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Abstract

Evolved resistance to fungicides is a major problem limiting our ability to control agricultural, medical and veterinary pathogens and is frequently associated with substitutions in the amino acid sequence of the target protein. The convention for describing amino-acid substitutions is to cite the wild type amino acid, the codon number and the new amino acid, using the one letter amino acid code. It has frequently been observed that orthologous amino acid mutations have been selected in different species by fungicides from the same mode of action class, but the amino acids have different numbers. These differences in numbering arise from the different lengths of the proteins in each species. The purpose of the current paper is to propose a system for unifying the labelling of amino acids in fungicide target proteins. To do this we have produced alignments between fungicide target proteins of relevant species fitted to a well-studied "archetype" species. Orthologous amino acids in all species are then assigned numerical "labels" based on the position of the amino acid in the archetype protein.

Background

Evolved resistance to fungicides is a major problem limiting our ability to control agricultural, medical and veterinary pathogens [1, 2]. Research over the last 30 years has often defined the mechanism conferring reduced sensitivity to the fungicide. Many cases of resistance have been ascribed to the activity of efflux pumps [3], or to over-expression of target genes [4] but the majority are due, at least partly, to substitutions (or indels) in the amino acid sequence of the target protein.

The convention for describing amino acid substitutions is to cite the wild type amino acid, the codon number and the new amino acid, using the one letter amino acid code (see [5] box 6.1 page 138 for a description of the system). A well-known example is the alanine (A) for glycine (G) substitution in the cytochrome b gene at position 143 conferring resistance to strobilurin fungicides, referred to as G143A [6]. Further alterations can be amino acid deletions designated with a **Δ** and insertions with an "ins".

Target site amino acid substitutions have been described for seven fungicide groups (named here according to the FRAC convention, [7]) and their target proteins. These are C3 and cytochrome b (Cytb) (Table 1); G1 and two sterol C14-demethylases (paralogs Cyp51A and Cyp51B), (Tables 2 and 3); B1/B2 and *b*-tubulin, (Table 4); C2 and three of the sub-units of the succinate dehydrogenase complex (SdhB, SdhC and SdhD) (Table 5, 6 and 7); H5 and cellulose synthase A3 (CesA3) (Table 8); E3 and the Os1 family histidine kinase (Os-1, synonyms Bos1, BcOS1, Daf1 and NIK1[8])(Table 9), G3 and the 3-keto reductase (Erg27). Where more than one species has been studied, it has frequently been observed that orthologous amino acid mutations have been associated with resistance to fungicides with the same mode of action.

In cases where the proteins are strongly conserved between species, the mutations have identical numbers. For example, the orthologous Cytb G143A mutation has been found in 22

species (Table 1). However in other cases orthologous mutations have different numbers – e.g. Cyp51B amino acid Y137 in *Zymoseptoria tritici* is orthologous to amino acids numbered from 132 to 145 in different species (Table 3). Similarly, SdhB amino acid H277 in *Pyrenophora teres* is orthologous to amino acids numbered from 249 to 278 (Table 5). These differences in numbers creates unnecessary confusion and obscures the relationships between mutations in different species.

The Proposal

These differences in numbering arise from the different lengths of the fungicide target protein in each species. The purpose of the current paper is to propose a system for unifying the labelling of mutant amino acids in fungicide target proteins. We propose that orthologous amino acids (i.e. ones presumed to be descended from the same amino acid in the common ancestor of these species) are given the same number in all species regardless of the actual position. The advantages of a unified system is that it would be easier to memorise common changes, to determine whether the changes were novel or were repetitions of what has already been seen in other species and to link changes to particular active ingredients. Orthologous mutations would be assigned the same 'mutation label'.

We distinguish between 'mutation labels' which refers to the orthology between proteins from different species from 'amino acid numbering', which remains the order of the amino acids in each protein in each species. To avoid confusion, we propose that mutation labels should be italicised and mutation numbers in regular lettering.

