

Review

Diurnal Leaf Starch Content: An Orphan Trait in Forage Legumes

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Abstract: Forage legumes have a relatively high biomass yield and crude protein content, but their grazed and harvested biomass lacks the high-energy carbohydrates required to meet the productivity potential of modern livestock breeds. Because of their low carbohydrate content, forage legume diets are typically supplemented with starch rich cereal grains or maize (*Zea mays*), leading to the disruption of local nutrient cycles. Although plant leaves were first reported to accumulate starch in a diurnal pattern over a century ago, leaf starch content has yet to be exploited as an agronomic trait in forage crops. Forage legumes such as red clover (*Trifolium pratense*) have the genetic potential to accumulate up to one third of their leaf dry mass as starch, but this starch is typically degraded at night to support nighttime growth and respiration. Even when diurnal accumulation is considered with regard to the time the crop is harvested, only limited gains are realized due to environmental effects and post-harvest losses from respiration. Here we present original data for starch metabolism in red clover and place it in the broader context of other forage legumes such as, white clover (*T. repens*), and alfalfa (*Medicago sativa*). We review the application of recent advances in molecular breeding, plant biology, and crop phenotyping, to forage legumes to improve and exploit a potentially valuable trait for sustainable ruminant livestock production.

Keywords: forage legumes; non-soluble carbohydrates; starch; water soluble carbohydrates

1. Introduction

In many areas of the world, ruminant-based livestock production has for centuries been a key component of sustainable agriculture and cultural traditions. These traditionally pasture and grassland-based agroecosystems maintain carbon balances, nutrient cycles, biodiversity, and water quality. However, in the past decades a growing global population with more purchasing power has placed a greater demand on livestock production. To meet demand, traditional forage-based production has ever-increasingly been intensified and replaced by confined feeding operations (CFOs) [1]. One of the primary reasons for this transition is that the high-energy feeds, which are required for maximum animal productivity, are difficult or too costly to distribute to livestock that graze on pasture lands. Because of the reduced efficiency of grassland-based livestock production, these traditional systems are not economically competitive with CFOs. The supplementation of local perennial forage with externally grown maize (*Zea mays*) and cereals has led to the disruption of the local nutrient, carbon, and water cycles that are maintained by the buffering capacity of perennial grassland agriculture.

Society is increasingly becoming aware of this environmental impact, and the challenges modern livestock production places on global water pollution, land use, and greenhouse gas emissions [1,2].

Although grassland-based forages can supply energy, protein, and fiber into animal diets, consistent nutritional quality required for optimal animal productivity is difficult to obtain. In the humid cool temperate to sub-tropic climates of Europe and Eastern North America, clovers and ryegrasses are major components of pastures and meadows. While perennial ryegrass (*Lolium perenne*) is valued for its high soluble sugar content, low lignin content, and high digestibility, red (*Trifolium pratense*) and white clover (*T. repens*) are valued for their high protein content. White clover is generally grown in pastures, while red clover in cut grasslands. Ryegrasses and clover are typically grown in mixed swards, because of their complementary nutritive and yield traits. Alfalfa (*Medicago sativa*) is the principle forage legume grown in dryer climates such as Western North America and the Mediterranean. It is typically grown in cut grasslands in monoculture for its high protein content and high digestibility. These legumes produce forage normally containing between 15% and 30% protein by dry weight (DW), depending on conditions during harvest [3,4].

The primary advantage of CFOs is the supplementation of readily transportable, starch rich grains such as barley (*Secale cereale*), wheat (*Triticum aestivum*), and maize into the animal's diet. Depending on animal species, age, and production type, the optimal total non-structural carbohydrate (NSC) concentration in the diet typically ranges between 25% and 60% [5,6]. Simple or soluble carbohydrates such as glucose and sucrose are readily broken down in the rumen, while NSCs with a more complex structure, such as starch, are more resistant to degradation. Due to their high digestibility, mono- and disaccharides improve animal performance by maintaining efficient rumen fermentation and the production of bacterial protein. Unlike simple sugars, up to 40% of ingested starch can pass through the rumen and eventually into the small intestine, because of its complex structure [6,7]. There, starch is broken down by glucosidases and amylases into glucose, which facilitates improved protein and energy uptake [6]. These combined effects on ruminal and intestinal digestion are the primary reasons why dietary soluble and insoluble carbohydrates are important for reaching the animal's productivity potential.

Although classically characterized as low in energy content, forage legumes are one of the most attractive crops for sustainable agriculture, because of their high cropping versatility, high digestibility, high leaf-to-stem ratio, high biomass yield potential, and high protein content [8]. Through their mutualistic relationship with soil rhizobia and their control of nodule number, forage legumes fix and furnish atmospheric nitrogen to companion species, buffer excess nitrogen to maintain soil quality with reduced input requirements, and subsequently reduce freshwater nitrate pollution [2,9]. Therefore, forage legumes are essential components of well managed pastures and grasslands that can coexist with native ecosystems more readily than modern farming systems based on arable crops [10]. As a major carbon sink, well managed perennial pastures and grasslands could play a major role in reducing global warming. Based on the modeling of global carbon balances, it was estimated that shifting annual crops toward managed perennial grasslands is part of a strategy to reverse current greenhouse gas levels using "climate smart soils" [11].

