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Rational identification of target enzymes for starch improvement through system-level analysis of a potato tuber model

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Abstract

In this study, identification of target enzymes for starch improvement through system-level analysis of a potato tuber model is presented. A kinetic model representing the conversion of sucrose to starch in potato tubers was employed. This model was used to predict the percentage of amylose content (AC%) and starch content in potato tubers in response to perturbations of enzyme activities. The predicted AC% (23%) was found to be in the range of the actual AC% reported in literature. The model could satisfactorily predict the response trend to down-regulation of enzymes on the starch content and AC% (Pearson's correlation coefficients > 0.9). To identify the target enzymes, the sensitivity of starch content and AC% to changes in the activity of each enzyme within the model was assessed by sensitivity analysis. The enzymes identified as targets were those to which starch content and AC% were found to be highly sensitive. The analysis revealed that the targets for increasing the starch content in potato tubers were inorganic pyrophosphatase (iPPtase), starch synthase (SS), granule-bound starch synthase (GBSS), and ADP-glucose pyrophosphorylase. Also, SS and GBSS were found to be targets for altering AC%. By dual perturbation studies, the increase of activities of both iPPtase and SS simultaneously was found to further improve the starch content. In addition, the model was applied to predict relative changes in tuber metabolite profiles to infer physiological changes of metabolically-engineered tubers. The suggested target enzymes and inferred tuber physiology are useful guidelines for rational metabolic engineering towards starch improvement in potatoes.

Keywords: amylose content; mathematical model; metabolic engineering; sensitivity analysis; starch improvement.
Abbreviations: AC-amylose content, AC%-the percentage of AC; ADP-adenosine diphosphate; ADPglc-adenosine diphosphoglucose or ADP-glucose; AL-amylose (unbranched glucan); AP-amylopectin (insoluble branched glucan); ATP-adenosine triphosphate; F6P-fructose-6-phosphate; Fru-fructose; G1P-glucose-1-phosphate; G1P-glucose-1-phosphate; G6P-glucose-6-phosphate; Glc-glucose; LG-linear glucan (unbranched glucan); PG-phytoglycogen (soluble branched glucan); Pi-inorganic orthophosphate; PP-inorganic pyrophosphate; S6P-sucrose-6-phosphate; Suc-Sucrose; Suc_supply-sucrose supply (external sucrose); UDP-uridine 5'-diphosphate; UDPglc-uridine diphosphoglucose or UDP-glucose; UTP-uridine 5'-triphosphate; AGPase-ADP-glucose pyrophosphorylase; ATPT-ADP/ATP transporter; bGly-glycolysis; DBE-debranching enzyme; FK-fructokinase; G6PT-G6P/Pi transporter; GBSS-granule-bound starch synthase; HK-hexokinase; INV-cytosolic invertase; iPPtase-inorganic pyrophosphatase; PGI-phosphoglucose isomerase; PGM-phosphoglucomutase; PiT-phosphate transporter; pPGM-plastidial phosphoglucomutase; SBE-starch branching enzyme; SPP-sucrose phosphate phosphatase; SPS-sucrose phosphate synthase; SS-starch synthase; ST-sucrose transporter; SuSy-sucrose synthase; UGPase-UDP-glucose pyrophosphorylase; cyt-cytosol; amp-amyloplast.

Introduction

Interest in starchy crop improvement has increased drastically, driven by the worldwide demand of starch for food, industries and especially biofuel production. In potato, cassava, maize, and rice, metabolic engineering has been employed to improve starch yield and physicochemical properties such as viscosity and clarity, which are dependent on the ratio of amylose (AL) to amylopectin (AP) or percentage of amylose content (AC%) (Raemakers et al., 2005; Smith, 2008). The metabolic pathway of starch synthesis, which is located in the amyloplast, has been the focus of considerable research. Glucose-6-phosphate (G6P) is taken up into amyloplasts and then converted to ADP-glucose (ADPglc) through reactions catalyzed by two enzymes: plastidial phosphoglucomutase (pPGM) and ADP-glucose pyrophosphorylase (AGPase). ADPglc is used as a precursor for the synthesis of the two glucose polymers that make up starch (AL and AP). This starch synthesis process requires the co-operation of at least four enzymes: starch

synthase (SS), granule-bound starch synthase (GBSS), starch branching enzyme (SBE), and debranching enzyme (DBE) (Smith, 2001; Fernie et al., 2002). Many enzymes involved in starch synthesis have been studied as targets for metabolic engineering in order to alter amylose content (AC) and/or improve starch content. GBSS is assumed to be responsible for amylose synthesis (Smith et al., 1997), and several studies have shown that inhibition of its expression leads to decreased AC (Visser et al., 1991; Kuipers et al., 1994; Raemakers et al., 2005). Also, the inhibition of SBE results in very high levels of AL (Schwall et al., 2000). AGPase has been of interest as the target for the improvement of starch content for decades. It drives the reaction that converts glucose-1-phosephate (G1P) to ADPglc, which is reported to have a relationship with the AC in pea embryos (Clarke et al., 1999). In addition, inhibition of AGPase activity led to a marked decrease of the starch content in potato tubers (Müller-Röber et al., 1992). However, other studies reported that the increase in AGPase activities in transgenic potatoes does not always result in the improvement of the starch content (Stark et al., 1992; Sweetlove et al., 1996).

