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The expression of a recombinant glycolate dehydrogenase polyprotein in potato (*Solanum tuberosum*) plastids strongly enhances photosynthesis and tuber yield

Greta Nölke[1], Marcel Houdelet[1], Fritz Kreuzaler[1], Christoph Peterhänsel[2] and Stefan Schillberg[1,3,*]

[1]*Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen, Germany*

[2]*Institute of Botany, Leibniz-University Hannover, Hannover, Germany*

[3]*Phytopathology Department, Institute for Phytopathology and Applied Zoology, Justus-Liebig University Giessen, Giessen, Germany*

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**Correspondence* (Tel +49 241 6085 11050;
fax +49 241 6085 10000;
email stefan.schillberg@ime.fraunhofer.de)

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Summary

We have increased the productivity and yield of potato (*Solanum tuberosum*) by developing a novel method to enhance photosynthetic carbon fixation based on expression of a polyprotein (DEFp) comprising all three subunits (D, E and F) of *Escherichia coli* glycolate dehydrogenase (GlcDH). The engineered polyprotein retained the functionality of the native GlcDH complex when expressed in *E. coli* and was able to complement mutants deficient for the D, E and F subunits. Transgenic plants accumulated DEFp in the plastids, and the recombinant protein was active *in planta*, reducing photorespiration and improving CO_2 uptake with a significant impact on carbon metabolism. Transgenic lines with the highest DEFp levels and GlcDH activity produced significantly higher levels of glucose (5.8-fold), fructose (3.8-fold), sucrose (1.6-fold) and transitory starch (threefold), resulting in a substantial increase in shoot and leaf biomass. The higher carbohydrate levels produced in potato leaves were utilized by the sink capacity of the tubers, increasing the tuber yield by 2.3-fold. This novel approach therefore has the potential to increase the biomass and yield of diverse crops.

Introduction

Potato is an important nongrain food commodity, with global production exceeding 373 million tonnes in 2011 (FAOSTAT, 2012). It is the primary staple food for more than one billion people worldwide, reflecting its high nutritional value, adaptability to diverse environments and potential yield (Wang, 2008). The tuber starch content also makes potato a versatile and sustainable raw material for the starch and biofuel industries. The additive effects of population growth, the emerging bioenergy economy and the loss of agricultural land to urbanization and land degradation means that greater agricultural productivity is required per hectare of land to meet the demands for food, feed, biomaterials and biofuel (FAO, 2009; OECD/FAO, 2011).

One way to increase the productivity of potato crops is to enhance their photosynthetic efficiency, because carbon metabolism in higher plants drives growth and helps to determine the yield (Long *et al.*, 2006). Previous studies have shown that carbon metabolism can be optimized by genetic engineering (Peterhansel *et al.*, 2008). Enhanced photosynthesis and biomass production has been achieved in tobacco by overexpressing the Calvin cycle enzyme SBPase/FBPase (Lefebvre *et al.*, 2005; Miyagawa *et al.*, 2001). Therefore, enhancing the efficiency of photosynthesis, and thus the amount of fixed carbon could be a straightforward approach to boost the productivity of important agronomic crops.

In C3 plants, photorespiration metabolizes the products of the ribulose-1,5-biphosphate (RuBisCO) oxygenation reaction, and thus reduces the efficiency of photosynthesis by removing carbon from the Calvin cycle, nitrogen and consuming reducing power (reviewed by Maurino and Peterhansel, 2010; Peterhansel and Maurino, 2011). Although the RuBisCO carboxylation reaction produces two molecules of phosphoglycerate that enter the Calvin cycle (ultimately to produce glucose, sucrose and starch and to regenerate ribulose-1,5-biphosphate), the oxygenation reaction produces single molecules of phosphoglycerate and phosphoglycolate, the latter being converted to phosphoglycerate by photorespiration (Leegood *et al.*, 1995; Tolbert, 1997). The balance between these two activities depends mainly on the CO_2/O_2 ratio in the leaves (Laing *et al.*, 1974). Abiotic stress, such as drought and heat, increase the rate of photorespiration. Although photorespiration is necessary for plant growth and survival (Peterhansel and Maurino, 2011), it is an inefficient and wasteful process that lowers the overall efficiency of photosynthesis by removing 25% of the fixed carbon and reduced nitrogen (Wingler *et al.*, 2000). Therefore, reducing metabolic flux through the photorespiration pathway should increase resource-use efficiency, promote growth and increase yields.