Despite their potential to contribute to sustainable agriculture, dedicated forage crops such as perennial grasses and legumes have not seen the same investment in research as maize or soy (*Glycine max*). Consequently, the genetic and genomic resources for breeding more productive forage crops have lagged behind high input crops. In the last two decades, forage crops have only seen yield improvements of 8%–10%, whereas in the same time period, yield improvements of maize and soy were in the range of 50%–60% [12,13]. This review aims to present the potential of integrating modern genetic tools and resources, the current knowledge of plant biology, and contemporary breeding strategies to address the low energy content in forage legumes. Although this review addresses concepts that in principle can be applied to all forage legumes, original research from red clover is presented as a model.

2. Energy Content in Forage Crops

As a quality trait in forages, NSCs are differentiated from structural carbohydrates, which are major components of digestible fiber. NSCs are further divided into water soluble carbohydrates (WSCs) and starch. In forage legumes, WSCs are primarily glucose, fructose, and sucrose, but osmolytes such as pinitol can make up a significant fraction of the WSCs during stress [14–16]. Although NSCs have received little to modest attention in forage legumes, perennial ryegrass varieties with high sugar content, such as “AberMagic” [17,18], have been readily accepted by the pasture livestock community and have been a commercial success. Unlike forage legumes, perennial ryegrass accumulates fructans in the vacuole. These high fructan varieties can accumulate up to 30% more WSCs than traditional varieties [19]. Although reported to increase animal productivity by up to 10%–15% in comparison to traditional varieties [20,21], high sugar grass diets only marginally increased animal productivity in comparison to grain supplementation, because the energy from the soluble sugars is rapidly converted to bacterial protein in the rumen [22]. Therefore, further increases in forage WSC concentration will not replace starch rich grain supplementation, because unlike starch, sugars do not contribute to the synergistic action in the animal’s small intestine required for maximum production.

Starch is an important form of assimilated carbohydrates in forage legume herbage, which diurnally accumulates in the leaf and nocturnally mobilizes to support growth. Reported starch concentration in forage legumes is inconsistent, but typically ranges from 0.5% to 10% of DW [23–25]. This 20-fold disparity is in part due to varying sampling strategies, weather conditions, the time of day the samples were harvested, postharvest treatment, and genotypic variation. The accuracy and standardization of quantification protocols also limits direct comparison of reported starch values. Moreover, genetic variation is often not addressed, as a majority of reports focus on bulked field samples and not on single genotypes. Recently, conventional breeding programs in alfalfa successfully selected for increased NSC concentration, and synthetics with a 10%–20% higher starch concentration were developed [26,27]. However, due to the low harvestable starch concentration of less than 10% the full benefit to animals’ diets cannot be realized and the trait has so far been elusive for forage legume breeders.

3. Physiology, Biochemistry, and Genetics of Starch Metabolism in Model Plants

Plant carbohydrates and their metabolism have received considerable research attention because of their high energy density, economic value, and dietary importance [28]. Leaf starch synthesis, structure, and degradation is best understood in the model plant *arabidopsis* (*Arabidopsis thaliana*), where ample knowledge on starch physiology, mutants, genes, and quantitation methods has been developed for over two decades. In leaves, starch and most of the protein is located in the chloroplast. The majority of leaf based protein content is chloroplast derived (from Rubisco and photosynthetic enzyme complexes) [29]. As a direct product of photosynthesis, starch accumulates in the chloroplast and it occupies a substantial proportion of the chloroplast volume at the end of the day (ED) (Figure 1). Starch can accumulate to significant levels, and up to 25% of the leaves’ dry biomass can be starch [30]. Ultimately, this starch is typically mobilized during the night to facilitate growth and cellular respiration, when, at the end of the night (EN), the diel (or daily) cycle repeats.

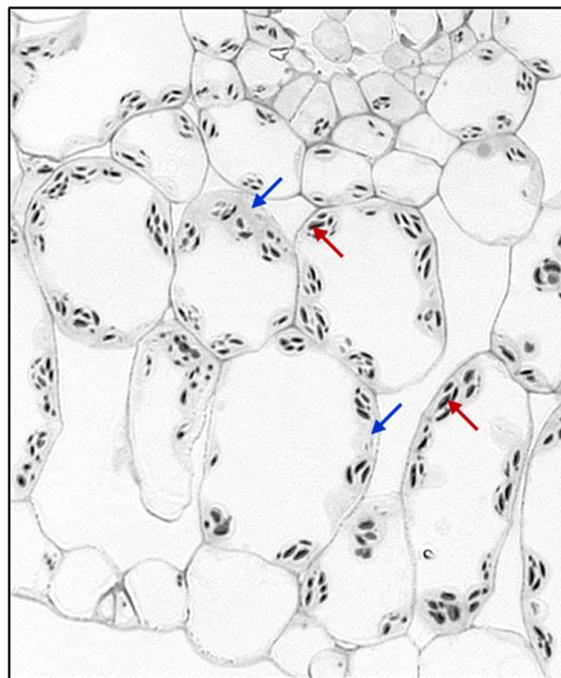


Figure 1. Light micrograph of starch granules in chloroplasts of *Arabidopsis thaliana* mesophyll cells harvested at the end of the day. Protein dense chloroplasts (the light-grey regions) and starch granules (the black regions) are indicated with a blue and a red arrows, respectively. Details are described in Supplemental Methods.