Besides enzymes in the starch synthesis pathway, the uptake rates of some precursors for starch synthesis, i.e., G6P and ATP, into the amyloplast have also been considered. Zhang et al. (2008) found that an increase in ATP uptake rate by an over-expression of ATP/ADP transporter (ATPT) protein did not significantly influence starch production in potato tubers, even though the level of this transporter was reported to positively correlate with the starch level (Tjaden et al., 1998; Geigenberger et al., 2001). Zhang et al. (2008) also found that an increase in G6P uptake by an over-expression of the G6P/Pi (inorganic orthophosphate) transporter, or G6PT, did not result in increased starch production. Instead, the starch content increased significantly when the uptake rate of both G6P and ATP was increased by double over-expression of both transporter proteins. Furthermore, the enzymes in the sucrose breakdown pathway converting sucrose to G6P in cytosol have been studied. Trethewey et al. (1998) reported that the combined over-expression of invertase and glucokinase, which was expected to enhance the capacity of sucrose breakdown flux into G6P, reduced the fluxes of the starch synthesis pathways in potato tubers. Increase in the cytosolic invertase activity was also found to have a negative effect on starch synthesis during potato tuber development (Hajirezaei et al., 2000). Consequently, the literature shows that there are many enzymes that can be candidates for starchy crop improvement. However, the effects of these enzymes on the starch quantity and quality of engineered plants are unpredictable and unclear. There are no tools currently available for the rational identification of target enzymes for starch production. To reduce time consumed by experiments in the laboratory for clarifying such effects and identifying enzymes, a mathematical model may be helpful. In plants, there have been models developed for a number of processes, including photosynthesis in leaves (Pettersson and Ryde-Pettersson, 1988; Poolman et al., 2000), sucrose accumulation in sugarcane (Rohwer and Botha, 2001), starch degradation in leaves (Nag et al., 2011), sucrose breakdown (Junker, 2004) and carbohydrate metabolism in potato tubers (Assmus, 2005). Recently, we have developed a sucrose-to-starch conversion model that incorporates the sucrose breakdown and the starch synthesis pathways in tubers (Liamwirat et al., 2009a; Liamwirat et al., 2009b). This model is a kinetic model that describes the starch synthesis in tubers through a series of enzymatic reactions involving 20 enzymes and having AL and AP as final products. As a result, this model has allowed the analysis of both starch quantity and quality, which could not be addressed using the previous models of Junker (2004) and Assmus (2005). We used the model to identify promising target enzymes for improvement of starch using enzyme relational network analysis (Liamwirat et al., 2009a) and hierarchical clustering analysis (Liamwirat et al., 2009b). AGPase and iPPtase were found to be promising targets by both techniques while G6PT was also suggested as another target by the hierarchical clustering. In this work, we applied sensitivity analysis to identify target enzymes for further metabolic engineering towards starch improvement in potatoes. Because our previous model failed to reach steady state conditions, we introduced another version of the sucrose-to-starch model that allows analysis at given steady state conditions. In addition, we used the model to explore how the starch quantity and quality would change with varying activities of dual enzymes and how the cellular physiology of engineered tubers would differ from a wild-type cultivar if the identified enzymes had been metabolically engineered.

Results

The constructed model of sucrose-to-starch conversion in potato tubers is composed of 25 reactions (with 97 kinetic parameters) and 28 metabolites incorporating sucrose breakdown in the cytosol and starch synthesis including the starch granule formation pathway in amyloplast (Fig. 1 and Table 1). The starch granule formation pathway contains reactions catalyzed by GBSS, SS, SBE, and DBE. Notice that the products of the enzymes in Fig. 1 (AL, AP, LG, and PG) are all polysaccharides but have different structures.

Model validation

The results of model validation are summarized in Table 2 and Fig. 2. The simulated data were found to be in the same order of magnitude as the experimental values, except for UDP_cyt, Fru_cyt, ADP_cyt, Glc_cyt, S6P_cyt and PP_amp (Table 2). Importantly, the simulated AC% (w/w) was 23.08%, which is within the range of 20.1-31.0% reported for natural tubers (Singh et al., 2003). The model validation under perturbed conditions compares the experimental and simulation values of the down regulation of AGPase (Müller-Röber et al., 1992), GBSS (Visser et al., 1991; Kuipers et al., 1994) and SBE (Schwall et al., 2000) (shown in Fig. 2). The starch content was compared for the case of the under-expression of AGPase, while the AC% was compared in the last two cases, which were under-expression of GBSS and SBE. It can be seen that the model can satisfactorily predict the response trend to down-regulation of these enzymes on the starch content and AC%, especially in the cases of under-expression of AGPase (r = 0.97) and GBSS (r = 0.96), even though the simulation output numbers do not equal the experimental values (Fig. 2). Consequently, the model was accepted for further analysis using sensitivity analysis and dual perturbation techniques which focused on relative changes rather than absolute values.

Rational identification of target enzymes by sensitivity analysis

The model was analyzed by sensitivity analysis in order to identify the most important enzymes for starch synthesis in terms of both quantity (i.e. the starch content) and quality (i.e. the AC%). These enzymes might be used as targets for future metabolic engineering and crop improvement. In this study, the enzyme parameters were perturbed one at a time. The perturbations that resulted in a significant change to

Table 1. The stoichiometric reaction system of sucrose degradation and starch biosynthesis pathways.

Name	Stoichiometric reaction
Enzymes:	
SuSy (Sucrose synthase)	*V2*: Suc_cyt + UDP_cyt ↔ UDPglc_cyt + Fru_cyt [a,b]
UGPase (UDP-glucose pyrophosphorylase)	*V3*: UDPglc_cyt + PP_cyt ↔ G1P_cyt + UTP_cyt [a,b]
PGM (Phosphoglucomutase)	*V4*: G1P_cyt ↔ G6P_cyt [a,b]
INV (Cytosolic invertase)	*V5*: Suc_cyt → Glc_cyt + Fru_cyt [a,b]
HK (Hexokinase)	*V6*: Glc_cyt + ATP_cyt → G6P_cyt + ADP_cyt [a,b]
FK (Fructokinase)	*V7*: Fru_cyt + ATP_cyt → F6P_cyt + ADP_cyt [a,b]
PGI (Phosphoglucose isomerase)	*V8*: G6P_cyt ↔ F6P_cyt [a,b]
SPS (Sucrose phosphate synthase)	*V10*: F6P_cyt + UDPglc_cyt ↔ S6P_cyt + UDP_cyt [a]
SPP (Sucrose phosphate phosphatase)	*V11*: S6P_cyt → Suc_cyt + Pi_cyt [a]
ATP consumption	*V12*: ATP_cyt → ADP_cyt + Pi_cyt [a,b]
ATP:UDP phosphotransferase	*V13*: ATP_cyt + UDP_cyt ↔ ADP_cyt + UTP_cyt [a,b]
pPGM (Plastidial Phosphoglucomutase)	*V15*: G6P_amp ↔ G1P_amp [b]
AGPase (ADP-glucose pyrophosphorylase)	*V16*: G1P_amp + ATP_amp ↔ ADPglc_amp + PP_amp [b]
GBSS (Granule-bound starch synthase)	*V17*: 5000 ADPglc_amp → AL_amp + 5000 ADP_amp *
SS (Starch synthase)	*V18*: 21 ADPglc_amp → LG_amp + 21 ADP_amp *
SBE (Starch branching enzyme)	*V19*: 15000 LG_amp → PG_amp *
DBE (Debranching enzyme)	*V20*: PG_amp → AP_amp [c]
iPPtase (Inorganic pyrophosphatase)	*V22*: PP_amp → 2 Pi_amp [b]
Transporters:	
ST (Sucrose transporter)	*V1*: Suc_supply → Suc_cyt [b]
G6PT (G6P/Pi transporter)	*V14*: G6P_cyt + Pi_amp ↔ G6P_amp + Pi_cyt [b]
ATPT (ADP/ATP transporter)	*V21*: ATP_cyt + ADP_amp ↔ ATP_amp + ADP_cyt [b]
PiT (Phosphate transporter)	*V23*: Pi_amp → Pi_cyt [b]
Summarized reaction:	
bGly (Glycolysis):	*V9*: F6P_cyt + 29 ADP_cyt + 28 Pi_cyt → 29 ATP_cyt [a]

Note: The characters '_cyt' or '_amp' appended on the metabolite abbreviation indicate that the metabolites are present in cytosol or amyloplast, respectively. A '*' represents the stoichiometric reaction adjusted from the previous developed model and added into the model in this study (see Supplementary Table 2 for more detail). References: [a] Junker (2004); [b] Assmus (2005); [c] Liamwirat et al. (2009b).

