVIER

FEMS Microbiology Letters 196 (2001) 183–187

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Cloning of the *CYP51* gene from the eyespot pathogen *Tapesia yallundae* indicates that resistance to the DMI fungicide prochloraz is not related to sequence changes in the gene encoding the target site enzyme

Henry M. Wood [a], Matthew J. Dickinson [a], John A. Lucas [b], Paul S. Dyer [a,*]

[a] *School of Biological Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK*
[b] *IACR-Long Ashton Research Station, University of Bristol, Long Ashton, Bristol BS18 9AF, UK*

Received 20 December 2000; received in revised form 24 January 2001; accepted 24 January 2001

Abstract

Resistance to sterol 14α-demethylase inhibitor (DMI) fungicides has been correlated with mutations in the *CYP51* gene encoding the target enzyme eburicol 14α-demethylase. *CYP51* was isolated from the eyespot pathogen *Tapesia yallundae* revealing a predicted 526-amino acid product exhibiting homology to other fungal CYP51s. *CYP51* was sequenced from four field isolates sensitive or resistant to the DMI fungicide prochloraz and partially sequenced from two further isolates and eight progeny from a cross between prochloraz-sensitive and -resistant parents. Two alleles of the gene were detected termed *CYP51-1* and *CYP51-2*. No correlation was found between sequence change and fungicide sensitivity. Therefore prochloraz resistance involved a mechanism other than mutation in the target site gene. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Fungicide resistance; CYP51; Prochloraz; *Tapesia yallundae*

1. Introduction

Sterol 14α-demethylase inhibitor (DMI) fungicides represent the largest and most important group of modern anti-fungal compounds. They have remained highly effective despite many years of use and their single-site mode of action [1]. However, decreased sensitivity and field resistance to certain DMIs has been reported in at least 13 species of plant pathogen [2].

Field isolates of the cereal eyespot pathogen *Tapesia yallundae* with reduced sensitivity to the imidazole DMI fungicide prochloraz (1-[*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]carbamoyl]-imidazole) have recently been detected in France and New Zealand [3,4]. Understanding the molecular genetic basis of resistance should aid in developing improved strategies for managing eyespot disease and predicting models of resistance to prochloraz in other pathogens [5]. Sexual crosses between field isolates of *T. yallundae* with varying levels of resistance to prochloraz, together with appropriate back and F_1 crosses, have indicated that resistance is conferred by a single major gene [4]. There was also evidence of polygenic components increasing levels of resistance in the most resistant isolates.

Given that DMI fungicides inhibit the enzyme eburicol 14α-demethylase in filamentous fungi, studies of DMI resistance have primarily investigated *CYP51*, the gene encoding this enzyme. Point mutations at amino acid residue 136 (in a highly conserved region thought to be responsible for substrate recognition) have been correlated with DMI resistance in field isolates of *Erysiphe graminis* and *Uncinula necator* [6–8]. Laboratory-generated mutants of *Candida albicans* and strains of *Penicillium italicum* resistant to DMIs also showed changes in this and two other regions [9–12]. Alteration(s) in the *CYP51* gene was therefore seen as a likely mechanism of prochloraz resistance in *T. yallundae*.

This study describes the isolation and sequencing of *CYP51* with comparisons made between sequences from field isolates sensitive and resistant to prochloraz. Progeny from a cross between sensitive and resistant parents were also analysed to determine whether differences in gene se-

* Corresponding author. Tel.: +44 (115) 9513203; Fax: +44 (115) 9513251; E-mail: paul.dyer@nottingham.ac.uk

0378-1097/01/$20.00 © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0378-1097(01)00066-0

Downloaded from https://academic.oup.com/femsle/article-abstract/196/2/183/720362 by Periodicals Assistant - Library user on 23 December 2019

quence segregated with resistance. Finally, the gene copy number within sensitive and resistant isolates was investigated as a possible basis for increased fungicide resistance.

