A peer-reviewed version of this preprint was published in PeerJ on 3 March 2015.

Núñez-López L, Aguirre-Cruz A, Barrera-Figueroa BE, Peña-Castro JM. 2015. Improvement of enzymatic saccharification yield in *Arabidopsis thaliana* by ectopic expression of the rice *SUB1A-1* transcription factor. PeerJ 3:e817 https://doi.org/10.7717/peerj.817

Improvement of enzymatic saccharification in *Arabidopsis thaliana* by ectopic expression of the rice *SUB1A-1* transcription factor.

Lizeth Núñez-López, Andrés Aguirre-Cruz, Blanca Estela Barrera-Figueroa, Julian Mario Peña-Castro

Saccharification of polysaccharides releases monosaccharides that can be used by ethanol-producing microorganisms in biofuel production. To improve plant biomass as a raw material for saccharification, factors controlling the accumulation and structure of carbohydrates must be identified. Rice *SUB1A-1* is a transcription factor that represses the turnover of starch and postpones energy-consuming growth processes under submergence stress. *Arabidopsis* was employed to test if heterologous expression of *SUB1A-1* or *SUB1C-1* (a related gene) can be used to improve saccharification. Cellulolytic and amylolytic enzymatic treatments confirmed that *SUB1A-1* transgenics had better saccharification yield than wild-type (Col-0), mainly from accumulated starch. This high saccharification yield was developmentally controlled since juvenile transgenic plants yielded 200-300% more glucose than Col-0. We measured photosynthetic parameters, starch granule microstructure, and transcript abundance of genes involved in starch degradation (*SEX4*, *GWD1*), juvenile transition (*SPL3-5*) and meristematic identity (*FUL*, *SOC1*) but found no differences to Col-0, indicating that starch accumulation may be controlled by down-regulation of *CONSTANS* and *FLOWERING LOCUS T* by *SUB1A-1* as previously reported. *SUB1A-1* transgenics also offered less resistance to deformation than wild-type concomitant to up-regulation of *AtEXP2* expansin and *BGL2* glucan-1,3,-beta-glucosidase. We conclude that heterologous *SUB1A-1* expression can improve saccharification yield and softness, two traits needed in bioethanol production.

Lizeth Núñez-López [1,2], Andrés Aguirre-Cruz[3], Blanca Estela Barrera-Figueroa[1*] and Julián Mario Peña-Castro[1*]

[1]Laboratorio de Biotecnología Vegetal, Instituto de Biotecnología, Universidad del Papaloapan, Tuxtepec, Oaxaca, México.

[2]División de Estudios de Posgrado, Universidad del Papaloapan, Tuxtepec, Oaxaca, México.

[3]Taller de Alimentos, Instituto de Biotecnología, Universidad del Papaloapan, Tuxtepec, Oaxaca, México.

*Corresponding authors:
Julián Mario Peña-Castro
Av. Circuito Central 200, Col. Parque Industrial, 68301, Universidad del Papaloapan, Tuxtepec, Oaxaca, México. Tel. + 52 287 875 9240. E-mail: julianpc@unpa.edu.mx, julianp@prodigy.net.mx

Blanca Estela Barrera-Figueroa
Av. Circuito Central 200, Col. Parque Industrial, 68301, Universidad del Papaloapan, Tuxtepec, Oaxaca, México. Tel. + 52 287 875 9240. E-mail: bbarrera@unpa.edu.mx, blanca_barrera_f@hotmail.com

1. Introduction

Ethanol produced by yeast and bacteria through fermentation of plant-synthesized carbohydrates is one of the oldest biotechnological applications, especially for beverage and food. Production of biological ethanol has emerged as an important means for substitution of traditional hydrocarbon-based fuels (Henry, 2010). Key to successful biofuel production is a net output of energy (Vanholme *et al*., 2013). The process of bioethanol production is currently under intense research to improve microbial fermentation efficiency, available microbial strains, industrial down- and upstream operations, plant stress tolerance and plant biomass quality (Chundawat *et al*., 2011; Karnaouri *et al*., 2013; Vanholme *et al*., 2013; Ribeiro *et al*., 2014).

A main goal of plant biomass improvement for biofuel production is an increase in a new trait called saccharification. It is defined as the solubilization of plant carbohydrate reservoirs, mainly starch, cell wall and free sugars (Fig. 1) through physical or enzymatic treatments to yield fermentable carbohydrates (Chuck *et al*., 2011; Chundawat *et al*., 2011). In this way, saccharification yield is the amount of fermentable sugars released from starch or cell walls after solubilization per unit of plant biomass (Petersen *et al*., 2012; Nigorikawa *et al*., 2012).

Many agricultural relevant plants have high saccharification yields with limited energy input. For example, sugarcane (*Saccharum sp.*) and sugar beet (*Beta vulgaris*) release a sucrose-rich juice after simple mechanical treatments, which is readily fermentable by microorganisms (Waclawovsky *et al*., 2010). Potato tubers and maize seeds require chemical or enzymatic hydrolysis of starch by amylase and amyloglucosidase to release glucose-rich extracts (Bahaji *et al*., 2013). These two processes are the core of first generation bioethanol production. However, each of these plants has a specific geographical growth range, limited saccharificable tissues (stems, tubers or seeds) and are traditionally employed as food staples, thus raising social and economical concerns (Henry, 2010; Stamm *et al*., 2012).

Second generation bioethanol production aims to use the abundant cellulose reserves present in agroindustrial waste, grasses and trees to increase plant saccharification yields (Stamm *et al*., 2012). Drawbacks found in this technology are poor enzymatic saccharification because of complex cell wall architecture, energy-consuming chemical and physical pretreatments for cell wall disruption and multiple genes involved in cell wall synthesis (Chundawat *et al*., 2011).

Different biotechnological strategies have been tested to change carbon allocation and improve raw plant biomass saccharification in the context of first and second generation bioethanol production. Maize and *Arabidopsis* plants with inducible silencing of genes encoding phosphate-metabolism enzymes glucan water dikinase (*GWD*) and phosphoglucan phosphatase (*SEX4*) increased starch saccharification yield by 50-300% when compared to WT (Weise *et al*., 2012). Increased cellulose saccharification yields of 20-250% have been achieved in different plant models by expressing peptide inhibitors of pectin synthesis (Lionetti *et al*., 2010), changing expression patterns of glycosyltransferases involved in xylan synthesis (Petersen *et al*., 2012), or over-expression of endogenous exoglucanases (Nigorikawa *et al*., 2012). Mutagenesis has also been applied to isolate *Arabidopsis* mutants with improved saccharification; while some remained uncharacterized, others were unexpectedly related to disrupted auxin transport (Stamaieu *et al*., 2013). Starch saccharification yield was increased by over-expressing

miRNA156 (Chuck *et al*., 2011), a factor downstream of the trehalose-6-phosphate (T6P) carbon flux sensing machinery (Wahl *et al*., 2013).

A plant abiotic stress in which carbohydrate consumption and signaling are crucial for survival is submergence stress. An excess of water around root and aerial organs excludes oxygen from cells, forcing an adjustment from aerobic to anaerobic metabolism (Bailey-Serres and Voesenek, 2008; Lee *et al*., 2011; Fukao and Xiong, 2013). Plants must finely control the consumption of starch to generate ATP and fuel energy demanding cellular processes because when this reserve is depleted, homeostasis is lost and cell death occurs (Bailey-Serres, Lee and Brinton, 2012).

In rice, the response in cultivars that have an increased tolerance to flooding stress is mediated by the *SUBMERGENCE1* locus (*SUB1*). *SUB1* contains three transcription factors from the *Ethylene Response Factor* (*ERF*) Group VII gene family, namely *SUB1A-1, SUB1B-1* and *SUB1C-1*; the main genetic factor for tolerance is *SUB1A-1* (Xu *et al*., 2006). *SUB1A-1* mRNA is rapidly induced when plants sense ethylene or low-oxygen conditions and redirects transcription relative to near-isogenic genotypes lacking *SUB1A-1* (Jung *et al*., 2010; Mustroph *et al*., 2010). Apparent roles of *SUB1A-1* include the repression of gibberellin-promoted starch consumption (Fukao *et al*., 2006; Fukao, Yeung and Bailey-Serres, 2012), inhibition of cell elongation (Fukao and Bailey-Serres, 2008) and delay of the progression to flowering (Peña-Castro *et al*., 2011). When floodwaters recede, *SUB1A-1* is down-regulated and normal growth processes resume. Collectively, these molecular and physiological activities leading to effective carbon and energy conservation under submergence to prolong survival are called the Low-Oxygen Quiescence Strategy (LOQS; Bailey-Serres and Voesenek, 2008). When compared to WT, rice plants ectopically expressing *SUB1A-1* have a delayed progression to flowering (Fukao and Bailey-Serres, 2008), and constitutive higher free sugars concentration in aerial tissue (mixed stem and leaves) but only show differential starch concentrations under dark-starvation stress (Fukao, Yeung and Bailey-Serres, 2012).