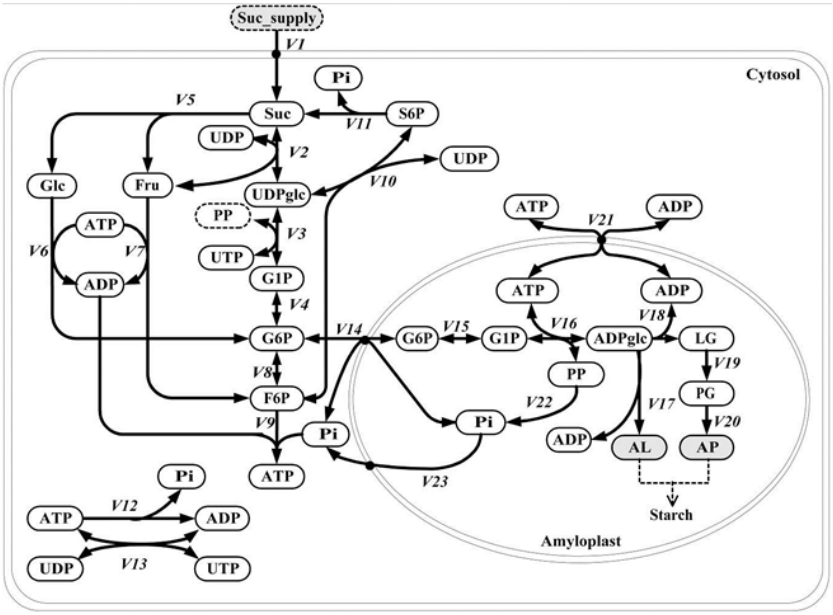


Fig 1. The schematic representation of sucrose degradation and starch biosynthesis pathways in potato tuber. Rounded rectangles represent metabolites. Shaded metabolites represent external metabolites. Metabolites represented with a dashed boundary used fixed concentrations for modeling. Final starch production output is represented with a dashed arrow. The stoichiometric reaction system of the pathways including abbreviations for metabolites and reactions is reported in Table 1.

starch content and AC% were noted and the corresponding parameter and enzyme were identified as targets for modification. The results from the sensitivity analysis are shown in Fig. 3 and 4. It can be seen that 11 out of 97 parameters were observed to significantly affect the starch content: $v_{max}^{iPPtase}$, $K_{m,ADPglc}^{SS}$, v_{max}^{SS}, $K_{m,ADPglc}^{GBSS}$, $K_{a,PGA3}^{AGPase}$, k_f^{ATPT}, v_{max}^{ATPT}, v_f^{AGPase}, $K_{i,Pi}^{AGPase}$, k_f^{G6PT}, and K_{eq}^{PPGM} (Fig. 3). iPPtase ($v_{max}^{iPPtase}$) is the most promising target enzyme by which starch content might be improved, followed by SS ($K_{m,ADPglc}^{SS}$ and v_{max}^{SS}), GBSS ($K_{m,ADPglc}^{GBSS}$), and AGPase ($K_{a,PGA3}^{AGPase}$). For the identification of target enzymes for starch quality, the results showed that the AC% was greatly affected by v_{max}^{GBSS}, $K_{m,ADPglc}^{SS}$, v_{max}^{SS}, and $K_{m,ADPglc}^{GBSS}$ (Fig. 4). These parameters correspond to SS and GBSS enzymes and therefore these enzymes are promising targets for the improvement of starch quality.

Exploring multiple targets for starch improvement by dual parameter perturbations

In addition to the single parameter sensitivity analysis, we studied the effect of perturbations of dual parameters belonging to the proposed enzymes on the content and quality of the starch. In this study, only the 11 significant parameters obtained from the sensitivity analysis were used, resulting in a total of (11x10)/2 = 55 pairs of parameters. Each pair consisted of 4 combinations of dual parameter changes, thus a total number of 220 cases were simulated. The predicted starch content and AC% in all cases are shown in Supplementary Table 3. Fig. 5A shows the pair of parameters whose perturbation resulted in the highest starch content was the 100% increase in both $v_{max}^{iPPtase}$ and v_{max}^{SS}, followed by the 100% increase in both $v_{max}^{iPPtase}$ and k_f^{ATPT} and the 100% increase in both $v_{max}^{iPPtase}$ and v_{max}^{ATPT}. It is not surprising that the starch content can be improved more efficiently through changes of multiple targets when compared with a single target alteration. Finally, it was found that the highest AC was achieved through the dual perturbation of a 50% decrease in v_{max}^{SS} and 100% increase in v_{max}^{GBSS}, while the lowest AC was obtained by the dual perturbation of a 50% decrease in $K_{m,ADPglc}^{SS}$ and 100% increase in v_{max}^{SS} (Fig. 5B).

Prediction of physiology of engineered tubers

Not only can the model help suggest the target enzymes for design and engineering of new engineered tubers with improved starch quantity and quality, it can also be used to help predict the cellular physiology of those new engineered tubers. Herein, we employed the model to simulate metabolite profiles of all proposed engineered tubers in relation to that of the wild-type tuber. This relative metabolite profiling was represented by the log ratio of the metabolite concentration in an engineered tuber to that of the wild-type tuber and shown in the form of a heat map plot. Fig. 6 shows the physiology of various engineered tubers whose target enzymes had been either over-expressed or under-expressed or structurally altered to improve the starch quantity (Fig 6A) or the starch quality (Fig. 6B). It can be seen that the metabolite profiles of all engineered tubers were different from the wild-type. Not surprisingly, the most significant change was observed on the metabolites present in the amyloplast, while little change was seen for the metabolites in the cytosol.