2. Materials and methods

2.1. Fungal isolates, media and DNA extraction

Field isolates of *T. yallundae* with differing levels of resistance to prochloraz (Table 1) were grown as previously described [4]. Eight progeny from a cross between 22-433 and PR11 [4] were also used: 37-53-4, 37-53-61, 37-53-101, 37-53-118 ($IC_{50} \leq 0.03$ mg l^{-1}, i.e. sensitive) and 37-53-34, 37-53-38, 37-53-44, 37-53-65 (IC_{50} 0.3–0.6 mg l^{-1}, i.e. resistant). Cultures for DNA production were grown as described [13] and DNA extracted using a DNeasy plant mini kit (Qiagen).

2.2. PCR of *CYP51* with degenerate primers

The amino acid sequences of published *CYP51* genes from the filamentous fungi *P. italicum* (GenBank accession number AFZ49750 [14]), *Ustilago maydis* (AFZ48164 [15]), *U. necator* (AFU72657 [6]) and *E. graminis* (AFO52515 [8]) were aligned and degenerate primers F2 (5′-[CT]TIACIACICCIGTITT[CT]GG-3′) and R2 (CCIGCCATIA[AG]IA[AG]IG[CT]IATCATCAT) designed to anneal to regions of high homology. These were used in 50 μl reactions containing 5 μl 10× buffer, 150 pmol each of F2 and R2, 200 μM dNTPs, 1 U Sigma Jumpstart polymerase and approx. 40 ng 22-432 genomic DNA. Cycle conditions were 94°C for 5 min; 45 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min 45 s; 72°C for 5 min. An appropriately sized band was excised and purified using a Qiaex II gel extraction kit (Qiagen) prior to sequencing.

Table 1
Characteristics of field isolates used in sequencing of *CYP51* from *T. yallundae*

Strain	Source	IC_{50} (mg l^{-1})[a]	Phenotype[b]
22-432	Wheat, Cambs., UK	≤0.03	Prc-S
22-433	Wheat, Beds., UK	≤0.03	Prc-S
22-445	Wheat, northern France	≤0.03	Prc-S
PR1	Wheat, northern France	1.0	Prc-MR
PR11	Wheat, northern France	0.5	Prc-LR
11-3-18	Wheat, Southland, New Zealand	2.4	Prc-HR

[a] IC_{50} value, prochloraz concentration required to inhibit mycelial growth by 50%.
[b] Prochloraz sensitivity/resistance. Prc-S indicates sensitive ($IC_{50} < 0.05$ mg l^{-1}); Prc-LR indicates low resistance ($IC_{50} = 0.2$–0.6 mg l^{-1}); Prc-MR indicates medium resistance ($IC_{50} = 0.7$–1.5 mg l^{-1}); Prc-HR indicates high resistance ($IC_{50} = 1.6$–5.0 mg l^{-1}).

2.3. Library screening, PCR and sequencing of *CYP51* from various isolates

A ^{32}P-labelled probe was made from the *CYP51* PCR product and hybridised at 65°C with plaque lifts of a Lambda FIX II library of 22-432 bound to Hybond-N membranes in three rounds of library screening (Amersham). Positive plaques were selected by PCR according to product formation with two specific primers, WK13 (CGGTTATGAGGTAGAGGAGC) and WK15 (AAGATTGGACTCACCACTGACG), derived from the sequenced PCR product. Further PCR was performed with 1 μl phage suspensions using Expand High Fidelity or Expand Long Template PCR systems (Boehringer Mannheim) with combinations of T3, T7, WK13 and WK15 primers according to the manufacturer. Resulting bands were gel-purified and sequenced. New primers were designed to allow double strand chromosome walking through the whole region of the putative *CYP51* gene.

Further primers flanking the *CYP51* gene were designed (ENDF, GGCACGAGCCAATCACACGGAGGC; and ENDR, GCTATCCCACCCCAAAGACGC) and used to amplify the complete gene from field isolates PR11, 22-433 and 11-3-18 with an annealing temperature of 55°C. Resultant products were gel-purified and sequenced. Later analysis identified differences between sensitive and resistant isolates upstream of, and at the 5′ end of the putative *CYP51* gene. This region was therefore sequenced from PR1, 22-445 and eight progeny from a cross between 22-433 and PR11.

