Determination of the genetic basis for phenological diversity in a heterozygous wild-type accession of *Lablab purpureus*

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Abstract
Lablab purpureus (lablab) is among the most diverse domesticated, multipurpose legume species in the world with forage, pulse, vegetable, green manure, ornamental and medicinal uses. There are 258 wild and domesticated accessions currently represented in the Australian Pastures Genebank. Prior to 2015, only two forage cultivars ('Rongai' [1962] and 'Highworth' [1974]) had been successfully released and commercialised in Australia. In 2012 and 2013, evaluation of 17 accessions and genotypes shortlisted from earlier work identified an Ethiopian accession (ILRI 13685) with high forage yields as heterozygous for multiple phenotypic traits (±anthocyanin pigmentation, flower colour, seed colour, hardseededness). Twenty-six spaced plants with anthocyanin pigmentation (the generationally unstable Purple phenotype) were progeny tested through two generations, confirming that 12 were homozygous for pigmentation, purple flowers and black seeds (the dominant phenotype). The progeny of the remaining 14 heterozygous plants of the dominant phenotype closely followed a 3:1 Mendelian ratio with the recessive and generationally stable White phenotype (anthocyanin-free, white flowers and brown seeds). The latter plants always bred true-to-type. Three new cultivars ('LLP-015', 'LLW-016', 'LLW-025') developed from the genetic diversity identified within ILRI 13685 have been registered for Australian Plant Breeder's Rights.

Keywords
lablab, heterozygous, anthocyanin, flower colour, seed colour

Introduction

Lablab purpureus (lablab) is among the most diverse domesticated multipurpose legume species with forage, pulse, vegetable, green manure, ornamental and medicinal uses (Maass et al. 2010; Maass 2016; Cook et al. 2020). Because wild plants are found only in eastern and southern Africa, the species is considered to be of African origin, but is now distributed globally through the tropics with a secondary centre of diversity in the Indian sub-continent. In Australia, lablab has mainly been used as an annual forage legume, though with increasing use as a rotational green manure fallow in sugarcane and vegetable production. Prior to 2015, only two forage cultivars – the brown-seeded 'Rongai' (1962) and the black-seeded 'Highworth' (1974) - had been successfully released and commercialised in Australia (Oram 1990). The most recent estimate of lablab seed sales in Australia put the market at around 600 tonnes per annum (Loch and Boyce 2003), more than 70% of which is 'Rongai' (English 1999).

Currently, there are 258 wild and domesticated accessions of *Lablab purpureus* in the Australian Pastures Genebank (APG) (https://apg.pir.sa.gov.au/gringlobal/), and more than 3000 accessions overall in germplasm collections around the world, albeit with an unknown number of duplicates across collections (Maass et al. 2010). WJ Scattini (personal communication) imported 32 accessions – now in APG - from the International Livestock Research Institute (ILRI) for a forage evaluation trial in 2005. Based on seed colour, 14 accessions (including ILRI 13685, a wild type lablab from the Ethiopian highlands) appeared to comprise mixed genotypes, so were divided into sub-accessions before trialling them. In 2012, the present author initiated further trials on 17 of the most promising accessions and genotypes shortlisted by Scattini. Sowings in both 2012 and 2013 showed inherent instability in the sub-accession of ILRI 13685 with black mottled brown seeds, which predominantly produced anthocyanin-pigmented, purple flowered plants as well as a proportion of anthocyanin-free, white flowered plants comparable to those from the separate brown-seeded sub-accession. This led to the three experiments presented in this paper, which were conducted in 2014 and 2015 specifically to test the hypothesis that ILRI 13685 was heterozygous for multiple linked traits.

Methods

All field experiments were conducted at Birkdale, QLD (Latitude 27°30'S, longitude 153°14'E) on a red volcanic ferrosol (krasnozem) soil. CK55(S) blended fertiliser (N:P:K:S = 12.8:14.2:11.9:6.4) was applied to

each experiment at 313 kg/ha following planting to give 40 kg N, 44 kg P, 37 kg K, and 20 kg S per hectare. Irrigation was applied through drip lines as required to maintain unstressed growth.

Experiment 1
Twenty-seven ILRI 13685 seedlings with anthocyanin pigmentation were germinated in forestry tubes (50 x 50 x 120 mm) and planted in the field as young seedlings spaced 3.0 m apart along a drip irrigation line in September 2013. The plants were allowed to grow unimpeded through to flowering and seeding in May/June 2014. One weak plant was discarded as it failed to produce any viable seeds. Ripe pods were hand-harvested separately from each of the remaining plants and hand threshed to give 26 single-plant seed samples.
A minimum of 40 seeds per parent plant were nicked with a scalpel to remove hardseededness and germinated in forestry tubes to determine their phenotype. The "Purple" phenotype with anthocyanin pigmentation present and the "White" phenotype without anthocyanin pigmentation can be determined definitively in seedlings within the first week after germination, typically within 1-2 days of emergence for strong seedlings. The first indication of anthocyanin pigmentation is a small purple spot in the middle of each petiole on the first unifoliate leaves. This pigmentation quickly spreads through the petioles and leaf veins, and later moves into the main stem (Figure 1).