Evolutionary analyses indicate that *SUB1A-1* is a descendent of gene duplication and neofunctionalization of *SUB1C* (Fukao, Harris and Bailey-Serres, 2009; Niroula *et al*., 2012; Pucciariello and Perata, 2013). However, *SUB1C-1* is repressed by *SUB1A-1* expression and its presence in rice is not associated with the LOQS. Its up-regulation by submergence, ethylene and GA led to the suggestion that it may be involved in promotion of carbohydrates consumption and cell elongation to enable submerged leaf tissue to grow to the surface of floodwaters (Fukao *et al*., 2006; Fukao and Bailey-Serres, 2008).

We previously employed *Arabidopsis thaliana* plants transformed with N-terminal FLAG-tagged *35S*:*SUB1A-1* (*OxSUB1A*) to evaluate the recapitulation of LOQS phenotypes observed in *SUB1* rice. This confirmed *OxSUB1A* confers abscisic acid (ABA)-mediated inhibition of growth and other traits, and exposed inhibition of flowering as a new integral trait of LOQS (Peña-Castro *et al*., 2011). In this work, we employed *Arabidopsis* as a functional prototype to explore if *SUB1A-1* over-expression can improve plant biomass saccharification. The rationale was that *Arabidopsis* plants constitutively expressing *SUB1A-1* may also display the LOQS low-starch consumption trait. We also included in the analysis 35S:*SUB1C-1* plants (*OxSUB1C*) to gain further insight on its function.

2. Materials and Methods

2.1 Plant material

Arabidopsis thaliana Col-0 accession was used as the wild-type (WT). Transgenic genotypes were described previously (Peña-Castro *et al*., 2011). Briefly, *SUB1A-1* or *SUB1C-1* cDNAs from *Oryza sativa* cv M202(*SUB1*) were expressed under Cauliflower Mosaic Virus 35S promoter with a N-terminal immunogenic FLAG-tag in Col-0. Two independent single-copy T4 generation transgenics were used for each transgene: *OxSUB1A-L5* and *-L12* and *OxSUB1C-L6* and *-L10*.

2.2 Plant growth conditions

Arabidopsis seeds were surface sterilized (70% v/v EtOH for 5 min followed by 6% v/v hypochlorite for 2 min and six 1-min rinse steps with ddH_2O) and germinated in half-strength Murashige and Skoog agar medium (MS, salts 0.215% w/v, 1% w/v sucrose, 1% w/v agar, pH 5.7) in vertical plates. Seedlings were transferred when 7-day-old to substrate (Sunshine Mix #3 plus 1:4 volume perlite:substrate, autoclaved for 2 h and mixed with 2% w/w slow liberation fertilizer NPK 12:12:17) and watered every 2 days. Germination and growth was under long-day conditions (16 h light / 8 h dark, 150 µE m^{-2} s^{-1}, 60% humidity) in a growth chamber (Conviron CMP6010). ZT0 (Zeitgeber Time) was the start of the light cycle (day). Genotypes were grown side-by-side in a randomized manner to minimize experimental noise.

2.3 Reducing sugars, cell wall digestibility and starch content.

Rosette leaves were harvested at the time described in each experiment, frozen in liquid nitrogen and stored at -80°C. For all experiments, leaves were ground to a fine powder in liquid nitrogen with mortar and pestle, weighted and further stored or processed. An experimental strategy was designed to quantify the three main components of saccharification yield, namely free reducing sugars, cell wall digestibility and starch content (Fig. 1).

To measure free reducing sugars, 100-120 mg FW of powdered leaves were incubated with ddH_20 for 5 min and centrifuged (13,000 rpm for 5 min) to remove debris. The supernatant (100 µl) was mixed 1:1 with DNS reagent (1% w/v 3,5-dinitrosalicilate, 30% w/v sodium potassium tartrate, 1.6% w/v NaOH) and incubated in a boiling water bath for 5 min, then diluted with 1 ml ddH_20 and absorbance was determined at 545 nm in a spectrophotometer (Miller, 1959). A glucose standard curve (0.1 to 5 mg Glucose / ml, R=0.985) was analyzed and used as reference.

Cellulose digestibility and starch content were enzymatically assayed as previously described (Chuck *et al*., 2011). To test cellulose saccharification yield, commercial cellulase enzyme complex Accellerase 1500 (Genencor, Cedar Rapids, USA) composed of proprietary exoglucanase, endoglucanase, hemicellulase and beta-glucosidase was used. Powdered leaves were weighted in 15 ml capped plastic tubes (100-125 mg FW) and 200 µl of 80% ethanol were added, and the sample vortexed. Next, 3 ml of acetate buffer plus 0.74% w/v $CaCl_2$ (pH 5.0) with 1.7% v/v Accellerase 1500 were added, mixed by inversion and incubated at 50°C for 24 h with rotation (11 rpm) in an oven. Saccharification was stable from 12-36 h as determined in a preliminary kinetics assay (Supplemental Figure 1). Reactions were stopped by incubation at 70°C for 15 min with rotation in an oven. To measure starch content, samples were treated as described above and further hydrolyzed using the manufacturer's instructions for the Total Starch K-TSTA kit (Megazyme, Bray, Ireland), which includes a thermostable α-amylase digestion in boiling water for 12 min with vigorous stirring every 4 min, and an amyloglucosidase digestion in a 50°C water bath for 30 min.

Glucose from cellulose and starch was quantified by glucose oxidase/peroxidase at 510 nm in a spectrophotometer as indicated in the commercial kit Total Starch K-TSTA kit. In parallel, Accellerase buffer (blank), carboximethylcellulose and soluble starch (efficiency probes) were processed. The blank was subtracted from calculations and only experiments with efficiency >93% based on the two probes were employed.

2.4 Iodine staining

Starch was visualized in rosette leaves by iodine staining as previously described (Bahaji *et al*., 2011; Ovecka *et al*., 2012) with the following modifications. Whole plants were harvested and immediately infiltrated under vacuum with 3.7% v/v formaldehyde in 0.1 M potassium phosphate buffer (pH 6.6) for ~10 min. Plants were incubated with hot 80% ethanol for 30 min under constant agitation, stained with iodine solution (KI 2% w/v, I_2 1% w/v) for 30 min in the dark and rinsed until the blue precipitate of starch was distinguishable from the yellowish background.

2.5 Hardness tests

To measure leaf mechanical strength, a texture analyzer was employed (Brookfield CT325k). The three largest rosette leaves from 23-day-old plants were stacked and placed in a fixture base and perforated in the middle of the left blade (avoiding the central vein) with a puncture test probe for fine films (TA-FSF). Resistance was expressed as the force (Newton) applied to break through the tissue.

2.6 Starch granule isolation and scanning electron microscopy.

Rosette tissue pulverized in liquid nitrogen (2.5 g) was hydrated in 40 ml of water, sonicated for 10 min (100% power, 20% amplitude, 50% intensity, Hielscher Ultrasonic Processor UP200ST) and centrifuged for 5 min at 4750 x *g*. The pellet was washed twice with 50 ml of water, resuspended and filtered through a 100 µm and then a 20 µm membrane. The filtrate was centrifuged again at 4750 x *g* and the pellet washed with 20 ml of 100% ethanol. Granules were covered with a gold coat and observed in a scanning electron microscope (Helios Nanolab 600).