Leaf starch directly accepts and stores fixed carbon from photosynthesis and the Calvin cycle, and its diurnal accumulation and depletion ultimately depends on the balance of starch synthesis and degradation. The enzymes required for starch synthesis and degradation are well understood in leaves, cereal endosperms, roots, and tubers in several plant species [28,31]. Although there are similarities between different starch forms, the regulation, structure, and utilization are distinct. Much of the detailed knowledge of leaf starch metabolism was derived from mutant studies in *arabidopsis*. As illustrated in Figure 2 in detail, starch metabolism consists of four basic processes; synthesis, remodeling, degradation, and sugar export. Mutants in starch synthesis accumulate less starch, mutants in starch remodeling loose starch structure, degradation mutants accumulate starch, and export mutants accumulate soluble sugars during granule degradation [28,32,33]. Degradation mutants are particularly interesting for developing a high starch trait, because mutants in the enzymes that solubilize the granule surface of starch or hydrolyze glucan chains, such as GWD1, SEX4, and BAM1/3/4, can accumulate up to 8-fold more starch than wild type *arabidopsis* [30,34,35].

Detailed knowledge of genes, enzymes, and processes involved in starch metabolism are key for developing modern breeding strategies. Analysis of QTL for primary leaf metabolism traits in *arabidopsis* have shown that a portion of the genetic variation causing the metabolite differences corresponds to genetic variants of known biosynthetic enzymes [36,37]. In the model forage legume birdsfoot trefoil (*Lotus japonicus*), this knowledge was used to guide a Targeted Induced Lesions In Genomes (TILLING) approach, which identified both *starchless* and *starch excess* mutants [38].

Remobilization of leaf starch serves to support both nocturnal respiration and facilitate continued leaf and root growth in the dark. Growth throughout the combined diurnal and nocturnal, or diel cycle is determined by carbohydrate availability, carbohydrate deposition, construction of the cell wall, and cell wall expansion. Since quantitation of leaf growth only directly measures cell wall expansion, diel growth patterns do not always reflect carbon availability [31]. For example in wild type *arabidopsis*, leaf relative growth rates (RGR) increase during the late night until dawn, when a burst of cell wall expansion is observed, presumably following carbohydrate deposition in the late

day and early night [39]. Alternatively in a *starchless* mutant, RGR increase in late afternoon when soluble sugars accumulate and carbohydrate availability is high, and decrease at night and dawn [39]. In soy, RGR correlates more directly with carbohydrate concentration, which peaks at ED, and linearly decreases to the lowest level at dawn [40]. Taken together utilization of leaf starch provides flexibility to plant growth strategies during the diel cycle, but how starch concentration and this added flexibility relates to overall biomass potential is still not well understood.