Finally, Southern analysis was performed using standard protocols [16] to determine gene copy number. Genomic DNA from 22-433 (Prc-S), PR11 (Prc-LR), and 11-3-18 (Prc-HR) was digested using *Bam*HI, *Eco*RI and *Eco*RV and probed using a 1019-bp fragment of the *CYP51* gene.

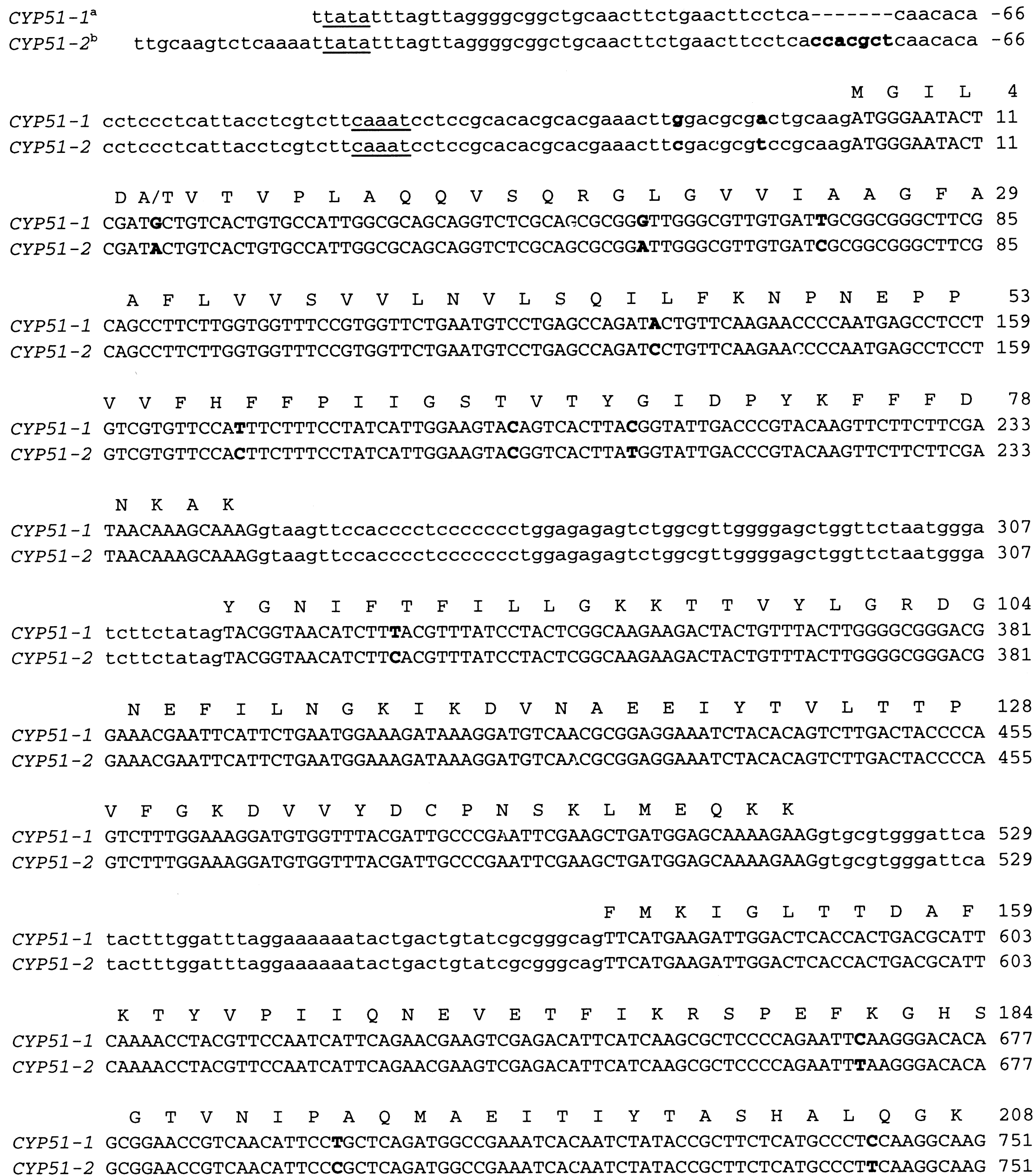
3. Results and discussion

The *CYP51* gene was sequenced from isolates of *T. yallundae* both sensitive and resistant to the DMI fungicide prochloraz in order to determine whether resistance was correlated with differences in sequence as reported elsewhere [7–9,11,12].

3.1. Isolation and sequencing of *CYP51*

PCR with primers F2 and R2 yielded a 608-bp product which was used as a probe to obtain a clone containing the putative *CYP51* gene from a genomic library of *T. yallundae*. The *CYP51* sequence was assembled from overlapping 3-kb and 12-kb fragments amplified using primers T7 and WK15 or T3 and WK13 respectively. The resulting gene (GenBank AF276660) was 1708 bp in

Downloaded from https://academic.oup.com/femsle/article-abstract/196/2/183/720362 by Periodicals Assistant - Library user on 23 December 2019



[a]Allele present in isolates 22-432, 22-433, 37-53-4, 37-53-34, 37-53-44, 37-53-61.

[b]Allele present in isolates 22-445, PR1, PR11, 11-3-18, 37-53-28, 37-53-65, 37-53-101, 37-53-118.

Fig. 1. Nucleotide sequences (upstream region and first 751 bp of open reading frame) of two allelic forms *CYP51-1* and *CYP51-2* in *T. yallundae* (GenBank accession numbers AF276660 and AF276661 respectively). Coding sequence in upper case with corresponding amino acids above, intervening sequences are indicated in lower case. Nucleotide differences (including deletions) between the two forms are shown in bold whilst putative promoter elements are underlined.

length, including two putative introns of 70 and 55 bp, and encoded a predicted 526-amino acid polypeptide. The amino acid sequence exhibited 68, 63 and 55% homology to *CYP51*s from the filamentous fungi *U. necator*, *E. graminis* [6,8] and *P. italicum* [14] respectively. Introns in the *T. yallundae* sequence were present at the same locations in other *CYP51*s, and six conserved regions were evident [6]. This provided strong evidence that the cloned gene was indeed *CYP51* since cytochrome P450-dependent enzymes (e.g. eburicol 14α-demethylase) which exhibit over 55% similarity are classified in the same functional gene subfamily [17].

Downloaded from https://academic.oup.com/femsle/article-abstract/196/2/183/720362 by Periodicals Assistant - Library user on 23 December 2019