2.7 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from *Arabidopsis* seedlings and qRT-PCR was performed as previously described (Peña-Castro *et al*., 2011). Primers for *TUBULIN2* (*TUB2,* At1g65480) were previously reported (Wenkel *et al*., 2006). Primers for *EXPANSIN2* (*AtEXP2*, At5g05290,

5'-TTACACAGCCAAGGCTATGGGCTA-3' and 5'-GCCAATCATGAGGCACAACATCGT-3') and *GLUCAN-1,3,-BETA-GLUCOSIDASE* (*BGL2,* AT3G57260, 5'-TCCTTCTTCAACCACACAGCTGGAC-3' and 5'-CCAACGTTGATGTACCGGAATCTGA-3') were obtained from the AtRTPrimer database (Han and Kim, 2006). Primers for *GLUCAN WATER-DIKNASE 1* (*GWD1*, At1g10760) and *STARCH EXCESS 4* phosphoglucan phosphatase (*SEX4*, At3g52180) were previously reported (Weise *et al*., 2013). Primers for *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3* to *5* (*SPL3-5*, At2g33810 At1g53160 At3g15270), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*, At2g45660) and *FRUITFUL* (*FUL*, At5g60910) were previously reported (Wahl *et al*., 2013).

3. Results and Discussion

Rice varieties that posses *SUB1A-1* are more tolerant to flooding stress than plants lacking this gene (Xu *et al*., 2006), the biochemical mechanism underling this tolerance is that plants express *SUB1A-1* during stress and conserve starch and free sugars reserves for longer periods improving survival (Fukao *et al*., 2006; Fukao, Yeung and Bailey-Serres, 2012).

In our previous work (Peña-Castro *et al*., 2011), *Arabidopsis* plants overexpressing *SUB1A-1* constitutively conserved several LOQS traits like hypersensitivity to ABA, reduced petiole cell elongation associated with hyponastic growth, decreased sensitivity to GA, increased lipid mobilization, and delayed flowering. In this work, we explored if *SUB1A-1* can allow *Arabidopsis* to preserve and accumulate starch and free sugars under normal growth conditions.

3.1 Improved production of fermentable sugars in OxSUB1A plants.

We analyzed two independent *Arabidopsis* over-expressing transgenic lines for each *SUB1* gene, *OxSUB1A*-L5 and *OxSUB1C*-L6 are weak over-expressing lines while *OxSUB1A*-L12 and *OxSUB1C*-L10 are strong over-expressing lines (Peña-Castro *et al*., 2011). As an experimental starting point for analysis of rosette leaves, we selected Col-0 (Wild-Type, WT) bolting time (22-day-old) since *OxSUB1A* lines have a late flowering-genotype (Peña-Castro *et al*., 2011). We hypothesized that flowering inhibition allows *OxSUB1A* plants to accumulate more carbohydrates at ZT16 (end of day).

To determine starch content and cell wall digestibility in rosette leaves of *OxSUB1A* and *OxSUB1C* transgenics, we followed a protocol reported to evaluate saccharification efficiency in

switchgrass (*Panicum virgatum*), where plant tissue is digested in two sequential steps (Chuck *et al*., 2011). In the first reaction, the plant material is used as substrate in a cellulolytic enzymatic cocktail (Accellerase 1500) to breakdown cellulose and hemicellulose into glucose and measure cell wall digestibility. In the second reaction, the tissue is digested with α-amylase and amyloglucosidase to quantify starch. For both digestions, saccharification is expressed as released glucose (Fig. 1).

The compartment that yielded most glucose in WT *Arabidopsis* was starch (8.5 mg of glucose g^{-1} FW), and then free reducing sugars (0.4 mg of glucose equivalents g^{-1} FW) followed by cell walls (0.1 mg of glucose g^{-1} FW).

When only free reducing sugars were determined (no enzymatic treatment), *OxSUB1A*-L5 had 37% more than WT whereas *OxSUB1A*-L12 did not show a significant difference (Table 1). If only cellulolytic treatment was applied, an improvement in cell wall saccharification was detected: *OxSUB1A*-L5 and *OxSUB1A*-L12 generated 16% and 23% higher yields than WT, respectively (Table 1, Supplemental Figure 1). *OxSUB1A*-L5 and *OxSUB1A*-L12 rosette tissue generated 88% and 36% more glucose from starch than WT, respectively (Table 1). These results indicate that ectopic expression of *SUB1A-1* allows plants to conserve carbohydrates, mainly starch, under non-stress conditions.

It has been reported similar saccharification improvement in switchgrass that over-express miRNA156, a strong inhibitor of the progression to flowering (Chuck *et al*., 2011). Weak miRNA156 over-expressing lines of switchgrass had better saccharification yield from starch than strongly expressing lines, probably because their growth was less impaired.

These data is consistent with previous research where rice UBI:*SUB1A-1* plants had a higher free sugar concentration when measured in aerial tissue (Fukao, Yeung and Bailey-Serres, 2012). However, these rice transgenics do not show a constitutive starch accumulation but the development is severely delayed. The effects of *SUB1A-1* on starch accumulation in rice and *Arabidopsis* may be due to different carbon allocation strategies among monocots and dicots (monocots use stems as storage organ), wild and cultivated plants, environmental cues and development stages (Bennett, Roberts and Wagstaff, 2012; Streb and Zeeman, 2013; Slewinski, 2012; Wang *et al*., 2013). For example, *sex1-1* (*gwd*) mutants in *Arabidopsis* accumulate starch and have severe developmental defects since they cannot efficiently match growth and anabolism (Weise *et al*., 2012; Paparelli *et al*., 2013), whereas development of rice *gwd* mutants is normal and only impacts grain yield even when they accumulate up to 400% more starch than WT

(Hirose *et al*., 2013). Overexpression of miRNA156 promoted starch accumulation in switchgrass but not in *Arabidopsis*, maize or tobacco (Chuck *et al*., 2011).

When the weak over-expressing line *OxSUB1C*-L6 was analyzed, it did not show a significant saccharification yield improvement in starch, cell wall or free reducing sugars (Table 1). The strong over-expressing line *OxSUB1C*-L10 showed decreased saccharification yield for starch and cell wall (-38% and -17%, respectively; Table 1) and lower free reducing sugars levels (-17%; Table 1). These data support the hypothesis that *SUB1A-1* and *SUB1C-1* control opposing biochemical mechanisms, despite belonging to the same ERF-VII gene family (Fukao *et al*., 2006; Fukao, Yeung and Bailey-Serres, 2012).

To visualize starch accumulation, we used iodine staining of 14-day-old plants of all transgenic lines and WT. ZT24 was selected as testing point to increase contrast and observe if accumulation was distinct at the end of night. The staining showed that both *OxSUB1A* lines leaves retained more starch in leaves. By contrast, the *OxSUB1C* lines retained less starch at ZT24 than WT (Fig. 2). Together these biochemical and histological data indicate that maintenance of significantly higher leaf starch is the main contributor to the improved saccharification yield of *OxSUB1A* plants. Differences in cell wall saccharification and free-sugar content are also distinct from WT but are less determining factors.

3.2 Diurnal and developmental starch accumulation patterns of OxSUB1A plants.

Leaf starch accumulation has a diurnal pattern with a peak at the end of day and consumption during the night (Bahaji *et al*., 2013; Ortiz-Marchena *et al*., 2014). To quantify if starch content could be maintained during the diurnal oscillations as suggested by iodine staining (Fig. 2), we collected 21-day-old *OxSUB1A* and WT plants at the start and middle of both day and night. WT plants accumulated starch in an expected pattern for transitory starch (Ortiz-Marchena *et al*., 2014) with a peak at the end of day (Fig. 3). *OxSUB1A* lines had the same normal accumulation pattern but conserved more starch than WT at all points tested. As previously observed at the end of the day, the weakly over-expressing *OxSUB1A*-L5 significantly doubled starch content relative to WT whereas the strongly overexpressing *OxSUB1A*-L12 had only 13-30% more (Fig. 3).