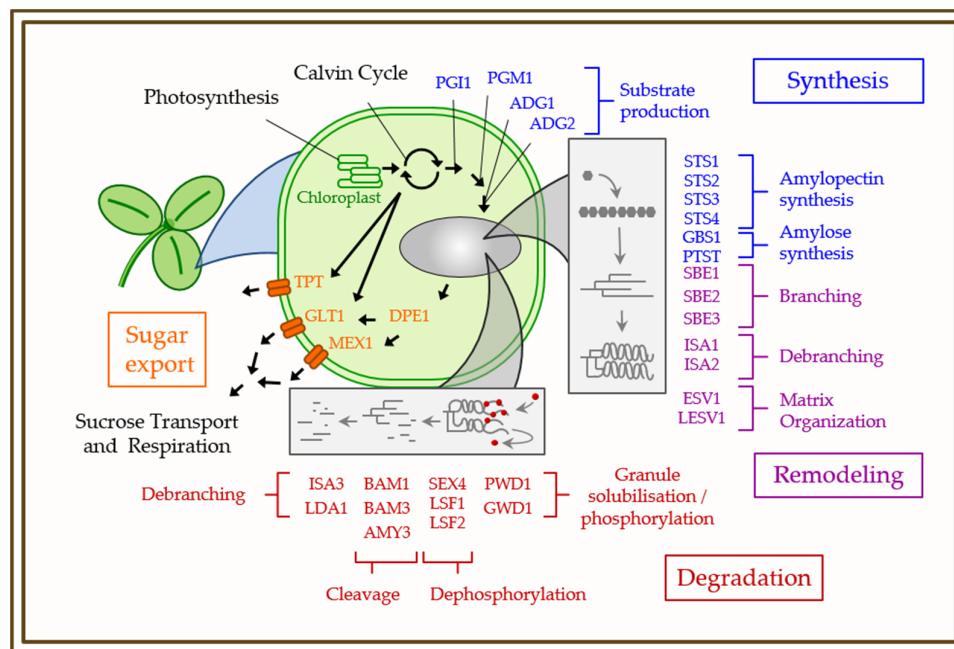


Figure 2. The synthesis and degradation of the complex semi-crystalline structure of starch is orchestrated by a series of enzymes that are involved in four basic processes. Synthesis: fructose-6-P from the Calvin cycle is isomerized (PGI1; PGM2) before it is activated with a UDP moiety (ADG1; ADG2). Soluble and granule bound starch synthases polymerize UDP-Glucose to produce amylose and amylopectin (STS1-4; GBS). Remodeling: the crystalline helical structure of starch requires the amylopectin to be remodeled, which is carried out by branching enzymes (SBE1-3) and debranching isoamylase (ISA1-2). Degradation: To release the soluble sugars from the starch granule, first the amorphization of the crystalline structure must be carried through glucan phosphorylation, which is done by glucan dikinases (GWD1; PWD1). Once amorphous, phosphatases remove the phosphate from the glucan chain (SEX4, LSF1-2), allowing hydrolyzing enzymes such as the β -amylases, (BAM1-3), α -amylases (AMY3), isoamylase (ISA3), and limit dextranase (LDA1) to cleave the glucan polymer. Export: Prior to export, oligomaltosaccharides are disproportionated to glucose and maltose (DPE1). These soluble sugars are exported out of the chloroplast by the maltose exporter (MEX1) and glucose transporter (GLT1). Enzyme nomenclature is based on *arabidopsis* for the enzymes that are conserved in higher plants.

In *arabidopsis*, a broad analysis of metabolites in natural accessions found that genetic variation in starch concentration inversely correlates with biomass. Because of this inverse correlation, starch concentration is hypothesized to be a major integrator of plant growth through a regulatory network that balances carbon availability with growth [41]. This hypothesis is supported by the reduced growth of both *starch excess* and *starchless* mutants, which has been observed in various species [31,38]. The reduction in growth of the *starchless* mutants is thought to come from nighttime starvation. In *starch excess* mutants, the unused sequestered carbon delays the exponential increase in biomass, which is observed during the development of wild type plants [31]. Because plant metabolism is highly plastic, one hypothesis would be that weak mutations that only moderately disrupt starch metabolism

would be tolerated without a growth penalty. In birdsfoot trefoil and maize, disruption of the starch synthesis and degradation, respectively, led to altered starch concentration, but without an observed biomass penalty, suggesting that the reliance of growth on starch is complex and likely varies among species [38,42]. Although starch is a central component of plant metabolism, the potential to develop a high starch trait could be achieved by exploiting this innate flexibility between starch metabolism and growth.

4. The Potential of Leaf Starch Content as a Trait in Forage Legumes

We adapted the knowledge from model species to assess the genetic potential of starch production in forage legumes. A controlled growth regime, optimized sampling strategy, and accurate quantification method was used to determine leaf starch concentration in single red clover genotypes. Leaf starch accumulation was estimated with a visual screen based on iodine staining of 32 plants derived from a genetically diverse population grown under simulated partially sunny conditions. To accurately quantify leaf starch, eight genotypes were selected based on their iodine stained phenotype and vegetatively propagated into biological replicates. These replicates were harvested at ED and EN (Figure 3). In these eight genotypes, the observed variation in leaf starch concentration at ED ranged between 6% and 35% by DW, and the variation in leaf starch concentration at EN ranged from 1% to 15% by DW. Moreover, two basic patterns of accumulation were observed in this population. The majority of the genotypes degraded almost all of their starch at night and their ED starch concentration was dependent on diurnal accumulation. These genotypes were designated diurnal dependent starch accumulation and two examples are presented, *TpDST05* and *TpDST20*. Two of the eight genotypes degraded less than 50% of their starch at night, and their ED starch concentration was more independent from diurnal accumulation and more dependent on starch retention at night (Figure 3). These genotypes were designated diurnal independent starch and two examples are presented, *TpDIST31* and *TpDIST27*. Although the diel usage of starch is well studied in model species, little is described about the genetic variation in diurnal starch accumulation and nocturnal mobilization. These diurnal dependent and independent starch concentration patterns illustrate the genetic potential of red clover to not only accumulate significant levels of starch during the day, but also the genetic potential of some genotypes to maintain high starch levels during the night.