Discussion

In this work, a model of sucrose-to-starch conversion in tubers was developed to identify the most important enzymes affecting the AC% and the starch content. First, the model was validated under both normal and perturbed conditions. Then the model was used to perform a sensitivity analysis in order to identify target enzymes for modification of starch production. In addition, dual parameter perturbation was used to determine whether modifying more than one enzyme at a time would result in greater starch quantity or quality. Finally, the model was used to predict the final physiological properties of the resulting engineered tubers. The validation of the sucrose-to-starch conversion model under normal conditions showed that the simulated values of steady state concentrations of most metabolites were in the same order of magnitude as the experimental values (Table 2). It should be noted that our model was able to predict the AC% (w/w) that is in the range reported by Singh et al. (2003). In addition, the model can also satisfactorily predict the response trend to down-regulation of these enzymes on the starch content and AC%, even though the simulation output numbers do not equal the experimental values (Fig 2). The difference between the simulation and experimental values may be attributed to lack of data on several pathways within the sucrose-to-starch conversion process in living organisms. Inability to predict the impact of enzyme modification on these pathways may have stimulated aggregated effects that emerge when subjected to perturbation. Also, the rough estimation methods used (Equations (2) and (3)) for the starch content or AC% might have affected the overall accuracy of the model. Target enzymes affecting the starch content and AC% were identified by conducting a sensitivity analysis on the validated model. The results revealed that iPPtase, SS, GBSS and AGPase were promising targets for improvement of starch yield (Fig. 3). These results are consistent with the results from our previous work that suggested iPPtase and AGPase (Liamwirat et al., 2009a) and iPPtase, AGPase and G6PT (Liamwirat et al., 2009b) as potential targets. However, the proposed target enzymes resulting from this study contradict those found by Junker (2004) and Assmus (2005). Both researchers analyzed their different potato models using metabolic control analysis (MCA). Junker (2004) and Assmus (2005) identified phosphoglucomutase and invertase, respectively, as good targets for increasing starch content. The disagreement between our results and those of Junker (2004) and Assmus (2005) can be attributed to the differences in the model characteristics and the analysis methods employed. The Junker model lumped reactions in the pathway of starch synthesis as a single reaction producing the starch from cytosolic G6P directly. The Assmus model included a starch degradation process, which may have affected the final content of starch accumulated. Another possible reason for the differences seen is that MCA focuses on infinitesimal changes in parameters, but sensitivity analysis explores the effect of both infinitesimal and large changes in parameters. It should be noted that the sensitivity analysis results also indicate the means for manipulating the target enzymes to achieve starch improvement through the parameters corresponding to the target enzymes. If the target parameters are v_f or v_{max}, we can do over- or under-expression of the enzyme. If the target parameters are K_a, K_i, K_m, or k_f, we must change the structure of the enzyme via directed mutagenesis technique to achieve the goal.

Table 2. The simulated data from the constructed model at steady state compared with the literatures.

Metabolites	Liturratures (mM)	Simulation (mM)
Suc_cyt	82.4[a]	82.1
UDP_cyt	0.0524[a]	0.0030
UDPglc_cyt	0.571[a]	0.454
Fru_cyt	0.516[b]	0.006
G1P_cyt	0.0427[a]	0.0431
UTP_cyt	0.238[a]	0.405
G6P_cyt	0.51[a]	0.82
ATP_cyt	0.292[a]	0.377
F6P_cyt	0.147[a]	0.405
ADP_cyt	0.0873[a]	0.0028
Glc_cyt	31[b]	0.0792
S6P_cyt	0.01[c]	0.10
G6P_amp	0.379[a]	0.297
G1P_amp	0.0174[a]	0.0157
ATP_amp	0.179[a]	0.153
ADPglc_amp	0.0215[a]	0.0491
PP_amp	0.00236[a]	0.01459
ADP_amp	0.149[a]	0.126
AC% (w/w)	20.1 - 31.0%[d]	23.08%

Note: The appended characters '_cyt' or '_amp' for abbreviation of metabolites indicate that the metabolites are present in cytosol or amyloplast, respectively. References: [a] Tiessen et al. (2002); [b] Farré et al. (2001); [c] Junker (2004); [d] Singh et al. (2003).

The manipulation of the target enzymes by over- or under-expression technique is more practical. The results of the sensitivity analysis suggest that modification for improving starch content should target iPPtase, SS, GBSS, and AGPase. However, it should be noted that these results do not necessarily agree with experimental data reported in other studies. Specifically, the sensitivity analysis identified $v_{max}^{iPPtase}$ as a significant parameter, but an experiment using iPPtase over-expressing transgenic tubers by Farré et al. (2006) showed no significant change in starch content. In the case of SS, GBSS, and AGPase, starch content was shown to be improved by either over-expression or changes in the structures of these proteins. For SS, there are many isoforms found in potato tubers, each operating with glucan chains of a specific length (Tetlow et al., 2004). SS in our model only represented the overall SS isoform (not individual isoforms); therefore, although our results indicated that the rate of LG production or ADPglc usage could be increased by enhancing SS expression, it remains to be seen which isoform(s) is/are responsible for the increase. Similarly, a change in the structure of SS or GBSS isoforms to increase their affinities for ADPglc could be performed. To increase the starch content by AGPase, our study suggests that a change in the structure of AGPase might increase its affinity for PGA3, a known AGPase activator ($K_{a,PGA3}^{AGPase}$). The model also suggests that v_f^{AGPase} was not a significant parameter, implying that over-expression of AGPase would not improve the starch yield. This seems to agree quite well with the experimental observation that AGPase's over-expression does not significantly increase starch production (Stark et al., 1992; Sweetlove et al., 1996). In addition to identifying target enzymes for starch yield improvement, the sensitivity analysis of the model also predicts the quality of starch, represented by the AC%. Since AC% determines the physicochemical properties of starch, it is used here to represent the quality of starch. Note that the desired quality of starch depends on how it will be used. Starch with low AC%, especially amylose-free starch, is rapidly digested, easily gelatinized, and can form a clear paste (Raemakers et al., 2005). This type of starch is often used as a stabilizer and