In several cases, amino acid substitutions have been found in the target protein but have not been definitively associated with any change in sensitivity either *in vitro* or in the field. It may be the mutation underlying the amino acid substitution is a random event and of no obvious relevance. Definitively linking a mutation to a sensitivity change can be technically very demanding. If resistance to the same class of fungicide is linked to mutations affecting orthologous codons in different species, this is strong, if still circumstantial, evidence of the importance of the mutation. Unifying the mutant labelling system will make it much easier to identify important codon changes. This would assist the prioritisation of research aiming to functionally characterise mutations.

Options for producing the alignments

We have produced a set of draft alignments of each target protein for which resistance to multiple species has been reported (Figures 1 to 9) and tables of putatively orthologous amino acids in other species where fungicide resistance has been reported (Tables 1 to 9). The species included in these tables and alignments have been referred to by their European and Mediterranean Plant Protection Organization (EPPO) codes[9] as listed in Table 10.

The alignments for *b*-tubulin and Cytb are essentially co-linear in fungi studied to date and hence there are no changes to be made on the current nomenclature. For the other genes, we have considered four possible methods to generate the alignment. The alignment could be;

1. Fitted to the longest gene in the gene set.
2. Fitted to a strict consensus alignment.
3. Fitted to the gene from the species that is currently the most researched species for the fungicide resistance concerned.
4. Fitted to the gene from the species that was the first species for the fungicide resistance concerned.

The aim is to create a set of alignments that would be stable into the foreseeable future and would invoke the least relabelling of mutations that have already been described and published. We favour method 3 (basing the alignment on the species with the most currently-described resistance mutations), but also taking into account method 1 (using the longer gene) when alternative species are candidates. We propose that Cyp51A is fitted to ASPEFU (*Aspergillus fumigatus)*, Cyp51B and Cytb are fitted to SEPTRI (*Zymoseptoria tritici)*, *b*-tubulin to ASPEND (*Aspergillus nidulans)*, the SDH proteins to PYRNTE (*Pyrenophora teres)*, CesA3 to PHYTIN (*Phytophthora infestans)* and Os-1 to BOTCIN (*Botrytis cinerea).* For Erg27, mutations associated with resistance have currently been described only in BOTCIN and thus we propose this species as the archetype.

The alignments have been summarised and fungicide resistance associated mutations are given in the Tables. By way of example, in Cyp51B the mutation Y136F in ERYSGH would be given the label *Y137F.* In CANDAL the orthologous amino acid is Y132 and has been mutated to both F and H. The Y132H mutation would therefore be given the label *Y137H*. V151 in SEPTRI is clearly demonstrated to be orthologous to I145F in PHAKPA (*Phakopsora pachyrhizi).* This mutation would be labelled *I151F* in PHAKPA and *V151F* in SEPTRI. The other proposed relabellings are listed in Tables 1 to 9.

By examining the species that have amino acids mutations with common labels, we can infer that positions 137, 148, 461, 467, 483 and 524 in Cyp51B are especially important in conferring resistance to triazole fungicides. This is consistent with numerous functional studies [10, 11]. We expect that the alignments should assist the identification of key amino acids in target proteins of newer fungicide classes.

The proposal in practice

The system must also allow for mutations to be discovered in new species. The parameters used to make the alignments are listed in the table legends and can be applied to an alignment between the new species and the archetype. We envisage regularly updating the alignments based on new published knowledge.

A potential problem with the system we propose might occur if an amino acid in a newly described mutant gene corresponded to a gap in the archetype protein's sequence. In such a case, the mutation could be labelled as X50.2Y if it concerned the second extra amino acid after number 50 in the archetype sequence. To our knowledge, no examples of mutations of such poorly conserved amino acids causing resistance have been described, but the possibility remains.

We hope that future studies will refer to the archetype by indicating that the mutation X123Y in the target protein associated with resistance corresponds to the archetype *X145Y* and refer to this paper or a related web page for support.

We commend this scheme to the community and seek comment and support. And we urge Journal editors to encourage authors to use this new system.

Note on the alignments

Amino acid sequences were downloaded from NCBI GenBank and annotated with reported amino acid substitutions[8, 12-14], using Geneious 6.1.8 software (Biomatters**)**. Alignments of sequences were generated using ClustalW[15] algorithm with Blosum scoring matrix, gap opening penalty 10, gap extension penalty 0.5, free end gaps.

The alignments are available as .doc file and as fasta files as supplementary data,

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