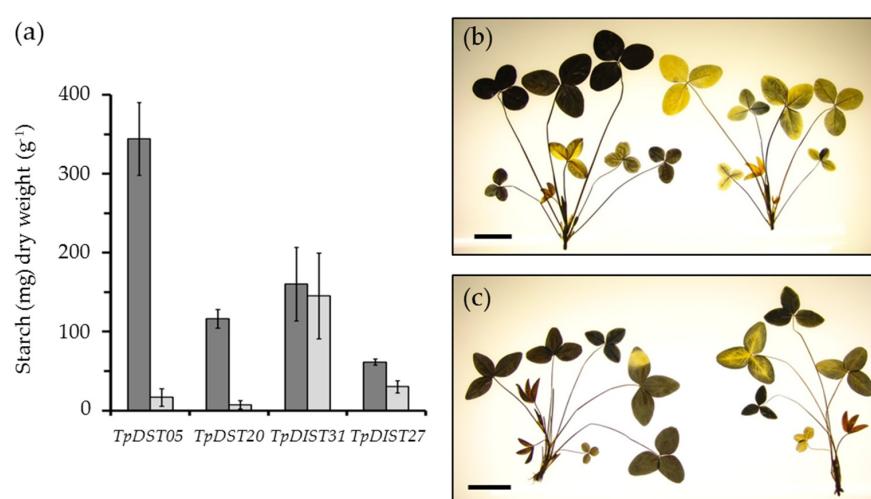


Figure 3. Diel patterns of leaf starch accumulation in selected red clover genotypes. (a) The starch concentration in four genotypes harvested at the end of the day (ED; dark-gray bars), and at the end of the night (EN; light-gray bars). Plants were grown at a fluence rate of $250\text{--}350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in a 14:10 light/dark and $14\text{--}16^\circ\text{C}/19\text{--}23^\circ\text{C}$ diel cycle. Error bars indicate 95% confidence intervals ($N = 8$); (b,c) Iodine staining of ED (left) and EN (right) plants of genotypes *TpDST05* (b) and *TpDIST31* (c). Details are described in Supplementary Methods.

The reduced nocturnal starch degradation observed in the genotypes with weak or no diurnal response is consistent with reduced mobilization, respiration, or growth [31]. Respiration in plants is temperature dependent, as reduced respiration rates are observed at lower temperatures [43]. Although in forage legumes little is known about the direct effect of night-time temperature on starch concentration, in other legume crops such as soy and white clover, studies have shown that lower temperatures lead to reduced degradation of starch at night. The temperature at which starch turnover is reduced differs between species, as reduced starch turnover occurs at 17 °C in soy, and at 7 °C in white clover [44,45].

One of the major challenges with assessing starch concentration is its dependency on the environment. Light is arguably the greatest determinant of starch concentration; During intense light, such as direct sunlight, starch accumulates to a greater degree, but under low light conditions, less starch is produced [46]. To determine the effect of light on starch concentration in red clover, four of the genotypes from above were re-grown in simulated cloudy conditions and compared to plants grown in simulated partially sunny conditions (Figure 4). The total leaf starch concentration in the plants grown in the cloudy conditions was between 0.5% and 1.0% by DW at ED and undetectable at EN, while it reached 16% and 4% at ED and EN, respectively under partially sunny conditions (Figure 4a). Taken together, the potential of red clover to accumulate starch is strongly dependent on light intensity.

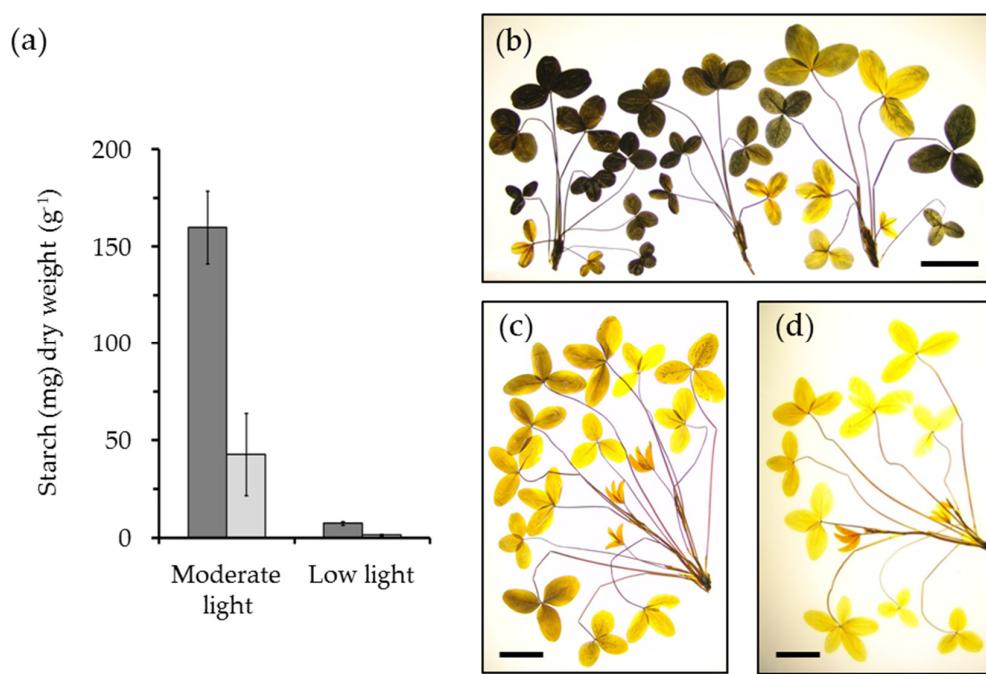


Figure 4. Starch concentration in red clover is dependent on the light intensity. (a) The starch concentration in *TpDST34* harvested at the ED (dark-gray bars), and at the EN (light-gray bars). Plants of *TpDST34* were grown under a moderate fluence rate of 250–350 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ or a low fluence rate of 50–150 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Other growth conditions were the same as described in Figure 3. Error bars indicate 95% confidence intervals ($N = 8$); (b–d) Iodine staining of moderate light grown ED (left 2 plants) and EN (right) *TpDIST34*, and low light ED (c) and EN (d) *TpDIST34*. Details are described in Supplementary Methods.