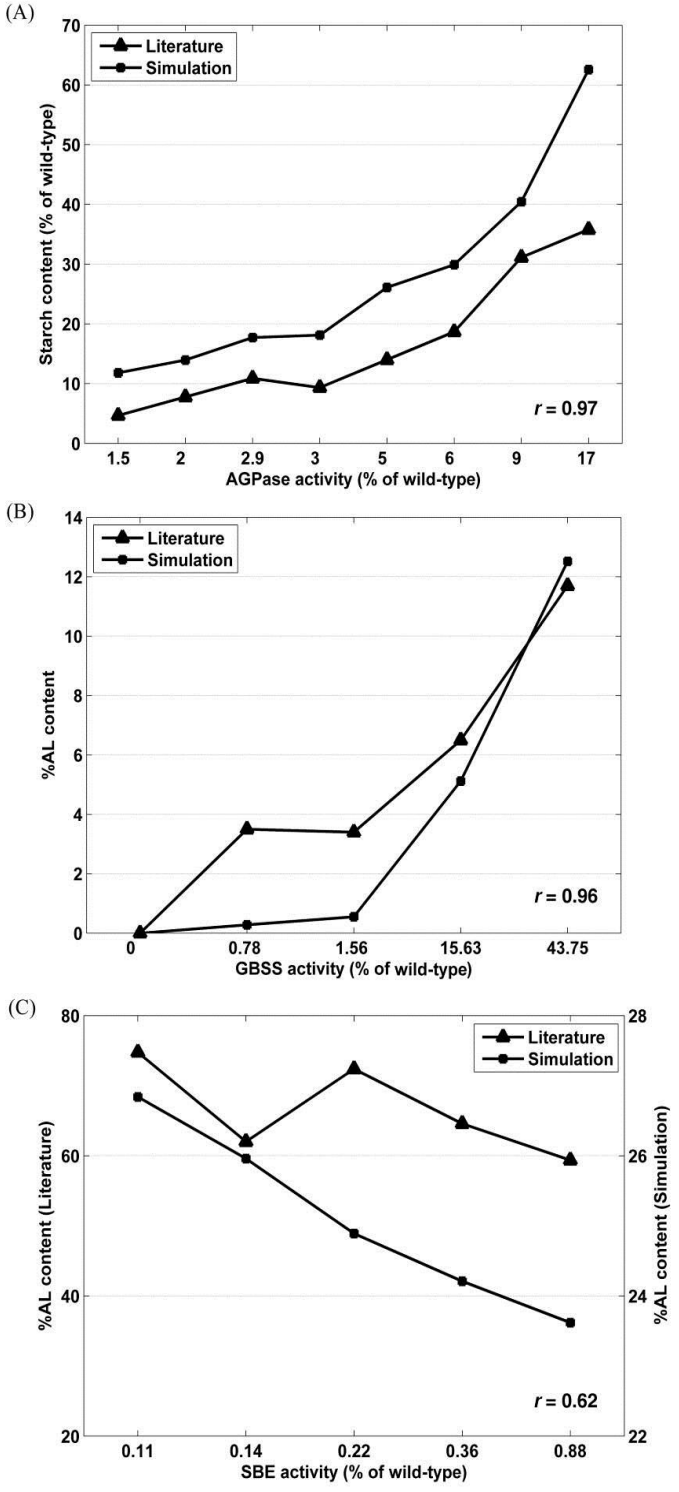


Fig 2. Comparison of literature and simulation values of starch content and AC% of transgenic lines under-expressing (A) AGPase, (B) GBSS, and (C) SBE. Note that % of wild-type = 100×enzyme activity (or starch content) in transgenic line / enzyme activity (or starch content) in wild-type.

thickener in food products (Jobling, 2004). On the other hand, the starch with high AC% is slowly digested, can rapidly form strong gel at relatively low concentrations and has low viscosity. This type of starch is good for several applications; for example, it can function as dietary fiber known as a resistant starch or used to produce the high-

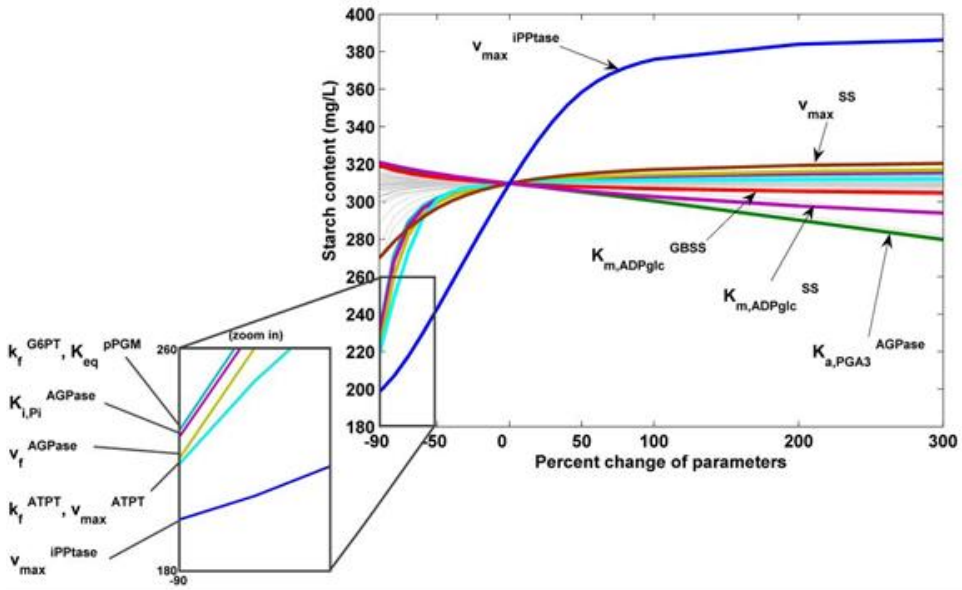


Fig 3. Sensitivity analysis for starch content. Each line represents the resulting starch content simulation value after the change of each parameter at steady state.