Plant development and starch accumulation are genetically coordinated, especially during floral transitions (Chuck *et al*., 2011, Ortiz-Marchena *et al*., 2014). To investigate the developmental stages where *SUB1A-1* can influence starch conservation improvement, we

collected leaves at ZT16 at two WT pre-flowering points (18 and 21-day-old) and two WT post-flowering points (27 and 31-day-old). In WT plants, starch increased as plants reached bolting time and decreased and stabilized after flowering time when cauline leaves begin to contribute to photosynthetic carbon gain (Early *et al*., 2009). Interestingly, starch content was higher in both juvenile *OxSUB1A*-L5 and -L12 lines (278% ± 23 S.E. and 189% ±9 S.E.). This difference decreased until all plants had the same starch content after flowering (Fig. 4A). Iodine starch staining at ZT24 of 14, 21 and 28-day-old rosette leaves matched the pattern of improved starch content (Fig. 4B-D).

This evaluation of diurnal and developmental kinetics further supports the conclusion that starch accumulation is responsible for the improved saccharification yield of *OxSUB1A* plants. The data also indicate that *SUB1A-1* is responsible for the starch conservation trait of the LOQS and that this phenotype is regulated in a developmental manner. Two factors involved in this developmental process are likely the flowering transcription factor *CONSTANS* (*CO*) and the florigen gene *FLOWERING LOCUS T* (*FT*); both transcripts are significantly down-regulated in *OxSUB1A* rice and *Arabidopsis* plants leading to a late transition to maturity even under an inductive flowering photoperiod (Peña-Castro *et al*., 2011).

Until recently an involvement of *CO*/*FT* in starch metabolism was not evident because *ft* and *co* mutants accumulate similar levels of starch as WT when grown under continuous light; however, mutants of *GIGANTEA (GI),* an upstream circadian regulator of *CO,* are strong starch hyperaccumulators (up to 300% of WT levels) (Eimert *et al*., 1995). Recently, the role of photoperiod in starch accumulation during the floral transition was studied and demonstrated that *CO* controls starch granule structure via differential diurnal DNA-binding patterns and developmental and diurnal regulation of *GRANULE BOUND STARCH SYNTHASE* (*GBSS*; Ortiz-Marchena *et al*., 2014). Through these events, *CO* promotes accumulation of starch granules with a higher amylose:amylopectin ratio that can be readily digested proposed to enable a carbohydrate burst that create an optimum metabolic state for flowering. With these results we hypothesize that down-regulation of *CO*/*FT* by *SUB1A-1* allows *OxSUB1A* transgenics to conserve starch that would be otherwise employed for developing inflorescence structures.

3.3 The mechanism of starch content improvement mediated by SUB1A-1.

Late flowering has been related to improved starch saccharification by mechanisms other than those directly regulated by *CO*. For example, in switchgrass engineered to over-express

miRNA156, young nodes accumulated more starch than WT mature nodes (Chuck *et al*., 2011). However, miRNA156 is a repressor of juvenile-maturity transition through a *CO* parallel pathway that was recently shown to be connected to T6P (Wahl *et al*., 2013; Yang *et al*., 2013), a repressor of starch catabolism through KIN10 signaling (Baena-González *et al*., 2007; Delatte *et al*., 2011). Defects in enzymatic starch mobilization also lead to late flowering, starch accumulation and size defects (Streb and Zeeman, 2012; Paparelli *et al*., 2013).

To obtain insight into mechanisms that are different or parallel to *CO* regulation of starch accumulation in *OxSUB1A* transgenics, we measured polyphasic fluorescence rise (OJIP kinetics). This method has been used to detect photosynthetically-improved plants with increased carbohydrate accumulation (Gururani *et al*., 2012). However, no significant differences were detected between the five genotypes analyzed in this study (Table S1), indicating that neither *OxSUB1A* nor *OxSUB1C* transgenics posses photosystem efficiency that differs from WT.

Although starch granule architecture and biosynthesis is not a well-understood process (Fettke *et al*., 2011; Stren and Zeeman, 2012), altered shape and size have been reported in some mutants (Zeeman *et al*., 2002; Zhang *et al*., 2008). We isolated and examined starch granules architecture by scanning electron microscopy and found that starch from *OxSUB1A* 21-day-old rosette leaves had the same size and characteristic ellipsoid-like shape of those of WT leaves of the same age (Supplemental Figure 2).

In our previously reported microarray studies at ZT8 of *OxSUB1A* and *OxSUB1C* seedlings (Peña-Castro *et al*., 2011), we did not detect a significant change in accumulation of mRNAs related to starch biosynthesis or catabolism. However, since these genes have a circadian oscillation, mostly peaking after midday (Smith *et al*., 2004) we searched our datasets for statistical outliers associated with this biological process that were up- or down-regulated and evaluated them in RNA from seedlings samples collected at ZT16 (end of day). We tested transcripts encoding starch degrading enzymes *GWD1* and *SEX4* between WT and *OxSUB1A* or *OxSUB1C* but found not significant difference in expression.

Recently, it was demonstrated that in parallel to *CO*, transcription factors of the *SQUAMOSA PROMOTER BINDING PROTEIN* gene family (*SPL3-5*) connect carbohydrate metabolism to the juvenile transition and also lead to late flowering phenotypes (Wahl *et al*., 2013). In switchgrass, down-regulation of *SPL3-5* by miRNA156 promotes late flowering and improvement of saccharification yield by both amylolytic and cellulolytic treatments, without modulation of *CO/FT* ortholog transcripts (Chuck *et al*., 2011), supporting the idea that *CO* and *SPL*/miRNA156 are parallel pathways in leaves that impact flowering time (Wahl *et al*., 2013).

To test if delayed juvenile-maturity transition in *OxSUB1A* is related to *SPL3-5*, we measured transcripts of *SPL3-5* and downstream genes *SOC1* and *FUL* in 7-d-old seedlings at ZT16. These transcripts were also statistical outliers down-regulated in our microarrays. The expression of all these transcripts was similar to that of WT plants suggesting independent activity from *SPL*/miRNA156.

In addition to *CO* and *SPL*/miRNA156, post-translational regulation of starch synthesis enzymes by reactive oxygen species (Lepisto *et al*., 2013) and T6P signaling through the stress integrating kinase *SnRK1* regulate starch levels (Baena-González *et al*., 2007; Mattos *et al*., 2013). T6P is of particular interest for further research since microarray studies of submergence stress response in different plants indicate there is a dynamic change in the transcripts of trehalose-6-phosphate synthase and trehalose phosphate phosphatase (Jung *et al*., 2010; Lee *et al*., 2011; Narsai and Whelan, 2013; van Veen *et al*., 2013; Tamang *et al*., 2014).

3.4 Hardness of OxSUB1A leaves.

In earlier transcriptome analysis we found that *SUB1A-1* promoted in *Arabidopsis* the up-regulation of 17 genes associated with modification of the cell wall and/or biotic stress response, including endotransglycosylase (*XTR3*, *XTR6*), expansin (*AtEXP2*) and glucan-1,3,-beta-glucosidase (*BGL2*, Peña-Castro *et al*., 2014). This latter gene was the most up-regulated transcript relative to WT in 7-d-old seedlings. In addition to their biological importance, cell wall associated proteins are also of technological interest for the development of bioethanol fuel. They consist of enzymes and proteins that can change the mechanical properties of cell wall polymers (cellulose, hemicellulose, lignin and callose) improving cell wall digestibility and saccharification yields (Arantes and Saddler, 2010).

To evaluate if the expression of cell wall associated genes in our transgenics was correlated with a phenotype with modified mechanical properties, we employed a texture analyzer to measure leaf resistance to puncture in 23-day-old rosette leaves. Both *OxSUB1A*-L5 and *OxSUB1A*-L12 leaves offered significantly less resistance to puncture stress than WT (67% ±18 S.D. and 70% ±11% S.D., respectively). *OxSUB1C* lines were not statistically different from WT (Fig. 5A). To confirm expression of *BGL2* and *AtEXP2* in *OxSUB1A* and *OxSUB1C*, RNA from 7-day-old seedlings at ZT8 were tested by qRT-PCR. *OxSUB1A*-L5 and *OxSUB1A*-L12 expressed more *BGL2* (Fig. 5B) and *AtEXP2* transcripts (Fig. 5C). WT and *OxSUB1C*

accumulated similar *BGL2* mRNA levels, whereas *OxSUB1C*-L6 had 3-fold more *AtEXP2* than WT; however this was not replicated in *OxSUB1C*-L10 (Fig. 5C).