Another major challenge with utilizing leaf starch content as a trait in forage legumes is the influence of harvest time and postharvest loss. As a diurnal trait, the peak starch concentration is observed at ED. This time is typically less convenient for farmers to cut forage fields, and the late cutting time also reduces the critical drying time in the sun. Although less feasible, studies have

demonstrated that hay produced from alfalfa fields harvested later in the evening contain 20% more NSCs than equivalent fields cut in the morning [47]. This higher NSC content was primarily attributed to higher starch concentration. The higher NSC content from the evening cutting resulted in higher digestibility, and when evening cut forage was fed to dairy cows, they produced up to 5% more milk compared to the morning cut [47,48]. Breeding for traits, such as diurnally independent starch turnover, as seen in *TpDIST27* and *TpDIST31* genotypes, would increase the harvestable starch content.

Unfortunately, maximizing starch concentration in forage legumes through breeding and adjustment of cutting time is only marginally useful, due to immense postharvest losses. After cutting, plants are unable to carry out transpiration and the production of carbohydrates from photosynthesis is not possible. Moreover, cut forage immediately begins to respire the produced energy. In forage crops, the yield loss due to postharvest respiration can be 10%–15% of the DW [49]. This yield loss is primarily due to a loss in total NSC concentration, as sugars and starch are readily mobilized after cutting to facilitate respiration [49]. Therefore, most of the energy potential of forage legumes is lost during harvest. Although the development of better varieties that accumulate more starch will be important, breeding for reduced postharvest respiration and loss will be crucial for successfully developing high starch content in red clover. Such strategies would include specifically targeting and selecting genotypes with reduced postharvest respiration rates, and reduced postharvest starch mobilization.

Because of starch's physiological relationship to growth, improvement of a leaf starch trait could appear counterproductive in forage crop species, since it could come at the cost of reduced biomass yield. To test the relationship between biomass and starch concentration in red clover, 128 genotypes were analyzed for ED starch concentration and biomass. Phenotypic variation in starch concentration ranged from 10% to above 40%, and a 10-fold difference in biomass was observed (Figure 5). Little to no correlation between starch concentration and biomass in this population was observed (Pearson $r = 0.1223$, $p = 0.169$) (Figure 5). Although under controlled conditions, phenotypic variation in starch concentration is less than the genetic variation observed between genotypes (Figure 3), the lack of phenotypic correlation between biomass and starch concentration does not imply a genetic correlation to distinguish these as independent traits. This observation, along with observations on starch mutants in birdsfoot trefoil [38], raises the hypothesis that forage legumes may have more flexibility in the regulatory and metabolic networks that link starch with growth.

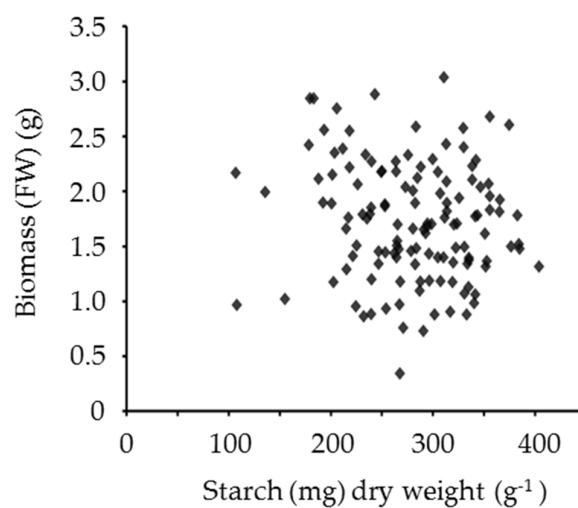


Figure 5. Biomass yield does not correlate with starch concentration in 128 red clover genotypes. The starch concentration and biomass were measured in 50 day old red clover plants grown under controlled growth conditions with a fluence rate of $200 \mu\text{mol}\cdot\text{m}^2\cdot\text{s}^{-1}$ in a 14:10 light/dark, $13\text{--}14^\circ\text{C}/18\text{--}19^\circ\text{C}$ diel cycle and harvested at the end of the day. Details are described in Supplementary Methods.

Although, diurnal accumulation of starch in the leaves facilitates diel growth, forage legumes also accumulate long term storage of starch in heterotrophic tissues, such as roots and stolons. This starch is important for the remobilization of carbon during stress or during specific stages of development to support growth [16]. In forage legumes, remobilization of starch in the roots and stolons is critical for regrowth after cutting, which is a key determinant of overall biomass potential [50]. Although there is overlap between the genes, and enzymes that control starch metabolism in leaves and heterotrophic tissue, their regulation is independent, leading to distinct traits, and allowing for the positive selection for leaf starch concentration, without impacting forage regrowth after cutting [28].