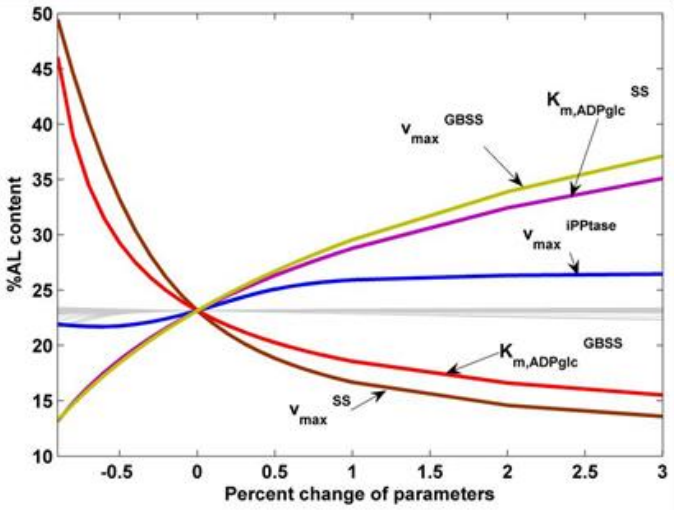
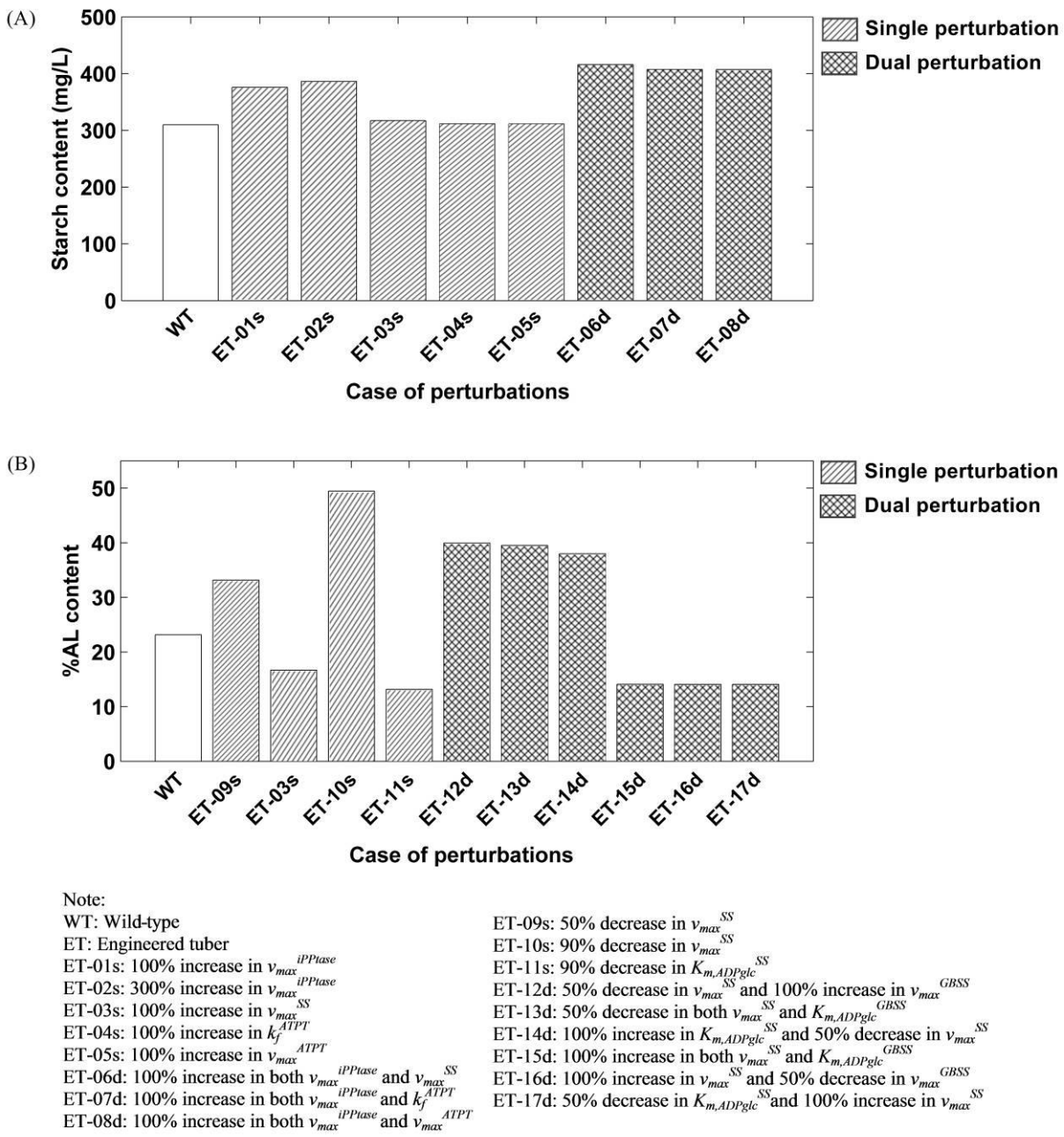


Fig 4. Sensitivity analysis for AC%. Each line represents the resulting AC% simulation value after the change of each parameter at steady state.

strength gels utilized to give candy shape and integrity (Richardson et al., 2000; Jobling, 2004). The results from the sensitivity analysis suggest that SS and GBSS are promising targets for altering the AC% in a tuber plant (Fig. 4). To obtain a low AC, our study suggested that the GBSS rate should be decreased. This could be effectively achieved by the under-expression of GBSS (V_{max}^{GBSS}) or increasing the affinity of SS ($K_{m,ADPglc}^{SS}$) for ADPglc to reduce the rate of amylose synthesis. Actually, the under-expression of GBSS was successfully done a few decades ago and the resulting transgenic cultivars showed a significantly low AC% (Visser et al., 1991; Kuipers et al., 1994). Inversely, to obtain high AC, our study suggested that the SS rate should be decreased. The decrease in the SS rate to produce the high AL starch could be achieved by under-expression of SS (v_{max}^{SS}) or by increasing the affinity of GBSS ($K_{m,ADPglc}^{GBSS}$) for ADPglc. Since double inhibition of two SS isoforms (SSII/SSIII) did not affect the amylose content in potato tubers (Lloyd et al., 1999), it is possible that the mechanism that exists *in vivo* might be more complicated than that being investigated in our study. Therefore, the effect on AC% in SS under-expressing potato tubers should be more thoroughly studied. Note that although our sensitivity analysis did not identify SBE as a target enzyme, the experimental observations of Schwall et al. (2000) indicates that it can be used to increase AC. This observation is in agreement with our model prediction (Fig. 2C), but the relative gain achieved by the SBE enzyme compared to the other enzymes is small. The results of the dual perturbation analysis indicated that the starch yield could be improved even further by the alteration of two target enzymes simultaneously. Dual perturbation shows that over-expression of iPPtase with either SS or ATPT by one fold each would improve the starch content of the modified tuber over that of the wild-type tuber by 1.34 and 1.31 fold, respectively. This improvement is greater than the 3 fold over-expression of iPPtase alone, which improves the starch content over the wild-type tuber only by 1.25 fold (Fig. 5A). Furthermore, it was demonstrated that the current



Note:
WT: Wild-type
ET: Engineered tuber
ET-01s: 100% increase in $v_{max}^{iPPtase}$
ET-02s: 300% increase in $v_{max}^{iPPtase}$
ET-03s: 100% increase in v_{max}^{SS}
ET-04s: 100% increase in k_f^{ATPT}
ET-05s: 100% increase in v_{max}^{ATPT}
ET-06d: 100% increase in both $v_{max}^{iPPtase}$ and v_{max}^{SS}
ET-07d: 100% increase in both $v_{max}^{iPPtase}$ and k_f^{ATPT}
ET-08d: 100% increase in both $v_{max}^{iPPtase}$ and v_{max}^{ATPT}
ET-09s: 50% decrease in v_{max}^{SS}
ET-10s: 90% decrease in v_{max}^{SS}
ET-11s: 90% decrease in $K_{m,ADPglc}^{SS}$
ET-12d: 50% decrease in v_{max}^{SS} and 100% increase in v_{max}^{GBSS}
ET-13d: 50% decrease in both v_{max}^{SS} and $K_{m,ADPglc}^{GBSS}$
ET-14d: 100% increase in $K_{m,ADPglc}^{SS}$ and 50% decrease in v_{max}^{SS}
ET-15d: 100% increase in both v_{max}^{SS} and $K_{m,ADPglc}^{GBSS}$
ET-16d: 100% increase in v_{max}^{SS} and 50% decrease in v_{max}^{GBSS}
ET-17d: 50% decrease in $K_{m,ADPglc}^{SS}$ and 100% increase in v_{max}^{SS}

Fig 5. Comparison of (A) starch improvement and (B) AC% between single and dual perturbations.