BGL2 belongs to a multigene family of hydrolytic enzymes involved in fungal pathogen defense and developmental processes related to callose, a transitory β-1,3-glucan relevant for cell wall maturation (Dokey *et al*., 2007; Park *et al*., 2014). The rice response during submergence stress includes the expression of genes associated to pathogen stress, and the presence of *SUB1A-1* improves this induction (Jung *et al*., 2010). In *Arabidopsis, SUB1A-1* also promoted the constitutive expression of these genes (Peña-Castro *et al*., 2011). The biotic stress component of the submergence stress response primes plants to resist the pathogens that may increase their access to plant tissue during submergence (Hsu *et al*., 2013).

When rice plants are submerged, plants encoding *SUB1A-1* induce *EXPANSIN* transcripts early in the stress and restrict them in later stages to conserve energy (Fukao *et al*., 2006). Expansins are cell wall morphogenic proteins that allow non-enzymatic loosening of cellulose and make it more accessible for enzymes during cell expansion (Arantes and Saddler, 2010). Our expression analysis indicates that *EXPANSIN* induction is conserved in *OxSUB1A* transgenics in non-stress growth conditions (Fig. 5C). AtEXP2 is a GA-responsive *EXPANSIN* normally active during seed germination (Yan *et al*., 2014).

Together, these data provide evidence that the expression of cell wall and biotic response associated genes mobilized by *SUB1A-1* is correlated to a phenotype with decreased mechanical strength and improved cellulose digestibility.

3.5 Further optimization of SUB1A-1 saccharification yield improvement

Strong constitutive starch conservation in plants is frequently accompanied with a growth penalty derived from their inability to efficiently use this energy reserve (Chuck *et al*., 2011; Weise *et al*., 2012; Paparelli *et al*., 2013); we could observe in our *OxSUB1A* transgenics such penalty, especially after flowering time (Fig. 4C-D). Weak cell walls also risk the plant to suffer pathogen attacks or suboptimal biomechanics (Nigorikawa *et al*., 2012; Petersen *et al*., 2012). These negative features would compromise the development of industrial applications based on plants with improved saccharification traits. A proposed solution to these drawbacks is the use of inducible promoters (Weise *et al*., 2012) or tissue-specific promoters (Petersen *et al*., 2012). Additional research focusing on these point is needed to further optimize and implement a

biotechnological strategy to improve biomass saccharification yield based on the promising *SUB1A-1* mediated starch conservation and cell wall digestibility.

4. Conclusion

The economies of both industrialized and developing nations are currently based on fuels obtained from petroleum and other hydrocarbon reserves. Plant biotechnology can help the transition towards renewable sources and make energy extraction a more sustainable activity. In this work we demonstrated that ectopic overexpression of the rice *SUB1A-1* gene in *Arabidopsis* confers phenotypes with desirable traits for bioethanol production (Supplemental Figure 3). *SUB1A-1* maintained the starch conservation phenotype of LOQS under normal growth conditions, improving the amylolytic saccharification yield. Additionally, up-regulation of cell wall associated transcripts associated with cell wall loosening by *SUB1A-1* improved cell walls deconstruction. With this information, we propose heterologous *SUB1A-1* expression as a new alternative for plant biomass improvement as raw material for bioethanol production.

Acknowledgements

We thank Dr. José Abad, Dr. Jacqueline Capataz, Dr. Sandra del Moral, Eng. Juan Hernández and Dr. Enrique Villalobos (UNPA-Tuxtepec) for sharing equipment, reagents and laboratory space, Ms. Fabiola Hernández and Lic. Héctor López (UNPA-Tuxtepec) for administrative assistance, Dr. Gladis Labrada (IPICYT) for technical assistance with SEM and Prof. Julia Bailey-Serres (UC-Riverside) for *OxSUB1* seeds, thoughtful discussions and reviewing the preliminary manuscript.

References

1. Arantes V, Saddler JN. 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotechnology for Biofuels* 3:4.
2. Baena-González E, Rolland F, Thevelein JM, Sheen J. 2007. A central integrator of transcription networks in plant stress and energy signalling. *Nature* 448:938-943.
3. Bahaji A, Li J, Ovecka M, Ezquer I, Muñoz FJ, Baroja-Fernández E, Romero JM, Almagro G, Montero M, Hidalgo M, Sesma MT, Pozueta-Romero J. 2011. *Arabidopsis thaliana* mutants lacking ADP-glucose pyrophosphorylase accumulate starch and wild-

type ADP-glucose content: further evidence for the occurrence of important sources, other than ADP-glucose pyrophosphorylase, of ADP-glucose linked to leaf starch biosynthesis. *Plant and Cell Physiology* 57:1162-1176.
4. Bahaji A, Li J, Sánchez-López AM, Baroja-Fernández E, Muñoz FJ, Ovecka M, Almagro G, Montero M, Ezquer I, Etxeberria E, Pozueta-Romero J. 2013. Starch biosynthesis, its regulation and biotechnological approaches to improve crop yields. *Biotechnology Advances* 32:87-106.
5. Bailey-Serres J, Lee SC, Brinton E. 2012. Waterproofing crops: effective flooding survival strategies. *Plant Physiology* 160:1698-1709.
6. Bailey-Serres J, Voesenek LACJ. 2008. Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology* 59:313-339.
7. Bennett E, Roberts JA, Wagstaff C. 2012. Manipulating resource allocation in plants. *Journal of Experimental Botany* 63:3391-3400.
8. Chuck GS, Tobias C, Sun L, Kraemer F, Li C, Dibble D, Arora R, Bragg JN, Vogel JP, Singh S, Simmons BA, Pauly M, Hake S. 2011. Overexpression of the maize *Corngrass1* microRNA prevents flowering, improves digestibility, and increases starch content of switchgrass. *Proceedings of the National Academy of Sciences USA* 108:17550-17555.
9. Chundawat SPS, Beckham GT, Himmel ME, Dale BE. 2011. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annual Review of Chemical and Biomolecular Engineering* 2:121-145.
10. Delatte TL, Sedijani P, Kondou Y, Matsui M, de Jong GJ, Somsen GW, Wiese-Klinkenberg A, Primavesi LF, Paul MJ, Schluepmann H. 2011. Growth arrest by trehalose-6-phosphate: an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway. *Plant Physiology* 157:160-174.
11. Doxey AC, Yaish MWF, Moffatt BA, Griffith M, McConkey BJ. 2007. Functional divergence in the *Arabidopsis* β-1,3-glucanase gene family inferred by phylogenetic reconstruction of expression states. *Molecular Biology and Evolution* 24:1045-1055.
12. Early EJ, Ingland B, Winkler J, Tonsor SJ. 2009. Inflorescences contribute more than rosettes to lifetime carbon gain in *Arabidopsis thaliana* (Brassicaceae). *American Journal of Botany* 96:786–792.
13. Eimert K, Wang S-M, Lue W-L, Chen J. 1995. Monogenic recessive mutations causing both late floral initiation and excess starch accumulation in *Arabidopsis*. *The Plant Cell* 7:1703-1712.
14. Fettke J, Malinova I, Albrecht T, Hejazi M, Steup M. 2011. Glucose 1-phosphate transport into protoplasts and chloroplasts from leaves of *Arabidopsis thaliana*. *Plant Physiology* 155:1723-1734.