The first attempts to increase yields by inhibiting photorespiration were unsuccessful because the photorespiration mutant phenotype was lethal under ambient CO_2 conditions (Somerville, 1984; Somerville, 2001). This is because photorespiration is necessary to protect plants from the accumulation of toxic photosynthesis inhibitors (Campbell and Ogren, 1990; Givan and Kleczkowski, 1992), the effects of excess light (Kozani and Takeba, 1996; Wingler *et al.*, 2000) and to provide reducing

power for nitrate assimilation (Bloom *et al.*, 2010; Rachmilevitch *et al.*, 2004).

Photorespiration is an inevitable consequence of the RuBisCO catalytic mechanism, as O_2 and CO_2 compete for the same active site on the RuBisCO enzyme, but its impact on the plant can be reduced by engineering a photorespiratory bypass in the chloroplast (Kebeish *et al.*, 2007; reviewed by Peterhansel *et al.*, 2012), or by the complete oxidation of glycolate to CO_2 in the chloroplast (Fahnenstich *et al.*, 2008; Maier *et al.*, 2012). Stepwise transformation has been used to introduce the bacterial glycolate catabolic pathway into *Arabidopsis thaliana* chloroplasts to convert glycolate into glycerate (Kebeish *et al.*, 2007). The plants were sequentially transformed with genes encoding the three subunits of glycolate dehydrogenase (D, E and F), glyoxylate carboxyligase (G) and tartronic semialdehyde reductase (T). The resulting transgenic plants showed higher levels of net photosynthesis and produced more shoot and root biomass. Similar results were achieved when only the genes encoding glycolate dehydrogenase (DEF), the first enzyme in the pathway, were introduced.

Here, we tested the ability of *Escherichia coli* glycolate dehydrogenase to increase photosynthetic efficiency and biomass accumulation in potato, which is an ideal model because of its high source-to-sink capacity. The three subunits of *E. coli* glycolate dehydrogenase are expressed at different levels (Pellicer *et al.*, 1996), and they assemble to form a functional GlcDH enzyme. The introduction of GlcDH into plants therefore typically requires multiple gene transfer. Data from the transgenic *Arabidopsis thaliana* plants mentioned above showed that the relative scarcity of the F subunit could limit the assembly of the complete DEF complex (Kebeish *et al.*, 2007). The three corresponding bacterial genes (*glc*D, *glc*E and *glc*F) were therefore fused to create a polyprotein construct with intervening flexible linkers. The activity of the engineered GlcDH polyprotein (DEFp) was initially verified in *E. coli*, and then by expression in the plastids of potato plants to test the impact of DEFp on carbon metabolism, photosynthetic efficiency and biomass accumulation.

Results and Discussion

DEFp displays glycolate dehydrogenase activity in *E. coli*

The D, E and F subunits of *E. coli* glycolate dehydrogenase have previously been introduced step-wise into *Arabidopsis thaliana* plants to reduce the loss of fixed carbon and nitrogen during photorespiration (Kebeish *et al.*, 2007). However, to avoid the time-consuming and cumbersome process of multigene transformation, we designed a recombinant glycolate dehydrogenase polyprotein (DEFp) by fusing the corresponding *E. coli glc*D (1493 bps), *glc*E (1046 bps) and *glc*F (1220 bps) cDNAs with intervening flexible $(Gly_4Ser)_3$ linkers (Figure 1a). This strategy ensured that the three subunits were expressed in stoichiometric amounts, which is not always possible when separate transgenes are expressed (Kebeish *et al.*, 2007). Prior to plant transformation, the engineered DEFp construct was overexpressed in *E. coli* allowing us to carry out rapid glycolate dehydrogenase activity assays and complementation analysis. The 3926 bp multisubunit fusion gene encoding the ~140 kDa DEFp polyprotein was therefore transferred to the bacterial expression vector pET22 downstream of the *pel*B leader peptide using the EcoRI and NotI restriction sites to generate pET-DEFp.