model could be used to predict the cellular physiology of engineered tubers whose target enzymes had been altered. The metabolite profiles represented in the form of heat maps can predict the traits of a given engineered tuber. For example, an engineered tuber with over-expressed iPPtase would exhibit a high starch trait while one with over-expressed SS would exhibit a waxy-like starch trait (Fig. 6A). Thus, the model serves as a helpful tool for the rational design for cultivar improvement. The resulting list of target enzymes for modifying starch yield or starch quality is not surprising. These enzymes are closely related to the starch content and quality. Some of these enzymes, such as AGPase and GBSS, have already been the targets for starch improvement, although the experimental results have been inconsistent. Nevertheless, our results show the predictive power of the sucrose-to-starch conversion model. In further work, the model might be used as a platform to study other modification effects, such as the effect of sucrose depletion on starch synthesis, in tubers or even other organisms, such as storage roots. More comprehensively, other pathways, such as amyloplastic glycolysis and starch degradation, should be added into the model to study allosteric regulation of starch synthesis and degradation. The role of energetic metabolites such as ATP, ADP, and phosphate in the sucrose-to-starch conversion should also be studied further.

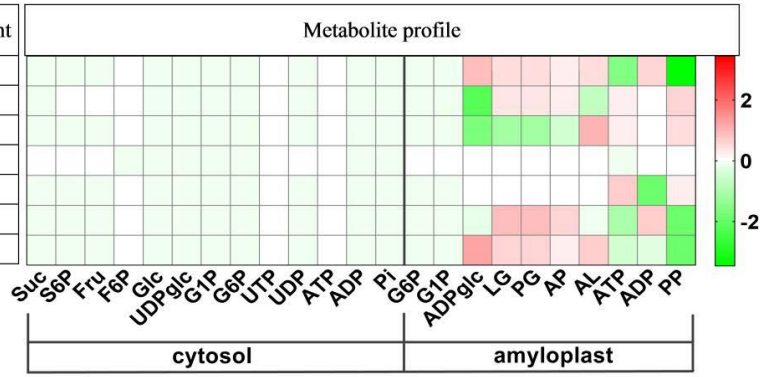
Materials and Methods

Model of potato tubers

The previously developed tuber model (Liamwirat et al., 2009a; Liamwirat et al., 2009b) was adopted in this study. This model incorporates metabolic pathways of sucrose-to-

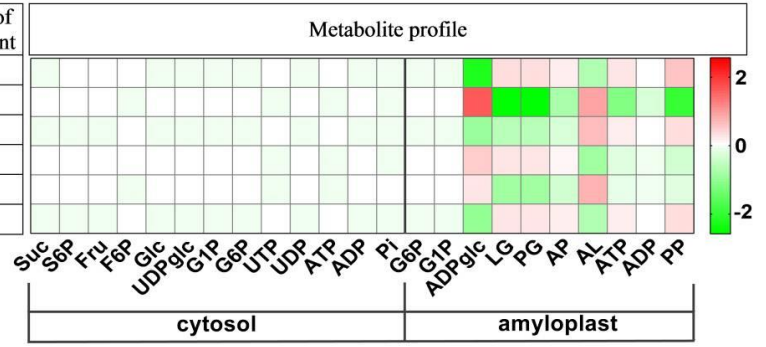
(A)

Target enzyme	Proposed approach	%improvement of starch	%AL content
iPPtase	Over-expression of protein	24.70	26.45
SS	Over-expression of protein	3.44	13.59
GBSS	Structural change to increase affinity for ADPglc	3.06	46.04
AGPase	Structural change to increase affinity for PGA3	0.01	23.14
ATPT	Over-expression of protein	0.74	23.22
iPPtase & SS	Over-expression of both proteins	34.26	16.55
iPPtase & ATPT	Over-expression of both proteins	31.41	27.64



(B)

Target enzyme	Proposed approach	Starch content	% change of %AL content
SS	Over-expression of protein	320.41	-41.28
SS	Under-expression of protein	270.02	113.66
GBSS	Over-expression of protein	316.24	60.38
GBSS	Under-expression of protein	302.94	-42.83
SS & GBSS	Under-expression of SS and Over-expression of GBSS	306.74	72.65
SS & GBSS	Over-expression of SS and Under-expression of GBSS	316.64	-39.18



Note: Starch content of wild-type = 309.75 mg/L
%AL content of wild-type = 23.14

% improvement of starch = (starch content in engineered tuber/starch content in wild-type) - 1
% change of %AL content = (%AL content in engineered tuber/%AL content in wild-type) - 1

Fig 6. Metabolite profiles of engineered tubers for (A) starch improvement and (B) alteration of AC%. Pseudo-color was used to represent the degree of metabolite concentrations different from tubers under normal conditions; dark red indicates a higher degree and dark green indicates a lower degree. White indicates little change (around zero).

starch conversion in potato tubers, which consists of 25 chemical reactions catalyzed by enzymes and transporters located in the cytosolic and amyloplastic compartments (Fig. 1). The pathways include only sucrose breakdown and starch synthesis to reduce the complexity of the model. Sucrose (Suc_supply) is the external metabolite that is constantly supplied to the tuber cells, while AL and AP are the final products accumulated within the amyloplastic compartment.
The stoichiometric system corresponding to the metabolic pathways (Table 1) was used to formulate a kinetic model, which is a set of ordinary differential equations (ODEs) systems representing the change in metabolite concentration with respect to time. In general, a change in concentration of a metabolite [*M*], where *M* is any metabolite, except Suc_supply and cytosolic PP, can be described as:

$$\frac{d[M]}{dt} = \sum s_p V_{production} - \sum s_c V_{consumption} \quad (1)$$

where [*M*] is metabolite concentration in mM; s_p and s_c are the stoichiometric coefficients of the production and consumption reactions, respectively; $V_{production}$ and $V_{consumption}$ are the rate laws of *M* production and consumption from a single reaction, respectively. The rate laws and kinetic parameters were obtained from literatures as provided in Supplementary Table 2. Most of the kinetic data used in this model were specific to potatoes; however, approximately 17% of the kinetic data used in the model were derived from other plants.

