Development of a high energy red clover

Conference Paper

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Abstract

Red clover (*Trifolium pratense* L.) is one of the most important forage legumes in grassland agroecosystems worldwide. Although red clover has a high biomass potential and is particularly valued for its high protein content, its herbage lacks the high-energy carbohydrates required to meet the productivity potential of modern livestock breeds. Like most plants, red clover accumulates diurnal starch in its leaves during the day as a temporary carbon store of photosynthesis, but harvesting this starch is challenging. To develop a harvestable high-starch agronomic trait in red clover, a reverse genetic pipeline was established to identify beneficial alleles in the gene encoding the starch degradation enzyme GWD1 which naturally accumulate starch. Within the targeted region, we were able to identify several rare and common variants. Of these variants, one that was present at a rate of approximately 25% in the original population contributed to a starch increase of 20% in selected populations enriched for this allele. Implementation of such a high-starch trait in forage production is envisioned to maximize the energy content and protein absorption of forage crops in order to deliver a higher proportion of the feed intake from environmentally sustainable and locally produced roughage.

Keywords: red clover (*Trifolium pratense* L.), digestibility, glucan water dikinase (GWD1)

Introduction

Ruminant livestock diets are often supplemented with high energy corn, cereals, and soy derived from foreign supply chains. These supplemented feeds are a major cause local nutrient and carbon cycles to be broken, that leads to increased water pollution and greenhouse gas emissions, and aspect the public's poor perception of modern livestock production practices. Starch granules are produced in the chloroplasts of forage legume leaves during the day as a carbohydrate storage for night-time growth and respiration. Forage legumes, such as red clover, have the potential to accumulate more than 10 to 15% starch by dry weight (Ruckle *et al.,* 2017; Claessens *et al.,* 2016; Ruckle *et al.,* 2018). This amount of starch is sufficient to promote ruminant microbial activity, better protein absorption, and ultimately better productivity with fewer emissions. Reports that 15% starch supplementation from grain into a pasture diet can lead to 25% more daily animal productivity, 30% reduced methane emissions, and 20% reduced nitrate emissions (Reis and Combs, 2000; Benchaar *et al.,* 2001; Van Dorland *et al.,* 2007; Kingston-Smith *et al.,* 2013; Huntington, 1997). *In vitro* analysis of red clover leaves collected at the end of day (ED) versus the end of the night (EN) had 25% more digestible organic material and 15% less methane gas produced, which is consistent with more leaf starch at ED (Ruckle *et al.,* 2017).

Materials and methods

The red clover accession TP1245 from Agroscope (Reckenholz, Switzerland) was used in this study. Plants were grown in the glasshouse. Intense direct light was shaded to 50 to 100 μmol m^{-2} s^{-1}, depending on external conditions. The sunlight was supplemented with 150 to 200 μmol m^{-2} s^{-1} light from Clean Arc© metal halide lamps (EYE Lighting International, USA). A 14:10 light/dark photoperiod with a 19 to 23 °C day 14 to 16 °C night temperature regime was used. Plants were organized in a random design within the growth area. Leaves were collected and flash frozen in liquid nitrogen after 60 days of growth post-germination at the ED and EN. Starch was measured as described by Ruckle *et al.* (2017). Difference in starch content was determined between 45 genotypes of TP1245 (parental population)

compared to 13 genotypes that were determined to be homozygous for the *TpGWD1-CBD1* allele. The difference was calculated by a one-way ANOVA per harvest time due to significant harvest time by genotype interaction. All statistical analyses were carried out in R statistical software version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria).

DNA was extracted using the Omega Mag-bind Plant DNA DS Kit 96 (Radnor, PA, USA). The 1 kb amplicon was generated by PCR with red clover gDNA as a template. PCR was carried out with Promega GoTaq Flexi (Promega; Madison, WI, USA) using the manufacturer's recommend protocol and 2.5 mM $MgCl_2$. Primers were designed to the published red clover genome (Phytozome v12.1; *Trifolium pretense* v2; De Vega *et al.,* 2015). Sanger sequencing (Microsynth, CH) was carried out to identify alleles by targeted resequencing of 380 genotypes of the TP1245 variety. The target amplicon specifically amplified a region with the primers 5'-CTTTAAATGCGGCATATTGTGTG-3' and 5'-TGACCTTCTCTTGCTCTGG-3'. A derived cleavage amplified polymorphic sequence (DCAPS) marker was designed to genotype plants for one of the identified alleles, *TpGWD1-CBD1*. DCAPS marker was amplified with 5'-TGATCCTTCTTCTGAGGTTTCTAC-3' and 5'-GTCCGTCTGATAGAATGACAAAAG-3' primers and digested with HPYCH4 (New England Biolabs, Ipswich, MA, USA).