Crude extracts of *E. coli* strain ER2566 overexpressing the DEFp polyprotein were used for enzyme activity assays, with the intact

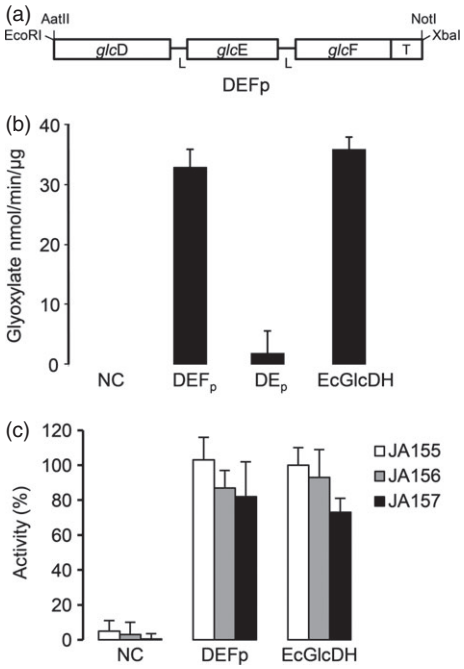

Figure 1 DEFp is functional in *Escherichia coli.* (a) Schematic presentation of the synthetic DEFp multisubunit fusion cassette. Abbreviations: *glc*D, *glc*E and *glc*F: coding sequences for bacterial glycolate dehydrogenase subunits D, E and F; L: $(Gly_4Ser)_3$ linker; T: His_6 polyhistidine tag. Restriction sites used for cloning of DEFp into bacterial and plant expression vectors are indicated. (b) DEFp shows glycolate dehydrogenase activity *in vitro*. (c) DEFp can complement the glycolate dehydrogenase activity of three *E. coli* glycolate oxidase mutants. Protein was extracted from (b) *E. coli* strain ER2566 and (c) the *E. coli* glycolate oxidase mutants JA155 (*glc*D), JA156 (*glc*E) and JA157 (*glc*F), and was tested for glycolate dehydrogenase activity by detecting glyoxylate formation directly. NC: overexpression of the empty pET (b) or pTrc (c) vectors. DEFp: overexpression of the glcD-glcE-glcF polyprotein. DEp: overexpression of the glcD-glcE polyprotein, EcGlcDH: overexpression of *E. coli* glycolate dehydrogenase subunits glcD-F. In (c), GlcDH activities were expressed as a percentage of the *E. coli* wild-type enzyme, which was set to 100%. The data represent the mean ± standard deviation of three biological replicates.

E. coli glycolate dehydrogenase (*Ec*GlcDH) and DEp polyprotein lacking the F subunit used as controls. As shown in Figure 1b, DEFp showed significant glycolate dehydrogenase activity comparable to that of the intact *E. coli* enzyme. The absence of the F subunit (DEp) completely abolished enzyme activity, confirming the importance of this subunit for the maintenance of GlcDH activity.

We next investigated whether DEFp was able to complement *E. coli* glycolate oxidase mutants carrying transposon insertions in the *glc*D, *glc*E and *glc*F subunits of the *glc* operon. These mutants cannot grow on glycolate as a sole carbon source and do not produce detectable glycolate dehydrogenase activity (Pellicer *et al.*, 1996). The overexpression of DEFp in all three mutant strains restored the ability of the bacteria to grow on glycolate-containing media (data not shown). Furthermore, all three mutants yielded detectable levels of glycolate dehydrogenase activity when transformed with DEFp (Figure 1c). Taken together, these results confirmed that the engineered polyprotein retained the functionality of the native *E. coli* glycolate dehydrogenase complex.

Generation and characterization of DEFp transgenic potato plants

To study the effects of DEFp accumulation *in planta*, the pTRA-35S-rbcs-cTP:DEFp construct (Figure 2a) was introduced into potato plants by *Agrobacterium*-mediated transformation. Transformed plants were regenerated under kanamycin selection and screened for the presence of the DEFp transcript and recombinant protein. Sixty-four transgenic lines (named DEFp-1 to DEFp-64) contained the transgene and produced DEFp with the anticipated molecular size of 142 kDa (Figure 2b). DEFp accumulated to levels between 0.05 and 0.15% of total soluble protein (TSP). Transgenic lines accumulating low (~0.05% TSP, DEFp-27), moderate (~0.09% TSP, DEFp-12) and high (~0.14% TSP, DEFp-21) levels of recombinant DEFp were selected for vegetative propagation and were analyzed for GlcDH activity and photosynthetic performance. The recombinant protein accumulated in the vegetatively propagated plants to similar levels as the parental lines (~0.04% TSP, DEFp-27; ~0.1% TSP, DEFp-12; ~0.15% TSP, DEFp-21).

To investigate whether the DEFp was active *in planta*, GlcDH activity was measured in chloroplast extracts of 6-week-old transgenic plants accumulating different amounts of the polyprotein (lines 12, 21 and 27). Wild-type potato plants assayed under the same conditions showed significant background activity as previously described in *Arabidopsis* (Kebeish *et al.*, 2007).