Figure 1. Comparison of Purple phenotype (Plus Anthocyanin) and White phenotype (Minus Anthocyanin) showing the development of pigmentation initially in the seedling petioles and spreading later to the main stem.

A total of 1211 seedlings were assessed for differences in anthocyanin pigmentation, 40-45 from each of 23 parent plants and 81-85 from each of the remaining three parent plants.

Experiment 2
Seeds from each of the 12 plants designated as homozygous Purple in Experiment 1 were nicked to remove hardseededness and sown in tubes on 5 March 2015. One seedling from each parent plant was planted in the field at 3.0 m spacings on 28 March 2015 and allowed to grow unimpeded through to flowering and seeding in May/June 2015. As in Experiment 1, single-plant seed samples were harvested and progeny tested for anthocyanin pigmentation based on 40-42 seedlings per field plant. A total of 492 seedlings were assessed.

Experiment 3
Seeds from Plant #24 (designated as heterozygous Purple in Experiment 1) were nicked to remove hardseededness and sown in tubes in early April 2015. Ten seedlings were transferred to the field at 3.0 m spacings on 9 May 2015 and allowed to grow unimpeded through to flowering and seeding in May/June 2015. Single-plant seed samples were again harvested and subsequently progeny tested for anthocyanin pigmentation based on 40-60 seedlings per field plant. A total of 506 seedlings were assessed.

Results

In chronological order, the three experiments conducted sought answers to the following questions:

- Is the generational instability shown by the Purple phenotype the result of mixed population of homozygous and heterozygous Purple genotypes;
- Do the homozygous Purple genotypes identified breed true-to-type in subsequent generations; and
- Do the heterozygous Purple genotypes identified continue to produce the mixed population of Purple and the White phenotypes in subsequent generations?

Progeny testing showed 12 of the 26 spaced plants in Experiment 1 to be homozygous Purple (zero White genotypes recorded from a total of 504 seedlings sown) with the remaining 14 genotypes determined as heterozygous Purple, all producing both Purple and White genotypes in the next generation. Overall, the heterozygous group produced 187 White progeny from a total of 707 seedlings, giving a Purple:White phenotypic ratio of 2.78:1.00; when individual ratios were calculated for each of the 14 parent plants (because the numbers of seedling progeny were not equally weighted), the average Purple:White phenotypic ratio was 3.24:1.00. Variation in other attributes was noted across both homozygous and heterozygous groups: the date of first flowering ranged from 8 May to 9 June 2014; the bulk of material produced differed considerably among individual plants; and their growth habit varied from prostrate plants with strong lateral spread to others that were more erect and did not spread as far laterally. The heterozygous Plant #24, for example, grew and spread vigorously to be c. 5 m across by the end of the growing season.

In Experiment 2, 492 seeds from second generation homozygous Purple plants produced only Purple seedlings with zero White genotypes recorded. Five of the 12 genotypes were selected based on their vigorous spreading growth habit and dates of first flowering (31 May - 5 June 2015) and bulked equally to form a new cultivar 'LLP-016' (Loch and Zorin 2016b) (Figure 2). While Experiment 2 included only one second-generation plant from each of the 12 homozygous parents identified in Experiment 1, no White genotypes have been observed subsequently following at least two more generations of seed multiplication.

Experiment 3 confirmed that heterozygous Purple genotypes produce a mixed population of Purple and White phenotypes, thereby perpetuating phenotypic instability between generations. Four of the 10 plants grown from the original Plant #24 were homozygous Purple genotypes (no White plants found in a total of 196 seedling progeny) and six were heterozygous Purple genotypes with 82 Whites recorded from a total of 310 seedlings assessed. For the heterozygous group, the overall Purple:White phenotypic ratio was 2.78:1.00, or 3.08:1.00 when averaged across calculations for each individual plant.

Similar detailed studies of the White phenotype are not warranted as no further segregation has been observed in 4 or more generations of multiplication since 2012. Like the Purples as discussed above, the White genotypes show variation in numerous other morphological and developmental attributes, which led to the release of 'LLW-015' (Loch and Zorin 2016a) and 'LLW-025' (Loch and Zorin 2020) (Figure 2). These two cultivars differ in their date of first flowering, leaflet shape, pod and seed size, and seed colour. Growing trials in 2015, 2016 and 2020 for Plant Breeder's Rights (PBR) registration of 'LLW-015' and 'LLW-025' confirmed that these differences are stable across generations.