15. Fukao T, Bailey-Serres J. 2008. Submergence tolerance conferred by *Sub1A* is mediated by SLR1 and SLRL1 restriction of gibberellin responses in rice. *Proceedings of the National Academy of Sciences USA* 105:16814-16819.
16. Fukao T, Harris T, Bailey-Serres J. 2009. Evolutionary analysis of the *Sub1* gene cluster that confers submergence tolerance to domesticated rice. *Annals of Botany* 104:143-150.
17. Fukao T, Xiong L. 2013. Genetic mechanisms conferring adaptation to submergence and drought in rice: simple or complex?. *Current Opinion in Plant Biology* 16:196-204.
18. Fukao T, Xu K, Ronald PC, Bailey-Serres J. 2006. A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. *The Plant Cell* 18: 2021-2034.
19. Fukao T, Yeung E, Bailey-Serres J. 2012. The submergence tolerance gene *SUB1A* delays leaf senescence under prolonged darkness through hormonal regulation in rice. *Plant Physiology* 160:1795–1807.
20. Gururani MA, Upadhyaya CP, Strasser RJ, Woong YJ, Park SW. 2012. Physiological and biochemical responses of transgenic potato plants with altered expression of PSII manganese stabilizing protein. *Plant Physiology and Biochemistry* 58:182-194.
21. Han S, Kim D. 2006. AtRTPrimer: database for *Arabidopsis* genome-wide homogeneous and specific RT-PCR primer-pairs. *BMC Bioinformatics* 7:179.
22. Henry RJ. 2010. Evaluation of plant biomass resources available for replacement of fossil oil. *Plant Biotechnology Journal* 8:288-293.
23. Hirose T, Aoki N, Harada Y, Okamura M, Hashida Y, Ohsugi R, Miyao A, Hirochika H, Terao T. 2013. Disruption of a rice gene for α-glucan water dikinase, *OsGWD1*, leads to hyperaccumulation of starch in leaves but exhibits limited effects on growth. *Frontiers in Plant Science* 4: 147.
24. Hsu F-C, Chou M-Y, Chou S-J, Li Y-R, Peng H-P, Shih M-C. 2013. Submergence confers immunity mediated by the WRKY22 transcription factor in *Arabidopsis*. *The Plant Cell* 25:2699-2713.
25. Jung KH, Seo YS, Walia H, Cao P, Fukao T, Canlas PE, Amonpant F, Bailey-Serres J, Ronald PC. 2010. The submergence tolerance regulator *Sub1A* mediates stress-responsive expression of AP2/ERF transcription factors. *Plant Physiology* 152:1674-1692.
26. Karnaouri A, Topakas E, Paschos T, Taouki I, Christakopoulos P. 2013. Cloning, expression and characterization of an ethanol tolerant GH3 β-glucosidase from *Myceliophthora thermophila*. PeerJ 1:e46
27. Lee SC, Mustroph A, Sasidharan R, Vashisht D, Pedersen O, Oosumi T, Voesenek LACJ, Bailey-Serres J. 2011. Molecular characterization of the submergence response of the *Arabidopsis thaliana* ecotype Columbia. New Phytologist 190:457-471.

28. Lepisto A, Pakula E, Toivola J, Krieger-Liszkay A, Vignols F, Rintamaki E. 2013. Deletion of chloroplast NADPH-dependent thioredoxin reductase results in inability to regulate starch synthesis and causes stunted growth under short-day photoperiods. *Journal of Experimental Botany* 64: 3843-3854.
29. Lionetti V, Francoccia F, Ferraria S, Volpi C, Bellincampi D, Galletti R, D'Ovidio R, De Lorenzo G, Cervonea F. 2010. Engineering the cell wall by reducing de-methyl-esterified homogalacturonan improves saccharification of plant tissues for bioconversion. *Proceedings of the National Academy of Sciences USA* 107:616-621.
30. Mattos Martins MC, Hejazi M, Fettke J, Steup M, Feil R. 2013. Inhibition of starch degradation in *Arabidopsis* leaves mediated by trehalose 6-phosphate. *Plant Physiology* 163:1142-1163.
31. Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 31:426–428.
32. Mustroph A, Lee SC, Oosumi T, Zanetti ME, Yang H, Ma K, Yaghoubi-Masihi A, Fukao T, Bailey-Serres J. 2010. Cross-kingdom comparison of transcriptomic adjustments to low-oxygen stress highlights conserved and plant-specific responses. *Plant Physiology* 152:1484-1500.
33. Narsai R, Whelan J. 2013. How unique is the low oxygen response? An analysis of the anaerobic response during germination and comparison with abiotic stress in rice and *Arabidopsis*. *Frontiers in Plant Science* 4:349.
34. Nigorikawa M, Watabnabe A, Furukawa K, Sonoki T, Ito Y. 2012. Enhanced saccharification of rice straw by overexpression of rice exo-glucanase. *Rice* 5: 14.
35. Niroula RK, Pucciariello C, Ho VT, Novi G, Fukao T, Perata P. 2012. *SUB1A*-dependent and -independent mechanisms are involved in the flooding tolerance of wild rice species. *The Plant Journal* 72:282-293.
36. Ortiz-Marchena MI, Albi T, Lucas-Reina E, Said FE, Romero-Campero FJ, Cano B, Ruiz MT, Romero JM, Valverde F. 2014. Photoperiodic control of carbon distribution during the floral transition in *Arabidopsis*. *The Plant Cell* 26:565-584.
37. Ovecka M, Bahaji A, Muñoz FJ, Almagro G, Ezquer I, Baroja-Fernández E, Li J, Pozueta-Romero J. 2012. A sensitive method for confocal fluorescence microscopic visualization of starch granules in iodine stained samples. *Plant Signaling and Behavior* 7:1146-1150.
38. Paparelli E, Parlanti S, Gonzali S, Novi G, Mariotti L, Ceccarelli N, van Dongen JT, Kolling K, Zeeman SC, Perata P. 2013. Nighttime sugar starvation orchestrates gibberellin biosynthesis and plant growth in *Arabidopsis*. *The Plant Cell* 25:3760-3769.
39. Park E, Díaz-Moreno SM, Davis DJ, Wilkop TE, Bulone V, Drakakaki G. 2014. Endosidin 7 specifically arrests late cytokinesis and inhibits callose biosynthesis,

revealing distinct trafficking events during cell plate maturation. *Plant Physiology* 165:1019-1034.
40. Peña-Castro JM, van Zanten M, Lee SC, Patel MR, Fukao T, Voesenek LACJ, Bailey-Serres J. 2011. Expression of rice *SUB1A* and *SUB1C* transcription factors in *Arabidopsis* uncovers flowering inhibition as a submergence-tolerance mechanism. *The Plant Journal* 67: 434-446.
41. Petersen PD, Lau J, Ebert B, Yang F, Verhertbruggen Y, Kim JS, Varanasi P, Suttangkakul A, Auer M, Loqué D, Scheller HV. 2012. Engineering of plants with improved properties as biofuels feedstocks by vessel-specific complementation of xylan biosynthesis mutants. *Biotechnology for Biofuels* 5:84.
42. Pucciariello C, Perata P. 2013. Quiescence in rice submergence tolerance: an evolutionary hypothesis. *Trends in Plant Science* 18:377-381.
43. Ribeiro Reis ER, Dias Brito da Cunha B, Kelly Martins P, Bazzo Martins MT, Alekcevetch JC, Chalfun-Júnior A, Carvalho Andrade A, Ribeiro AP, Qin F, Mizoi J, Yamaguchi-Shinozaki K, Nakashima K, Corrêa Carvalho JF, Ferreira de Sousa CA, Lima Nepomuceno A, Kenji Kobayashi A, Correa Molinari HB. 2014 Induced over-expression of *AtDREB2A CA* improves drought tolerance in sugarcane. *Plant Science* 221-222:59-68.
44. Slewinski TL. 2012. Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and biofuel production. *Journal of Experimental Botany* 63:4647-4670.
45. Smith SM, Fulton DS, Chia T, Thorneycroft D, Chapple D, Dunstan H, Hylton C, Zeeman SC, Smith AM. 2004. Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in *Arabidopsis* leaves. *Plant Physiology* 136:2687-2699.
46. Stamatiou G, Vidaurre DP, Shim I, Tang X, Moeder W, Bonetta D, McCourt P. 2013. Forward genetic screening for the improved production of fermentable sugars from plant biomass. *PLOS One* 8: e55616.
47. Stamm P, Verma V, Ramamoorthy R, Kumar PP. 2012. Manipulation of plant architecture to enhance lignocellulosic biomass. *AoB Plants* 2012:pls026.
48. Streb S, Zeeman SC. 2012. Starch metabolism in *Arabidopsis*. Arabidopsis Book 10, e0160.
49. Tamang BG, Magliozzi JO, Maroof MAS, Fukao T (2014) Physiological and transcriptomic characterization of submergence and reoxygenation responses in soybean seedlings. *Plant Cell and Environment* 37:2350-2365.
50. van Veen H, Mustroph A, Barding GA, Vergeer-van-Eijk M, Welschen-Evertman RAM, Pedersen O, Visser EJW, Larive CK, Pierik R, Bailey-Serres J, Voesenek LACJ,