Results and discussion

Different genotypes of red clover utilize their diel starch content differently. Some genotypes degrade less starch at night, and maintain relatively high starch contents in the morning (Ruckle *et al.* 2017). Such traits are interesting for increasing red clover energy content. One possible explanation for the difference in starch usage observed between genotypes is a deficiency in starch degradation. *TpGWD1* (Glucan Water Dikinase) encodes an important enzyme in the starch degradation pathway, and mutants in *GWD1* of several species are known to accumulate starch, because they do not degrade starch at night. To identify genotypes with higher starch content, a 1 kb fragment of *TpGWD1* was sequenced in a genetically diverse population of red clover. Targeted resequencing enabled the discovery of rare alleles in a genetically diverse population, allowing us to discover several allele variants in the coding space of a 1 kb region of *TpGWD1*.

One of the identified allele variants was in one of the carbohydrate binding domains (CBD) of *TpGWD1*, and was named *TpGWD1-CBD1*. Although this allele causes a non-synonymous change, there are possibly other alleles in TpGWD1 that are associated with the haplotype that contains this allele. To rapidly identify plants that were homozygous for this allele a molecular marker was developed that identified this allele within our genetically diverse population (Figure 1A). Plants that were homozygous for the *TpGWD1-CBD1* allele had 20% more mean leaf starch at ED (*P*=0.00686) and 60% more at EN (*P*=0.0205) compared to plants from the parental population that were not selected for the *TpGWD1-CBD1* allele (Figure 1B). The amount of dry matter produced by the plants homozygous for the *TpGWD1-CBD1* had a mean value of 2.00 g DW compared to 2.06 g DW of the parental population (*P*=0.674). Therefore, the increase in starch content does not appear to affect biomass production.
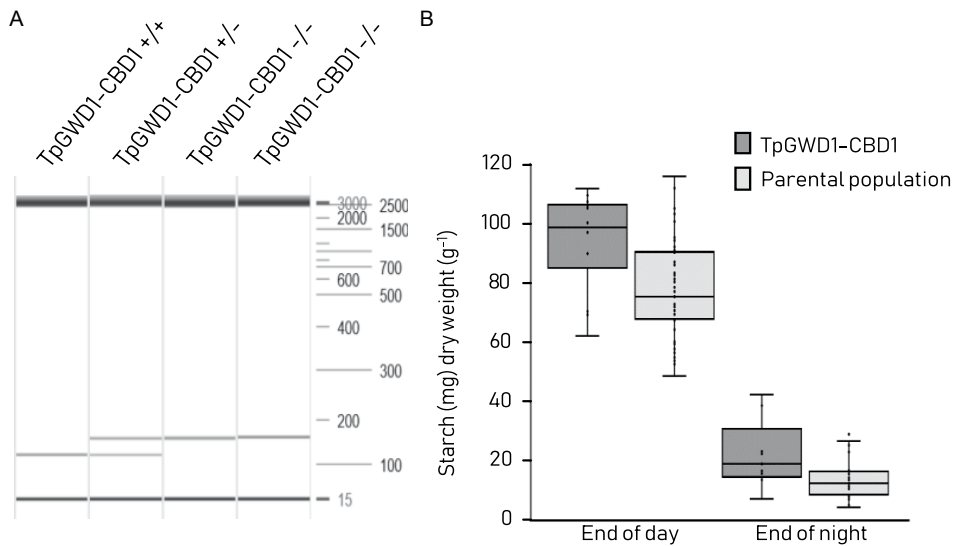
Figure 1. (A) Marker developed to identify the TpGWD1-CBD1 allele. (B) Starch content of the allelic variant *TpGWD1-CBD1* compared to the parental population, at ED and EN.

Conclusion

We have overcome preconceived restrictions based on the central dogma that starch content and growth are tightly linked to maximize biomass potential. There is likely a large degree of flexibility in carbohydrate metabolism that red clover utilizes to adapt to its environment or specified growth conditions. Genetic variation in the genes that encode for the enzymes in starch metabolism has the potential to increase starch content.

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