Based on the analysis of single genotypes in red clover, it is likely that forage legumes share a similar genetic potential to produce different amounts of leaf starch. Although a leaf starch concentration of over 30% by DW was observed in some red clover genotypes, to be a practical trait, the dependence on the environment and harvest loss must be feasibly addressed.

5. The Potential of Modern Genomic and Phenomic Tools to Develop Leaf Starch as a Trait in Forage Legumes

In the past decade, the scientific resource gap between forage crops, model species, and high value crops has narrowed due to an ever-increasing plant biology knowledge-base and better genomic tools. Therefore, many benefits of functional genomics and molecular breeding techniques are now accessible to forage crop breeders. Alfalfa, red clover, and white clover have described genetic linkage maps and partial genome sequences based on assembled contigs and scaffolds [51–55]. Contig mapping and gene annotation of these genomes has been aided by cDNA sequencing, and genomic synteny mapping using well defined legume genomes, such as *M. truncatula* [53,55–57]. Given these genomic tools, modern breeding methods such as genotyping by sequencing (GBS), genome-wide association study (GWAS), marker assisted selection (MAS), and TILLING can be integrated into traditional trait selection pipelines [58]. Combined with progress made in the field of starch metabolism, these methods have the potential to address the issues that have made starch content so far an elusive trait.

For complex traits such as leaf starch content, MAS can play an important role to overcome the limitations of conventional selection [59]. A basic prerequisite for MAS is the identification of genomic regions linked to the trait of interest. Under controlled conditions, quantitative trait loci (QTL) for leaf starch concentration have been identified in *arabidopsis* [36]. In the field, GWAS are challenging for complex traits, because the identified QTL are dependent on environmental context, but strategies have been developed to identify QTL for diel traits that are influenced by light and temperature, similar to starch [60]. Using ecophysiological modeling, QTL were identified for diel leaf elongation rates in maize [61]. Such ecophysiological modeling could be useful to apply the appropriate context to starch concentration QTL identified in the field.

An alternative approach would be to influence starch metabolism through mutagenesis. Starch degradation mutants would have less variation in genetic background, time of day, and environment [62]. Starch degradation mutants identified through TILLING in birds-foot trefoil maintained high levels of leaf starch concentration throughout the diel cycle [38]. TILLING has been adapted to breeding programs in many crop species, but it remains challenging to identify induced mutations in populations that are genetically heterogeneous, such as forage legumes [63,64]. Because transformation methods exist for alfalfa and red clover, CRISPR/Cas9 mediated mutagenesis has the potential to replicate well described alleles in starch degradation from other species and overcome challenges that TILLING has in heterogeneous genomes [65,66]. Such approaches have been used to specifically target branched amino acid metabolism to confer herbicide resistance in maize [67].

In addition to targeted genetic approaches, a rapid, cost-effective, high-throughput, phenotyping method is needed to allow for developing starch content into a selectable trait. Such a method would have to be field based and allow for assessing the trait throughout the season to compensation for seasonal variation. Due to the lack of uniformity of starch concentration between leaves (Figure 3b,c), sample homogenization is a major bottleneck for accurate inferred or biochemical analysis. Therefore,

technologies such as hyperspectral imaging offer the greatest promise for efficiently and accurately measuring starch in the field as has been shown for WSC in wheat [68].