3.2. Comparison of *CYP51* from isolates with different sensitivities to prochloraz

CYP51 was completely sequenced from 22-433, PR11 and 11-3-18 (GenBank AF276660, AF276661 and AF276662 respectively). The sensitive isolate 22-433 had an identical sequence to 22-432 (Prc-S) derived from the genomic library. In contrast, differences were evident with the resistant isolates PR11 and 11-3-18, which had identical sequences. A substitution (threonine for alanine) was present at residue 6, and there were 10 further silent substitutions, all within the first 750 bp (Fig. 1). Differences were also evident in the 125-bp sequence upstream of the start codon, with two substitutions and a deletion of seven nucleotides. Thus, two allelic forms of the gene, termed *CYP51-1* and *CYP51-2* (from 22-432 and 22-433, or PR11 and 11-3-18 respectively) appeared to be present. These alleles were also detected in isolates 22-445 and PR1, and the 37-53 sexual progeny. However, no correlation was evident between change in sequence and increased resistance to prochloraz with sensitive isolate 22-445 having the identical *CYP51-2* sequence as the resistant isolates PR1, PR11 and 11-3-18 in the region sequenced (Fig. 1). Furthermore there was no correlation between nucleotide sequence and alteration in fungicide sensitivity in the 37-53 sexual progeny with resistant and sensitive progeny exhibiting the same *CYP51* sequence (Fig. 1). Thus, resistance to prochloraz in *T. yallundae* is not due to a mutation in the gene encoding the target enzyme, at least for the isolates examined in this study. This is an important finding because it contrasts with results for *U. necator*, *E. graminis* [7,8], *P. italicum*, [12] and *C. albicans* [9–11] in which resistance to DMI fungicides is linked to point mutations in *CYP51*. In particular, no point mutation was evident at codon 136 in *T. yallundae*, unlike the other species. However, our results do not preclude the possibility that prochloraz resistance in other species or field isolates of *T. yallundae* may arise from mutations in *CYP51*. One other *CYP51* sequence from *T. yallundae* became available at the end of the study period (AF208658), differing from *CYP51-1* only at amino acid position 13 (threonine instead of glutamine).

Hybridisation results revealed no clear differences in signal intensity between 22-433, PR11 and 11-3-18 (results not shown). All appeared to have a single copy of *CYP51*, although restriction pattern polymorphisms were evident. Thus, increased copy number of *CYP51* did not provide a mechanism for increased fungicide resistance, although this has been reported elsewhere [16].

Previous work has established that prochloraz resistance in *T. yallundae* is primarily conferred by a single major gene for resistance [4]. Assumptions that resistance is due to mutation(s) in the gene encoding the target protein, as may be hypothesised from comparable DMIs, are invalid. The mechanism of resistance may alternatively involve an alteration in a gene upstream in the sterol biosynthesis pathway, as reported for *Saccharomyces cerevisiae* [18] or expression of a gene conferring ability to detoxify prochloraz as in *Rhizoctonia solani* [19]. Genes involved with multidrug resistance (notably ABC transporter genes) may also confer fungicide resistance [20–22]. However, preliminary screening suggests that DMI fungicide resistance in *T. yallundae* is not associated with resistance to fungicides of different chemical classes (H.M. Wood, unpublished results). Future work to identify the genetic basis of prochloraz resistance in *T. yallundae* will now involve the identification of molecular marker(s) [23] linked to resistance genes and examination of sterol biosynthetic pathways.

Acknowledgements

Financial support from the Lawes Trust, UK and the Biotechnology and Biological Sciences Research Council, UK is gratefully acknowledged.

References

[1] Russell, P.E. (1995) Fungicide resistance: occurrence and management. J. Agric. Sci. 124, 677–680.

[2] de Waard, M.A. (1994) Resistance to fungicides which inhibit sterol 14α-demethylation, a historical perspective. In: Fungicide Resistance (Heaney, S., Slawson, D., Hollomon, D.W., Smith, M., Russell, P.E. and Parry, D.W., Eds.), pp. 1–10. BCPC Monograph no. 60, British Crop Protection Council, Farnham.

[3] Leroux, P. and Gredt, G. (1997) Evolution of fungicide resistance in the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* in France. Pestic. Sci. 51, 321–327.

[4] Dyer, P.S., Hansen, J., Delaney, A. and Lucas, J.A. (2000) Genetic control of resistance to the DMI fungicide prochloraz in the cereal eyespot pathogen *Tapesia yallundae*. Appl. Environ. Microbiol. 66, 4599–4604.

[5] Georgopoulos, S.G. and Skylakakis, G. (1986) Genetic variation in the fungi and the problem of fungicide resistance. Crop Protect. 5, 299–305.

[6] Délye, C., Laigret, F. and Corio-Costet, M.F. (1997) Cloning and sequence analysis of the eburicol 14α-demethylase gene of the obligate biotrophic powdery mildew fungus. Gene 195, 29–33.

