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Variation for constitutive flavonols and morphological traits in a new white clover population

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ABSTRACT

Breeding forage legumes combining high levels of stress-protective secondary metabolites and high herbage yield are possible. Previous findings suggested a trade-off between flavonol glycosides and biomass production in white clover (*Trifolium repens* L.), with population specific evidence indicating that association. The present study used a novel white clover first filial (F_1) reciprocal cross ($n = 130$, 3 replications in pots) between the productive cultivar "Kopu II" and the cold- and drought stress-resistant population "Tienshan". The conditions were non-limiting as the plants were watered at regular intervals as needed, to establish a baseline for both constitutive flavonol glycosides and above-ground biomass production. This study showed that the phenotypic correlation between the traits quercetin glycosides (Q) and shoot dry matter (SDM) although significant ($P < 0.001$) was weak with $r^2 = 0.0903$ (9%). This suggests the possibility of improving white clover performance by increasing the levels of stress protective metabolites in tandem with selection for high yield. At a genotype level, constitutive quercetin (Q) glycoside accumulation in this white clover line is not a major constraint on DM production in the absence of moisture stress. This indicates that combining high DM yield and high constitutive levels of Q glycosides for abiotic stress protection is possible. This finding is significant to overcoming a key challenge in plant breeding: combining stress tolerance with increased herbage production.

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Breeding forage legumes combining high levels of stress-protective secondary metabolites and high herbage yield are possible. Until recently, it was unknown if there was a trade-off between flavonol glycosides and biomass production in white clover individuals, with population level evidence suggesting there may be one. This study showed that white clover improvement is possible by increasing the levels of stress protective metabolites in tandem with selecting for high yield.

1. Introduction

The combination of high yield potential and abiotic stress tolerance is a longstanding challenge in plant breeding. White clover (*Trifolium repens* L.) is a perennial forage legume that adds value to many temperate pastoral production systems (Baker and Williams, 1987). White clover is also an effective break crop in arable crop rotations (Kanneganti and Kaffka, 1995) with the added benefit of fixing nitrogen in the soil to benefit following crops (Williams et al., 2000, 2003). In pastoral systems, the amount of white clover in grazed mixed swards combined with grass species should remain at around one third (Elgersma and Li, 1997). However, many pastoral systems fail to consistently achieve this level, for a variety of reasons including moisture limitation in dry summer periods (Knowles et al., 2003; Lane et al., 2000a).

In the field, white clover shows large phenotypic plasticity due to the ability to adapt to different environmental conditions (Collins et al., 2002). Most morphological traits, such as stolon density, stolon branching, plant spread, plant height, stolon thickness and leaf length often express strong genotypic correlation with average herbage yield in different environments (Jahufer et al., 1994; Lane et al., 2000b).

Improving drought tolerance contributing to yield and persistence in white clover is a long standing breeding objective (Marshall et al., 2004; Woodward and Caradus, 2000).

Recent research (Ballizany et al., 2012b; Hofmann and Jahufer, 2011) has shown associations at the population level between accumulation of the secondary metabolite Q glycosides and plant

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adaptive response to abiotic stresses, and plant biomass production under non-stress conditions. These studies found that induced accumulation of Q glycosides was positively associated with abiotic stress tolerance, and negatively associated with biomass production at the population and individual level. It is unknown whether this observed relationship is maintained at the individual plant level, and if it is possible to select for plants with both high levels of Q glycosides associated with increased abiotic stress tolerance, and high biomass yield potential production under non-stress conditions.

This paper reports on an experiment using plants randomly sampled ($n = 130$) from an F_1 full-sib population (Ballizany et al., 2012a). The objective of the research was to investigate in these white clover plants the variation and relationships among Q glycoside accumulation, DM production and other traits under summer growth conditions in the absence of moisture limitation, to provide information on the possibility for plant breeding to combine the positive characteristics of abiotic stress tolerance and high yield potential in white clover.

2. Materials and methods

2.1. Plant material

2.1.1. A new white clover full-sibling cross

A population of plants ($n = 190$) was generated by a hand pollinated pair cross between an individual plant from population C20133 of "Grasslands Kopu II", and an individual plant from population C8749 of "Tienshan", as reported in Ballizany et al. (2012a,b). There were 95 crosses made with Kopu II as the maternal parent (K×T), and 95 reciprocal crosses with Tienshan as the maternal parent (T×K). From each progeny collection 65 F_1 genotypes were randomly sampled by using a random list function in Microsoft Excel adding up to a total of 130 progeny. The population of progeny plus the two parental genotypes were cloned by stolon cuttings and grown in 7.5 L plastic pots in the nursery at Lincoln University. From these stock plants three clones (stolon cuttings with a uniform length of 10 cm) each were then derived for the three replications.

