Epiheterodendrin in Malting Barley: Molecular Evidence for Cytochrome P450-Mediated Production

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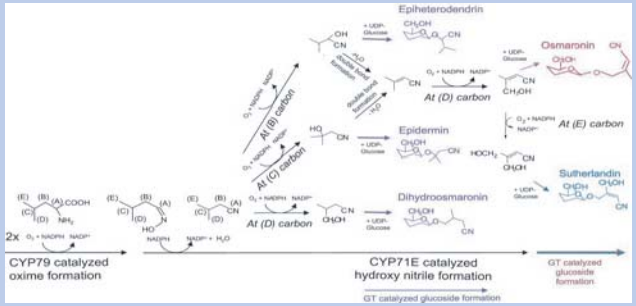
Cyanogenesis in barley: a problem for Scotch Whisky

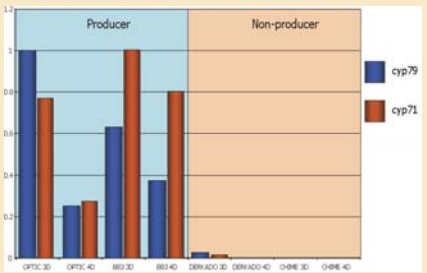
Many plant species produce cyanogenic glucosides which, through release of the breakdown product hydrogen cyanide (HCN), have been implicated in the natural defence response to herbivores and pathogens. These compounds are also believed to have a role in nitrogen storage and osmoregulation. In certain cultivars of barley a leucine-derived cyanogenic glucoside, epiheterodendrin (EPH), is present at high levels in young seedling (malt) leaf tissue. Fermentation of malted barley leads to hydrolysis of EPH, via action of yeast-derived β-glucosidase and subsequent heating during distillation, to form HCN. A reaction within the distillate between HCN and ethanol, in the presence of copper and oxygen, leads to trace but significant levels of the potentially carcinogenic compound ethyl carbamate. Low ethyl carbamate varieties of barley are therefore high priority for the Scotch Whisky industry, indicating a necessary requirement for both detailed biochemical and molecular characterisation of EPH pathways, and development of an unambiguous marker for varietal selection by malting barley breeders.

Epiheterodendrin biosynthetic pathway

Cytochrome P450 enzymes form the largest family of plant proteins, with over 1,000 members identified, catalysing a wide array of both simple and complex reactions, generating a diverse range of natural plant products. EPH has been proposed to be derived from the amino acid leucine through action of two cytochrome P450 enzymes, cyp79 and cyp71E (Nielsen et al., 2002). By screening the HarvEST barley database, which contains around 400,000 expressed sequence tags (ESTs), we identified a small number of barley genes whose derived amino acid sequences showed significant homologies to previously characterised cyp79 and cyp71E proteins. Using these barley homologues we were able isolate and characterise full-length P450 gene sequences and provide molecular evidence for their implication in EPH production.

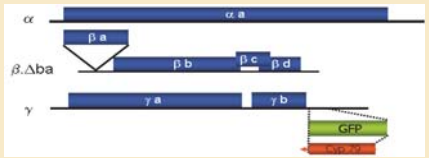


Validation of putative gene function



Expression of cyp79 and cyp71 genes were determined by real-time RT-PCR. Both genes were clearly up-regulated in EPH 'producers' compared to 'non-producers', and showed similar patterns of temporal decrease in expression levels between 3 day and 4 day old seedling leaf material.