[7] Délye, C., Laigret, F. and Corio-Costet, M.F. (1997) A mutation in the 14α-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl. Environ. Microbiol. 63, 2966–2970.

[8] Délye, C., Bousset, L. and Corio-Costet, M.F. (1998) PCR cloning and detection of point mutations in the eburicol 14α-demethylase (CYP51) gene from *Erysiphe graminis* f.sp. *hordei*, a 'recalcitrant' fungus. Curr. Genet. 34, 399–403.

[9] Löffler, J., Kelly, S.L., Hebart, H., Schumacher, U., Lass-Flörl, C. and Einsele, H. (1997) Molecular analysis of *cyp51* from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol. Lett. 151, 263–268.

[10] Marichal, P., Koymans, L., Willemsens, S., Bellens, D., Verhasselt, P., Luyten, W., Borgers, M., Ramaekers, F.C.S., Odds, F.C. and Van den Bossche, H. (1999) Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology 145, 2701–2713.

[11] Sanglard, D., Ischer, F., Koymans, L. and Bille, J. (1998) Amino acid

Downloaded from https://academic.oup.com/femsle/article-abstract/196/2/183/720362 by Periodicals Assistant - Library user on 23 December 2019

substitutions in the cytochrome P450 lanaosterol 14 alpha demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. 42, 241–253.

[12] de Waard, M.A. (1996) Molecular genetics of resistance in fungi to azole fungicides. Am. Chem. Soc. Symp. Series 645, 62–71.

[13] Dyer, P.S., Nicholson, P., Rezanoor, H.N., Lucas, J.A. and Peberdy, J.F. (1993) Two-allele heterothallism in *Tapesia yallundae*, the teleomorph of the cereal eyespot pathogen *Pseudocercosporella herpotrichoides*. Physiol. Mol. Plant Pathol. 43, 403–414.

[14] van Nistelroy, J.G.M., van den Brink, J.M., van Kam, J.A.L., van Gorcom, R.F.M. and de Waard, M.A. (1996) Isolation and molecular characterisation of the gene encoding eburicol 14α-demethylase (CYP51) from *Penicillium italicum*. Mol. Gen. Genet. 250, 725–733.

[15] Hargreaves, J.A. and Keon, J.P.R. (1996) Isolation of an *Ustilago maydis* ERG11 gene and its expression in a mutant deficient in sterol 14α-demethylase activity. FEMS Microbiol. Lett. 139, 203–207.

[16] Dyson, N.J. (1991) Immobilisation of nucleic acids and hybridisation analysis. In: Essential Molecular Biology – A Practical Approach (Brown, T.A., Ed.), Vol. II, pp. 111–156. IRL/Oxford University Press, Oxford.

[17] Gonzales, F.J. (1993) Cytochrome P450 evolution and nomenclature. In: Cytochrome P450 (Schenhman, J.B. and Greim, H., Eds.), pp. 211–219. Springer-Verlag, Berlin.

[18] Watson, P.F., Rose, M.E., Ellis, S.W., England, H. and Kelly, S.L. (1989) Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. Biochem. Biophys. Res. Commun. 164, 1170–1175.

[19] Kapteyn, J.C., Pillmoor, J.B. and de Waard, M.A. (1992) Biochemical mechanisms involved in selective fungitoxicity of two sterol 14α-demethylation inhibitors, prochloraz and quinconazole: accumulation and metabolism studies. Pestic. Sci. 36, 85–93.

[20] de Waard, M.A. (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic. Sci. 51, 271–275.

[21] Nakaune, R., Adachi, K., Nawata, O., Tomiyama, M., Akutsu, K. and Hibi, Y. (1998) A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Appl. Environ. Microbiol. 64, 3983–3988.

[22] Del Sorbo, G., Schoonbee, H-J. and de Waard, M.A. (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fund. Genet. Biol. 30, 1–15.

[23] Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Homes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.

Downloaded from https://academic.oup.com/femsle/article-abstract/196/2/183/720362 by Periodicals Assistant - Library user on 23 December 2019