Sasidharan R. 2013. Two *Rumex* species from contrasting hydrological niches regulate flooding tolerance through distinct mechanisms. *The Plant Cell* 25:4691-4707.
51. Vanholme B, Desmet T, Ronsse F, Rabaey K, Van Breusegem F, de Mey M, Soetaert W, Boerjan W. 2013. Towards a carbon-negative sustainable bio-based economy. *Frontiers in Plant Science* 4: 174.
52. Waclawovsky AJ, Sato PM, Lembke CL, Moore PH, Souza GM. 2010. Sugarcane for bioenergy production: an assessment of yield and regulation of sucrose content. *Plant Biotechnology Journal* 8:263-276.
53. Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, Feil R, Lunn JE, Stitt M, Schmid M. 2013. Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana*. *Science* 339:704-707.
54. Wang J, Nayak S, Koch K, Ming R. 2013. Carbon partitioning in sugarcane (*Saccharum* species). Fronters in Plant Science 4,201.
55. Weise SE, Aung K, Jarou ZJ, Mehrshahi P, Li Z, Hardy AC, Carr DJ, Sharkey TD. 2012. Engineering starch accumulation by manipulation of phosphate metabolism of starch. *Plant Biotechnology Journal* 10:545-554.
56. Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, Coupland G. 2006. CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering in *Arabidopsis*. *The Plant Cell* 18:2971-2984.
57. Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ. 2006. Sub1A is an ehtylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* 442:705-708.
58. Yan A, Wu M, Yan L, Hu R, Ali I, Gan Y. 2014. *AtEXP2* is involved in seed germination and abiotic stress response in *Arabidopsis*. *PLOS One* 9:e85208.
59. Yang L, Xu M, Koo Y, He J, Poethig RS. 2013. Sugar promotes vegetative phase change in *Arabidopsis thaliana* by repressing the expression of *MIR156A* and *MIR156C*. *eLife* 2:e00260.
60. Zeeman SC, Tiessen A, Pilling E, Kato KL, Donald AM, (2002) Starch synthesis in *Arabidopsis*. Granule synthesis, composition, and structure. *Plant Physiology* 129: 516-529.

61. Zhang X, Szydlowski N, Delvallé D, D'Hulst C, James MG, Myers AM. 2008. Overlapping functions of the starch synthases SSII and SSIII in amylopectin biosynthesis in *Arabidopsis*. *BMC Plant Biology* 8:96.

Table 1. Free reducing sugars, cell wall digestibility and starch content of 22-day-old rosette leaves *Arabidopsis* WT and transgenics expressing rice *SUB1A-1* or *SUB1C-1*.

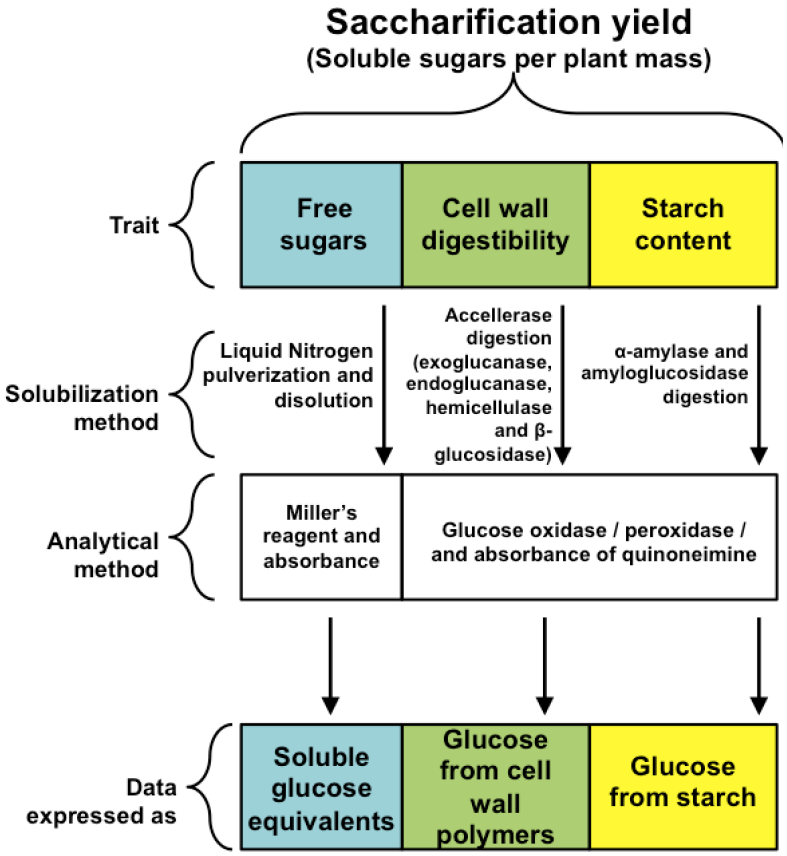
	Free reducing sugar[1] (mg of reducing sugars g^{-1} FW) / % of WT	**Cell wall digestibility[2] (mg of glucose g^{-1} FW) / % of WT**	**Starch content[3] (mg of glucose g^{-1} FW) / % of WT**
Col-0 WT[4]	0.40 ± 0.01 a	0.100 ± 0.004 a	8.5 ± 2.7 a
***OxSUB1A* L5**	0.54 ± 0.02 / +37 b	0.116 ± 0.008 / + 16 b	15.9 ± 0.7 / +88 b
***OxSUB1A* L12**	0.44 ± 0.03 / +10 a	0.123 ± 0.010 / +23 b	11.6 ± 0.8 / + 36 c
***OxSUB1C* L6**	0.42 ± 0.02 / +6 a	0.101 ± 0.012 / +1 a	10.6 ± 1.1 / + 25 ac
***OxSUB1C* L10**	0.33 ± 0.01 / -17 c	0.062 ± 0.005 / - 38 c	7.05 ± 0.5 / -17 d

[1] As measured by Miller's reagent (dinitrosalicylic acid).

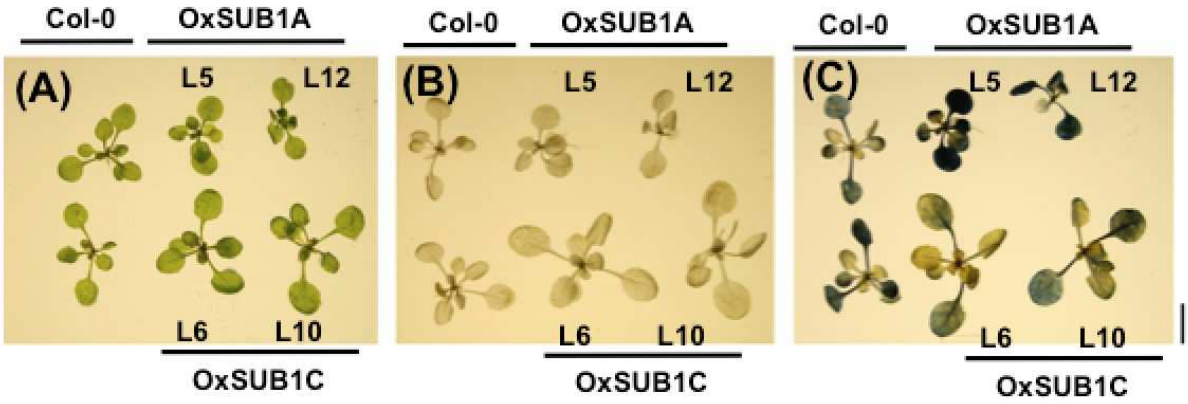
[2] After 24 h saccharification with Accellerase enzyme mix

[3] After amylase/amyloglucosidase digestion.

[4] Different letters indicate a significant difference between genotypes ($P<0.05$, means comparison, Student's *t* test). Values are means ±S.E. of three independent experimental replicates, each with n=5 plants.



423 **Figure 1.** Experimental strategy followed to quantify the contribution of three different plant
424 carbohydrates compartments to saccharification yield.



425 **Figure 2.** Iodine staining of 14-day-old rosette leaves at the end of night. (A) Formaldehyde
426 infiltrated plants. (B) 80% hot ethanol destained plants. (B) Stained plants show starch as a dark-
427 blue precipitate. Black bar is 1cm.