Discussion and Conclusions

The data presented are consistent with anthocyanin pigmentation being determined by a single locus with two alleles (*P*,*p*) where *P* is the dominant allele conferring anthocyanin pigmentation and *p* is the recessive allele for the White phenotype. Heterozygous genotypes segregated close to the classical Mendelian phenotypic ratio of 3:1 based on $PP + 2Pp + pp$. The homozygous dominant plants identified (and the homozygous recessive plants) have remained genetically stable for at least four generations, both in their anthocyanin pigmentation and in multiple other traits (flower colour, seed colour, hardseededness) associated with the pigmentation allele.

In trials by WJ Scattini (personal communication, 2005) and DS Loch (unpublished data, 2012 onwards), both homozygous groups from ILRI 13685 have consistently ranked among the lablab lines with the highest forage yields, equal to or better than 'Rongai' and 'Highworth'. They have also shown good drought tolerance (perhaps related to the origin of the parent accession) and resistance to damping-off fungi in recent trials in northern and southern Queensland (2019-21 – DS Loch, unpublished data). The dominant Purple phenotype may offer the prospect of limited short-term perenniality, but would require treatment to reduce hardseededness (c. 50 % in hand-harvested samples) to facilitate more widespread commercial use.

In addition to the major differences, there is significant variation in numerous other attributes within each of the two homozygous groupings (including date of first flowering, growth habit, leaflet size and shape, pod and seed size, and seed colour) This provides further opportunities to select potentially the best commercial genotypes to challenge the current market predominance of 'Rongai' (as noted with concern by Maass et al. 2010) and increase the level of genetic diversity among commercially-grown lablab. Moreover, the inherent instability in the heterozygous Purple component can be exploited to generate on an ongoing basis further diversity in these other attributes.

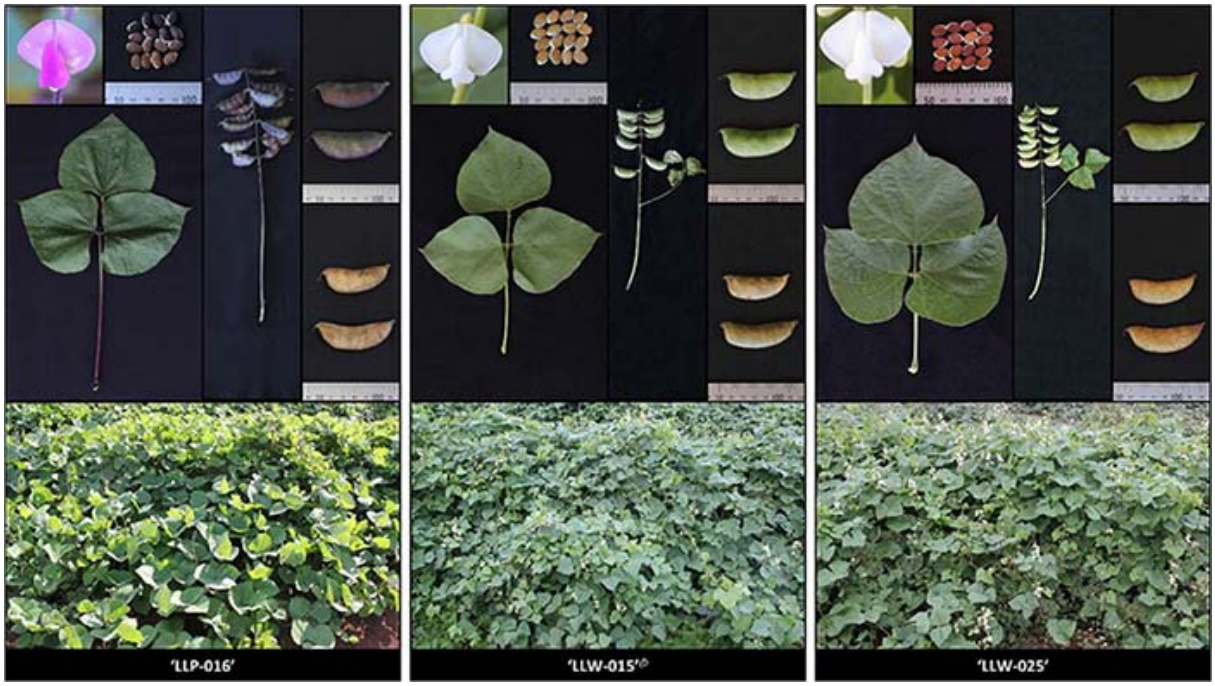

Figure 2. Composite photographs showing morphological differences in 'LLP-016' (Purple genotype), 'LLW-015' and 'LLW-025' (White genotypes) (Loch and Zorin, 2016a, 2016b, 2020).

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