Model simulation

Several assumptions were made to successfully simulate the metabolic process that is taking place in developing tubers. First, the sucrose supplied from leaves (Suc_supply) was assumed in excess and was not affected by the diurnal cycle, and PP in the cytosol was used as a buffer within the model. Their concentrations were therefore fixed. Second, the starch content was not limited by the tuber volume or size. Third, the transcriptional regulation of all enzymes was excluded from this analysis, thus the concentrations of enzymes/transporter proteins were kept constant during simulation. Additionally, the effects of temperature and pH on the functions of enzymes/transporters were not taken into account. In essence, the structures and kinetic properties of these components were not changed during the course of simulation. For simulation, the model was implemented and performed by using the SimBiology™ toolbox in MATLAB® (R2006a). By this toolbox, the stoichiometric reactions, rate laws and values of kinetic parameters were entered into the toolbox (Table 1 and Supplementary Table 2). For reversible reactions, the toolbox considered metabolites at the right side of reaction equations to be produced, i.e. the direction of production reaction was from left to the right sides of reaction equations. The ODEs system was solved using ode15s (stiff/NDF) solver with absolute tolerance of 1×10^{-6} and relative tolerance of 0.0010. The model output provided metabolite concentrations with respect to time. Starch content and AC% were then calculated from AL and AP concentrations. First, units of mM of AL and AP concentrations obtained from model simulation were converted into the units of mg/L. The weight-average molecular weight of AL and AP used were 8.1×10^5 g/mole and 5.1×10^7, respectively. These values were calculated by multiplying the molecular weight of ADP-glucose by the number of ADP-glucose units in AL and AP, respectively. These estimated values were in the range of the weight-average molecular weight of AL and AP in potato reported by Buléon et al. (1998). Then the starch content and the AC% were calculated by the summation of the weight of AL and AP contents and by the weigh fraction of AL (w/w), as shown in Equations (2) and (3), respectively.

$$[starch] = (8.1\times10^5)[AL\_amp] + (5.1\times10^7)[AP\_amp] \quad (2)$$

$$AC\% = \frac{(8.1\times10^5)[AL\_amp]}{[starch]} \times 100 \quad (3)$$

Where, [*starch*] is the starch content in mg/L; [*AL_amp*] and [*AP_amp*] are amyloplastic AL and AP concentrations in mM, respectively.

Model validation

The model was validated using data from the literature in order to decide whether it could represent the actual cellular activities of starch synthesis under normal and perturbed conditions. First, the simulated concentrations of metabolites at steady state achieved from the initial conditions (as given in Supplementary Table 1) were compared with experimental data in the literature. Those initial conditions which could not be set using experimental values found in the literature were assigned arbitrarily such that the model could run and reach a steady state. Second, the model was validated under perturbed conditions by under-expression approach. The reaction steps were perturbed through the fold change of their reaction rates and then the starch content or AC% was compared with the literature. In order to imitate under-expression of proteins in transgenic cultivars, multipliers (α_i) were added into all rate laws to represent fold numbers in Equation (4):

$$V_i' = \alpha_i V_i \quad (4)$$

where V_i' is a new rate law, α_i is a multiplier, and V_i is an original rate law (*see* Supplementary Table 2) for reaction *i*. Under normal conditions, α_i was 1 as the default for each reaction. The assignment of an α_i value was used to determine a fold number of the reaction rate that represented the degree of change in enzyme activities found in the literature. Once the α_i of the reaction of interest was assigned, the model was simulated until a new steady state was reached. The final concentrations of AL and AP were then used for calculation of starch content and AC% following Eqs (2) and (3), respectively. These simulated contents were compared with literature values. In addition, the comparison of the response trends to the perturbations between simulation and experiments was evaluated by Pearson's correlation coefficient (*r*), using the linear correlation function in Statistics toolbox™ on MATLAB.

Sensitivity analysis

Sensitivity analysis refers to the parameter sensitivity analysis commonly used in engineering fields to explore the system response to the changes in parameters (Voit, 2000). First, the model was run until the steady state was reached using the initial conditions given in Supplementary Table 1. The concentrations of all metabolites were collected at the end of the simulation and used as initial concentrations to begin the sensitivity analysis. The value of a single parameter was changed in proportion to the original value, following:

$$p_{new} = p_{original} + (\alpha_{proportion} \times p_{original}) \quad (5)$$

where p_{new} and $p_{original}$ are the new and original values of the parameter, respectively, and $\alpha_{proportion}$ is the degree of proportion, i.e. the degree of increase or decrease. In this study, $\alpha_{proportion}$ was varied from -0.9 (90% decrease) to 3 (300% increase). For illustration, an $\alpha_{proportion}$ of -0.5 represented a 50% decrease or a half-fold decrease and an $\alpha_{proportion}$ of 1 represented a 100% increase or two-fold increase in the parameter value. Once the value of the parameter was changed, the model was run until a new steady state was reached. The parameter was reset to the original value before another parameter was changed. Steady state concentrations of all metabolites obtained from the sensitivity analysis of all parameters were collected and analyzed further.

Dual parameter perturbation studies

The effect of dual parameter perturbation on the content and quality of starch was studied in the same manner as the sensitivity analysis, except that a pair of parameters was simultaneously changed in proportion to their original values. The values of $\alpha_{proportion}$ in Equation (5) used were -0.5 (50% decrease) and 1 (100% increase) for each parameter. Hence, there were four combinations of dual parameter perturbations for a given pair of parameters. The model was run for each dual perturbation, and the steady state concentrations of AP and AL were collected and analyzed further. The parameters were reset to their original values before a new pair of parameters was considered.

Simulation of cellular physiology of engineered tubers

The cellular physiology of the proposed engineered tubers was observed in comparison with that of a wild-type. In this study, cellular physiology refers to the steady state metabolite profiles. The steady state metabolite profiling of an engineered tuber was performed by first adjusting the $\alpha_{proportion}$ of a given target enzyme and then simulating the steady state concentrations of all metabolites present in the tuber. For engineered tubers, $\alpha_{proportion}$ was set to 1 to achieve over-expression of the target enzyme, while $\alpha_{proportion}$ set as -0.5 gave an under-expression of the target enzyme. For all remaining enzymes and all enzymes within the wild-type comparison tuber, $\alpha_{proportion}$ values were set to 0. For the comparative analysis, log ratio was calculated and plotted as a heat map. The log ratio was defined as $\log_2(C_{i,1}/C_{i,0})$, where $C_{i,1}$ was the concentration of metabolite i in an engineered tuber and $C_{i,0}$ was the concentration of metabolite i in the wild-type tuber.

Conclusion

In this work, our previously developed model of sucrose-to-starch conversion comprising sucrose breakdown and starch synthesis pathways in tubers was improved, thus enabling the analysis and prediction of target enzymes for the improvement of starch yield and AC%. The sensitivity analysis of the system parameters revealed that iPPtase, SS, GBSS, and AGPase are promising targets for improvement of starch yield. Additional analysis also revealed that the starch yield could be improved further by simultaneously increasing the activities of iPPtase and SS or iPPtase and ATPT. On the other hand, SS and GBSS were found to be promising targets that can alter the amylose content within the tuber cell. Genetic modifications of these enzymes should be further explored in laboratory either by gene over-expression or directed mutagenesis to realize these predictions. We believe that the successful identification of key enzymes by this model can be used towards the development of metabolically-engineered potatoes, leading to designer starches for food, industry and biofuel.

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