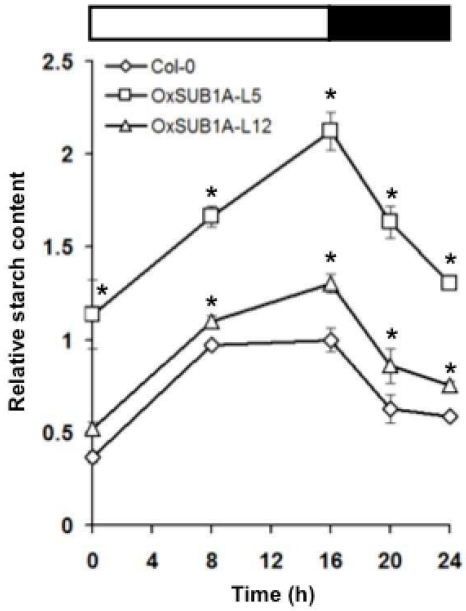


Figure 3. Diurnal oscillation of starch content of 21-day-old *Arabidopsis* plants expressing rice *SUB1A-1*. Upper bar indicates day (open) and night (black) time (16 h day / 8 h night). Asterisks indicate a significant difference between genotypes ($P<0.05$, Student's *t* test). Data were normalized to Col-0 maximum value at the end of the day ZT16 (6.6 mg of glucose g^{-1} FW). Values are means of three independent experimental replicates, each with n=5 plants. Error bars are ±S.E.

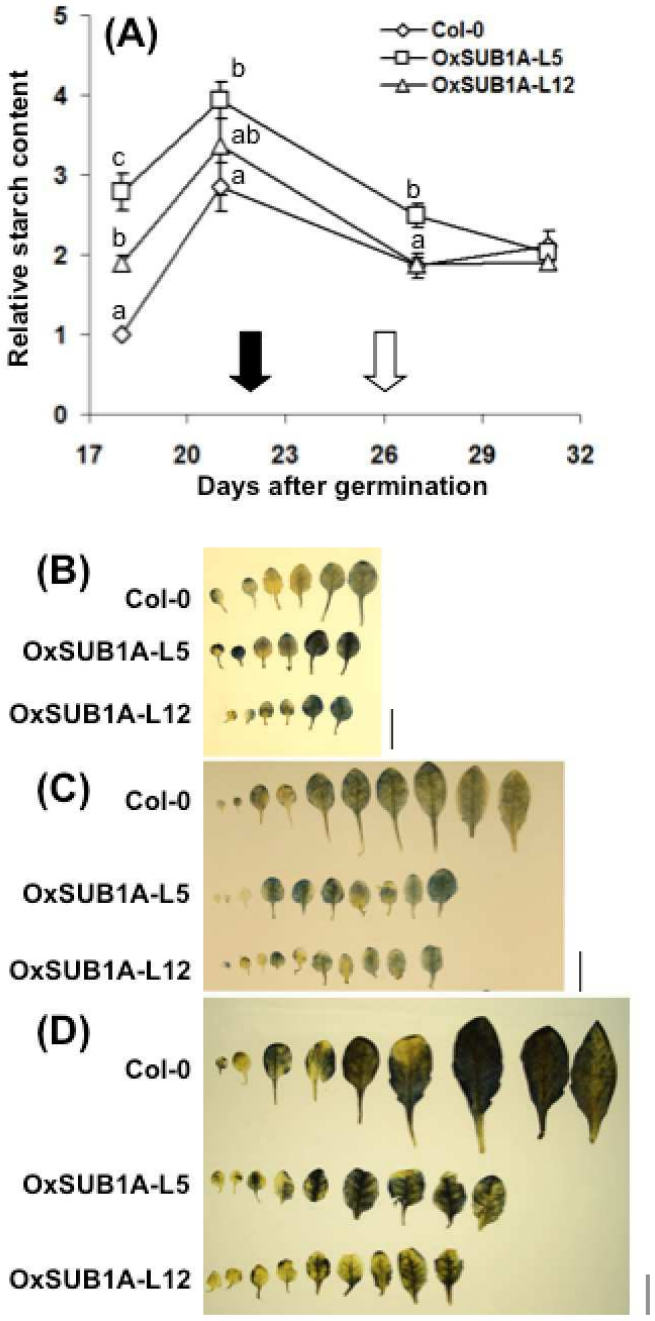
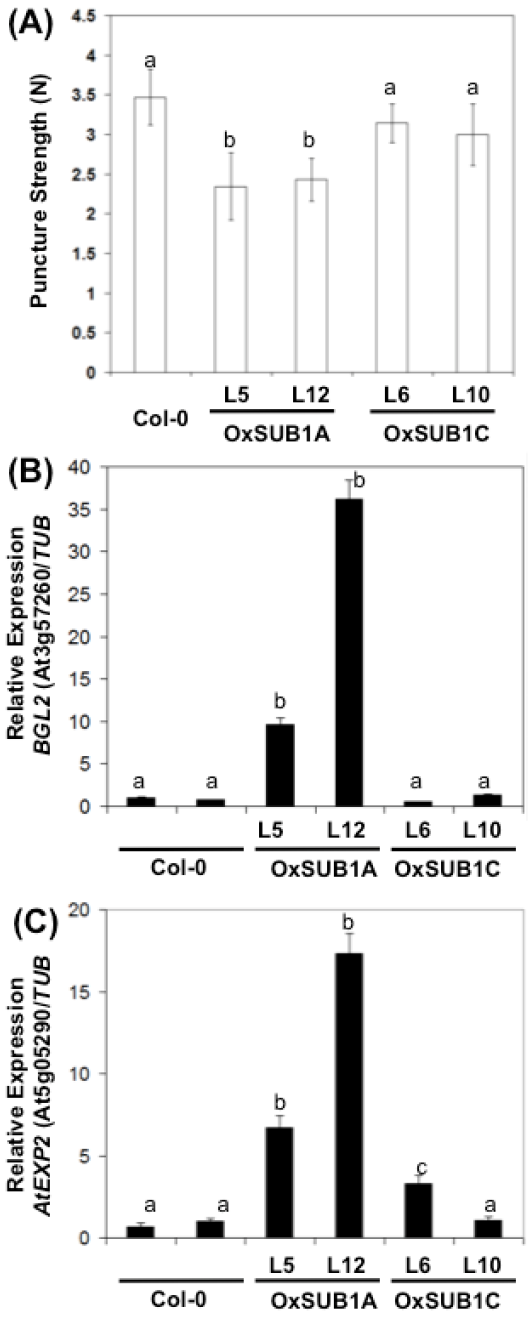


Figure 4. Developmental progression of starch accumulation of *Arabidopsis* plants expressing *SUB1A-1*. (A) Plants were grown (16 h day / 8 h night) and collected at ZT16. Black and white arrows indicate Col-0 and *OxSUB1A* budding day, respectively. Different letters indicate a significant difference between genotypes on the same day ($P<0.05$, Student's *t* test). Data were normalized to 18-day-old Col-0 value at ZT16 (6.1 mg of glucose g^{-1} FW). Values are means of three independent experimental replicates, each with n=5 plants. Error bars are ±S.E. (B-D) Iodine staining of Col-0 and *OxSUB1A* rosette leaves at (B) 14 day, (C) 21 day and (D) 28 day after germination. Black bar is 1cm.



442 **Figure 5.** Leaf hardness phenotype of *OxSUB1A* and *OxSUB1C* transgenics. **(**A) Hardness
443 comparison of rosette leaves from *Arabidopsis* Col-0 and plants expressing rice *SUB1A-1* and
444 *SUB1C-1* genes was determined by a puncture resistance test on 23-day-old plants. Different
445 letters indicate significant difference with Col-0 ($P<0.01$, Student's *t* test). Values are means of
446 n=7 to 13 plants. Error bars are ±S.D.
447 (B-C) Transcript accumulation in 7-day-old seedlings at ZT8 (middle of the day) of Col-0,
448 *OxSUB1A* and *OxSUB1C* transgenics. (A) *BGL2* transcript, (B) *AtEXP2* transcript. Transcript

449 abundance was determined by quantitative RT-PCR and normalized to abundance in Col-0.
450 Experiments were performed twice with similar results. Values are means ± S.E.