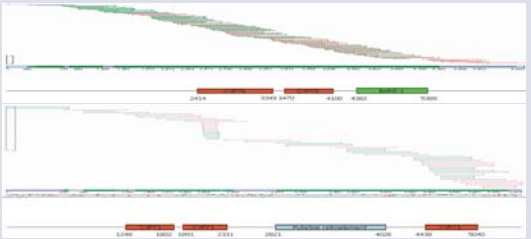
In order to unambiguously link cytochrome P450 genes with EPH production, it is necessary to take a reverse genetics approach. Here, we have utilised a virus-induced gene silencing (VIGS) procedure developed at SCRI, based on a barley stripe mosaic virus (BSMV) vector. Initially, susceptibility to BSMV infection of different 'high-producer' EPH genotypes were determined to select the most responsive barley variety. To monitor the level of gene silencing following the systemic spread of the virus, a fraction of the endogenous phytoene desaturase (pds) gene was cloned in antisense direction (BSMV:PDS), resulting in a well-characterised photobleached phenotype. Sequence derived from the 3' end of cyp79 was cloned in antisense direction into BSMV (BSMV:Cyp79) and the effects of BSMV replication in the plant monitored using BMSV:GFP, which contains a fraction of non-endogenous green fluorescent protein (GFP). Leaf samples have been harvested and are currently being measured for EPH levels in both BSMV:GFP and BSMV:Cyp79 infected plants. We are currently developing quantitative phenotyping procedures (LC-MS) to accurately assess the levels of EPH silencing.



Characterisation of P450 genes from barley

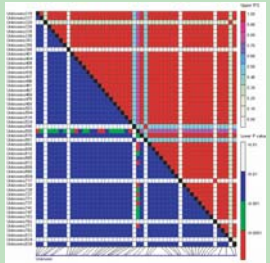
Barley ESTs with homologies to cyp79 and cyp71 were identified, representing partial 3' cDNA sequences, and the corresponding gene fragments were subsequently isolated using PCR. These gene probes were used to screen a barley (cv. Morex) bacterial artificial chromosome (BAC) library to isolate full-length gene sequences. Fingerprinting of the BAC clones, utilising SNaPshot-based fluorescent labeling of restriction-digested fragments, clustered the clones into two distinct groups. Single BAC clones for each gene were subcloned and sequenced, revealing full-length genes encoding cyp79 and cyp71, which contain two and three exons respectively and also indicate the presence of putative retroelement sequences, which are common features of the barley genome.

Using polymorphic barley populations, both genes genetically mapped to the same position, which was also confirmed as the Eph locus on chromosome 1H, first identified by Swanston et al. (1999).



Molecular diversity of EPH-associated P450 genes

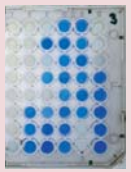
Oligonucleotide primer sets were designed to re-amplify regions of the cyp79 and cyp71 genes in a range of cultivated, landrace and wild barley germplasm. In general, lower levels of polymorphism were detected in the cyp71 gene compared to cyp79. A single nucleotide polymorphism (SNP) haplotype was identified in cyp79 which shows complete association with the EPH phenotype. In a total of 860nt sequenced across cyp79, 45 SNPs were detected, which were organised into 5 different haplotypes, 4 representing 'producers' and only 1 haplotype representing 'non-producers'. Almost all of the SNPs distinguish the two groups, with very little variation within 'producers' and no variation within 'non-producers'.



The majority of SNPs are in linkage disequilibrium (LD) across the cyp79 region in barley, establishing the basis for an association between haplotype and phenotype across this region. Pairwise comparison between SNPs across cyp79 is represented in a LD matrix, where the upper triangle represents LD measured by r^2, and the lower triangle those pairwise comparisons that are significant.

Development of EPH molecular marker

Central to selection of barley varieties with low levels of potential ethyl carbamate generation is the development of a robust unambiguous molecular marker. Previously at SCRI, a simple-sequence repeat (SSR) marker (BMAC213) was generated which showed good, but not complete, association with the EPH phenotype, as determined by the biochemical assay (Swanston et al., 1999). The SNP haplotype identified for the cyp79 gene shows complete (100%) association with the EPH phenotype, clearly distinguishing 'producers' from 'non-producers' and was therefore used as the basis for development of a multiplex PCR-based screen. This assay can therefore be used for efficient, reliable and cost-effective marker-assisted varietal selection in malting barley breeding programmes.



Acknowledgements

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References

Nielsen et al., 2002. Plant Physiology, 129: 1066-1075;
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