# BACKGROUND

QUESTIONS ON THE ORIGIN AND SELECTIVE MAINTENANCE OF ADAPTIVE POLYMORPHISMS.
Characterizing the molecular evolution of adaptive polymorphism requires detailed understand-ing of both the molecular origin of phenotypic variants and the selective forces that maintain them in contemporary populations.  This breadth of knowledge is exceedingly difficult to obtain for a single study system, as it requires a species that is both genetically tractable at the molec-ular level (the traditional realm of model organisms) and also ecologically well characterized, such that the fitness consequences of phenotypic variation are well documented in nature.  The wealth of ecological data on the clover cyanogenesis polymorphism, together with the genetic tractability of this system, allows for the integration of molecular and ecological genetic analyses within this single study system.

WHITE CLOVER AND THE CYANOGENESIS POLYMORPHISM
*Trifolium repens* is an outcrossing perennial legume found in temperate regions worldwide.  A native of Eurasia, it has been widely dispersed as a forage crop, lawn plant and weed, so that it is now extremely common throughout the world.  White clover is an allotetraploid with strictly disomic inheritance (45); the two loci underlying the cyanogenesis polymorphism (see below) are both single-copy genes, present in only one of the two ancestral genomes (45).  The species is easily propagated either vegetatively or by crossing, and it can be genetically transformed (46).
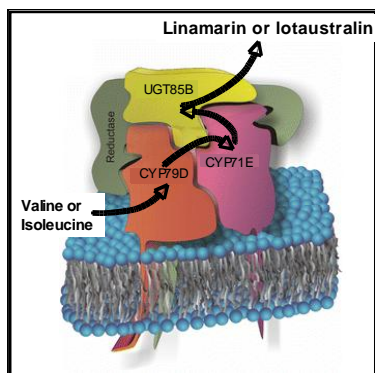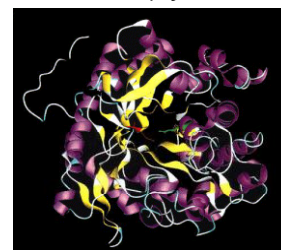


**Fig. 1.**  Cyanogenic glucoside biosynthetic pathway.  Modified from Bak *et al.* (2006). The *Ac/ac* biochemical polymorphism arises through the presence/absence of *CYP79D15*, the gene encoding the first enzyme in the pathway (Olsen *et al.*, 2008).

●**Biochemistry and molecular genetics.**
Cyanogenesis has evolved repeatedly across the plant kingdom and has been shown to function as a chemical defense against herbivores (e.g., 47).  In white clover, cyanogenesis arises through the interaction of two compounds that are separated in intact tissue and brought together with cell rupture: cyanogenic glucosides (cyanide-containing sugars), and their hydrolyzing enzyme linamarase (reviewed in 31).  The cyanogenic glucosides occur in two chemically similar forms (linamarin and lotaustralin) that are synthesized in a single biosynthetic pathway (Fig. 1); these are stored in vacuoles of photosynthetic tissue.  Linamarase (Fig. 2) is present in the cell wall.  Hydrolysis of the cyanogenic glucosides following cell rupture leads to the spontaneous liberation of hydrogen cyanide (HCN), a potent toxin.



**Fig. 2**  White clover linamarase. Modified from Barrett *et al.* (1995).

The clover cyanogenesis polymorphism arises through two independently segregating biochemical polymorphisms for the presence/absence of each of the two required cyanogenic components.  Inheritance of these biochemical polymorphisms follows that of two simple Mendelian genes: *Ac/ac* (presence/absence of cyanogenic glucosides) and *Li/li* (presence/absence of linamarase) (reviewed in 31).  To be cyanogenic a plant

must possess at least one functional (dominant) allele at both genes (*Ac_*, *Li_*); the acyanogenic phenotype can arise either through the absence of cyanogenic glucosides (*acac* genotype), or linamarase (*lili* genotype), or both compounds (*acac*, *lili*). The presence/absence of functional alleles at *Ac* and *Li* can be easily determined through a colorimetric cyanide assay, which I have adapted for high-throughput use in the lab (described in 3). The assay uses 48-well plates to hold leaf tissue and HCN test paper (48), which turns bright blue upon exposure to cyanide (see Fig. 3). For acyanogenic plants, exogenous addition of cyanogenic glucosides or linamarase reveals which cyanogenic component(s) is/are lacking. Heterozygotes at *Ac* and *Li* produce cyanogenic components at levels approximately half that of homozygous dominants (see 31), a difference that is often detectable in the HCN assay (e.g., sample 5 in Fig. 3, a likely *Acac* heterozygote). However, quantitative variation in cyanogenesis is also affected by many other factors (e.g., age of leaf, drought stress, nutrient availability; 41) and it is not a focus of this research.

In recent work, I have determined the genomic DNA sequences corresponding to *Li* and *Ac*, and I have determined the molecular basis of both biochemical polymorphisms. The *Li* gene, encod-ing linamarase, is 3.9 kb in length, comprising 13 exons and 12 introns (3). The *Ac* gene corresponds to the locus *CYP79D15*, which encodes a cytochrome P450 protein catalyzing the first step in cyanogenic glucoside biosynthesis (4; see Fig. 1); *CYP79D15* is 1.7 kb in length and comprises 2 exons and 1 intron (4). The *Li/li* and *Ac/ac* polymorphisms arise through two unlinked gene presence/absence polymorphisms at the loci *Li* and *CYP79D15*, respectively (3,4). Gene presence/absence polymorphisms have been shown to underlie adaptive polymorphisms in a number of other systems (e.g., 12, 39-40, 49).
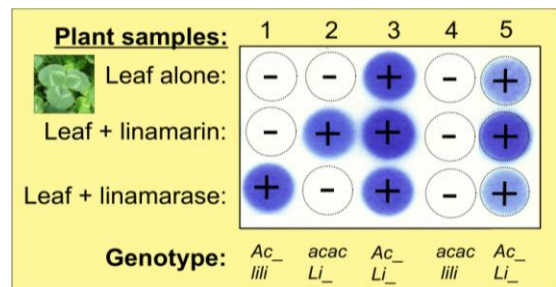


**Fig. 3** Colorimetric HCN Assay. Leaf tissue is frozen to rupture cells, then exposed to Feigl-Anger test paper. Addition of cyanogenic glucosides or linamarase reveals the biochemical basis of acyanogenic phenotypes.