All experimental individuals (parental and progeny) were derived as clones from stock plants, not seedlings. In this way we were able to compare genetically identical individuals between replications and treatments. Sanderson et al. (2003) pointed out that the competitive capacity of white clover in the sward is limited due to stress-induced fragmentation into individuals. In view of that, all of the measured traits were based on independent clonal plants. The white clover plant in the field normally transforms into clonal plants rooted from stolon fragments when the taproot dies (Caradus and Woodfield, 1998). Thus, the plants used in this experiment were 1–2 years in advance of experiments which would use seedlings with tap-roots. In contrast to the latter, in the clonal plants used in this study the shorter secondary root systems on the stolon fragments had taken over (Brock and Tilbrook, 2000). The secondary roots of most white clover cultivars are not as deep as the original taproot (Baker and Williams, 1987).

The plant material used for this experiment was a random sample of 130 F_1 genotypes from the total population. The parental genotypes were also included as repeated checks.

Timing: Stolon cuttings from stock plants were transplanted in pots placed outdoors on 29 February 2008. Plants were maintained by watering and weeding throughout the experiment. Harvest of leaves for HPLC analysis was done on 14 April 2008 and the plants were destructively harvested for dry matter from 15 April 2008.

2.2. Experimental design

The experimental design was a randomised complete block design (RCBD) with three replicates as blocks. Blocks were randomised in Excel (Clewer and Scarisbrick, 2001). Each replication consisted of 130 randomly sampled F_1 genotypes (65 with Kopu II as the maternal parent (K×T), and 65 with Tienshan as the maternal parent (T×K) with the addition of the two parents Tienshan and Kopu II (a total of 132 clones per replication). The plants were grown in 7.5 L pots, the treatment structure was single factor (genotype), under Canterbury, New Zealand outdoor summer conditions, well-watered without induced moisture stress and after the maximum UV stress in the spring and early summer had passed.

2.3. Soil mix

A multi-purpose soil-mix was used for white clover testing in pots under outdoor conditions. The soil had the following qualities: reduced shrinking or clotting of the local silt loam, a fine, crumby structure that allows the establishment of roots throughout the soil-mix and washes well from the roots at the end of the experiment. Ideally, white clover experiments in pots outdoors should reflect the situation in the field as closely as possible, so the pot size (7.5 L) should not be limiting for plant growth.

The soil mix used in this experiment consisted of 75% Wakanui silt loam (Francis and Kemp, 1990) obtained locally from a paddock in the Lincoln University Horticultural Research site, New Zealand, and 25% mortar sand 0–3 mm. 30 g/L horticultural gypsum (Winstone Wallboards, calcium sulphate), 1 g/L wetting agent (Hydraflo, Scotts Australia Pty Ltd) and 2 g/L fertiliser (Osmocote Exact Standard 3–4 months, N–P–K 16–5.0–9.2 + 1.8 mg trace elements) were added to the mix to improve soil structure, water holding and rewetting capacities and nutrient content.

2.4. Measurements

Biomass in the form of dry matter (DM, g) was destructively measured for above ground parts or shoot DM (SDM, g) and below ground parts or root DM (RDM, g) separately. The above ground parts (shoots) were rinsed, cut off at 10 mm above soil height, put in bags and oven-dried. The roots were washed out of the silt loam and oven-dried for 48 h at 70 °C separately from the shoots. Root-washing was greatly facilitated by the improved soil structure. Roots and shoots were weighed separately for total DM (TDM = SDM + RDM) and root-to-shoot ratio (RSR = RDM/SDM) determination.

The flavonol glycosides (mg g^{-1} DM) of quercetin (Q) and kaempferol (K) were measured with HPLC analysis as described below. Quercetin-to-kaempferol glycoside ratio (QKR) was subsequently determined by Q/K.

2.4.1. Quantitative flavonoid analysis with high performance liquid chromatography

Sample preparation and HPLC analysis follows established methods used in other experiments with this population (Ballizany et al., 2012b). Six weeks after planting, just before destructive harvest, ten white clover laminae from fully expanded leaves were harvested at the nursery and put in vials containing liquid nitrogen prior to transport to the laboratory for preparation.

2.5. Data analysis

The genotypes used in this experiment were a random sample representing the population. Therefore, analysing the genotypes as random effects gave the opportunity of estimating the genotypic variation within the population. The data were analysed using the Restricted Maximum Likelihood analysis procedure in GENSTAT ver. 12 (VSNInternational Ltd., Hemel Hempstead, United Kingdom). The final genotypic means were based on Best Linear Unbiased Predictors (BLUPs) (Piepho et al., 2008; White and Hodge,