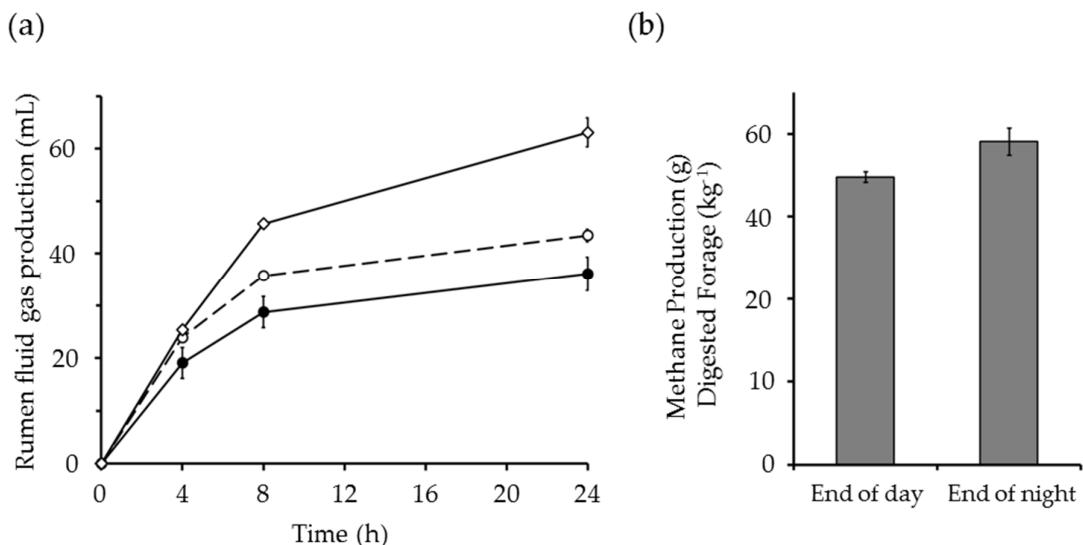


Figure 6. Diurnal starch accumulation increases the digestibility of red clover. The same tissue that was analyzed in Figure 3a for genotype *TpDST20* was analyzed by the Hohenheim gas test. (a) Gas production from rumen fluid digestion of *TpDST20* samples harvested at the ED (empty circles), or the EN (filled circles). Gas production from the Hohenheim concentrate standard is presented as a reference (empty diamonds); (b) Methane production from ED and EN samples from Figure 6a after 24 h of digestion. Error bars represent 95% confidence intervals ($N = 5$). Details are described in Supplementary Methods.

6. The Potential to Reduce Concentrate Feeding

Forage legumes such as red clover have the potential to accumulate one third of their dry matter biomass as starch, but due to environmental, harvest, and post-harvest limitations, a 10%–15% final starch proportion in post-harvest biomass is optimistic. An amount of 10%–15% starch is 2- to 10-fold greater than the starch concentration reported in bulk field samples of red clover [23,24,69]. This concentration of starch in an animal's diet is sufficient to promote ruminant microbial activity, better protein absorption, and ultimately better productivity with less nitrate and methane emissions [13,70]. Although leaf starch has been reported to be completely fermented during in vitro rumen assays, much of the research regarding the role of starch in the small intestine has focused on grain supplements [6,23]. An amount of 10%–15% dietary starch is adequate to support the ruminant microbial activity required for better protein absorption, and for the high protein content of forage legumes to be fully utilized [3,4]. Higher protein absorption rates facilitated by 10%–15% grain starch supplementation can lead to 25% more daily animal productivity, 30% reduced methane emissions and 20% reduced nitrate emissions [71–73].

To determine whether red clover leaf starch improves rumen fermentation, we tested the in vitro digestibility of one particular red clover genotype which accumulates around 12% of its DW as starch at ED and little to no starch at EN under standard growing conditions (Figure 3a). The ED and EN samples were assessed for digestibility in comparison to a high energy concentrate using the Hohenheim Gas Test [74]. Based on the gas produced after 24 h of rumen fluid digestion, the starch containing ED samples had 20% more digestible organic material than the EN samples (Figure 6a). Because the rumen degrades compounds at different rates, the major class of compounds leading to the digestibility gain can be estimated. As observed in the concentrate control, soluble sugars and proteins are rapidly digested in the first 4 h, starch is degraded for up to 8 h, and structural carbohydrates such as cellulose are degraded beyond 8 h [74]. Therefore, the increased digestibility observed between 4 h

and 8 h, is consistent with higher starch concentration of the ED sample compared to the EN sample (Figure 6a). The slower rumen digestion of leaf starch should allow it to move to the small intestine, where it is hypothesized to have more impact on animal productivity [6]. Moreover, because the ED sample is more efficiently digested than the EN sample, the ED sample produced almost 15% less methane per unit of digested of organic material than the EN sample (Figure 6b). Since in vivo rumen digestibility is a strong measure of the productivity potential of a feed, diurnal leaf starch has the potential to increase productivity and reduce grain starch supplementation.

7. Conclusions

The goal of this review was to illustrate the potential that forage legumes have to produce starch, and to dissect the major factors that currently limit the development of a high starch trait. For decades, traditional breeding strategies to increase NSC content, decrease post-harvest dry matter loss, and increase digestibility have resulted in minimal gains in alfalfa starch content [26]. Arguably, these minimal gains are due to the complexity of the trait. The lack of success in selecting a high starch trait has led it to receiving much less attention than other forage legume traits in recent years. Today, the merging of novel phenotyping platforms, molecular breeding tools, knowledge of plant biology, and genetic resources with traditional breeding allows for a greater level of creativity to develop novel traits that have never seen selection, or were considered too challenging to develop. Theoretically, the tools and knowledge are available to develop high starch content in forage legumes, but there is still significant investment required to merge basic biology with modern breeding.

Investment into developing a high starch trait in forage legumes is particularly imperative to address negative aspects of modern agriculture. Traditionally, the driving force behind the development of a high NSC trait was the economic benefit it offered to farmers. These economic incentives have increased with increased awareness of sustainable livestock production by the public. Because a high-starch content in forage legumes would have the potential to reduce grain supplementation in the livestock's diet, the demand for high-input grain crops, such as maize and soy would be reduced. Therefore, livestock production based on sustainable pastures and grasslands would be more economically competitive. Because of the potential value and impact that a high starch trait could have on agriculture, the concerns about the feasibility of this orphan trait should be re-evaluated.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/7/1/16/s1. Detailed growth conditions and experimental methods are supplied as supplemental Materials and Methods.

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