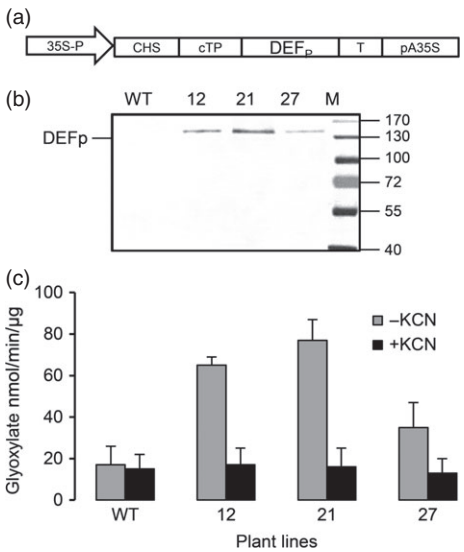

Figure 2 Generation of DEFp transgenic potato plants. (a) Plant expression cassette targeting DEFp to the chloroplast. Abbreviations: 35S-P, double-enhanced CaMV 35S promoter; CHS, 5′-untranslated region of the chalcone synthase gene; cTP: potato *rbc*S1 gene coding for the chloroplast targeting peptide; T: His_6 polyhistidine tag; pA35S: CaMV terminator sequence. (b) Immunoblot analysis of 10 µg crude total soluble protein (TSP) extracted from the leaves of DEFp transgenic potato plants. The anti-His_6 antibody was used for detection of the 140 kDa DEFp. M: prestained protein marker; 12, 21, and 27: TSP extracts from representative transgenic lines; WT: TSP extract from wild-type potato leaves. (c) DEFp is active in potato chloroplasts. The chloroplast extract was assayed for glycolate dehydrogenase activity by direct measurement of glyoxylate formation in the presence and absence of KCN. WT: wild-type potato plant (n = 3); 12, 21, 27: Transgenic potato lines producing DEFp (n = 3). The data represent means ± standard deviation.

Chloroplast preparations from the DEFp plants produced significantly more glyoxylate from glycolate than the wild-type plants (Figure 2c), indicating that the engineered DEFp was correctly localized in the plastids and possessed GlcDH activity. The addition of cyanide inhibited GlcDH activity, confirming that the observed activity was due to the recombinant DEFp and not endogenous peroxisomal glycolate oxidase (Nelson and Tolbert, 1970). The DEFp activity correlated with the level of recombinant protein accumulation, i.e. highest in line 21, lower in line 12, and lowest in line 27 (correlation coefficient R^2 = 0.8935, data not shown). Therefore, we concluded that the novel DEFp polyprotein was targeted to the chloroplast and was functional *in planta*.

Photosynthetic activity of DEFp transgenic potato plants

The impact of recombinant DEFp on the photosynthetic performance of the transgenic potato plants was determined by monitoring gas-exchange parameters in the youngest fully expanded leaf. The apparent photosynthetic rate (A) in DEFp lines 12 and 21 (with the highest GlcDH activity) increased by 18% and 34%, respectively, under ambient conditions (400 ppm CO_2, 21% O_2, 24 °C) (Table 1). The maximum CO_2 fixation rate did not change significantly in any of the lines at saturating CO_2 concentration, indicating that the photosynthetic rates under ambient conditions is dependent on the competition of oxygen and CO_2 for binding to RuBisCO, suggesting that the increased photosynthetic rate in the transgenic lines reflects the greater availability of CO_2 in the vicinity of RiBisCO. To estimate the amount of CO_2 competing with O_2 for RuBisCO binding in the plastids, the O_2 inhibition of photosynthesis was calculated in the control plants and DEFp lines 12 and 21 (Table 1). Oxygen inhibition was significantly lower in the transgenic plants, falling 12% in line 12 and 19% in line 21 ($P < 0.05$ and $P < 0.005$, respectively) compared to the control, providing evidence for a

Table 1 Photosynthetic performance of wild-type and DEFp transgenic potato plants

Photosynthesis parameter	Potato lines		
	WT	DEFp12	DEFp21
A_{max} (µmol/m^2/s^1) (at C_a = 400 p.p.m)	16.5 ± 0.9	19.8 ± 0.5*	22.7 ± 1.3**
A_{max} (µmol/m^2/s) (at C_a = 2000 p.p.m)	40.6 ± 2.7	41.5 ± 2.4	40.2 ± 1.6
O_2 inhibition (%)	34.9 ± 4.2	30.75 ± 2.1**	28.25 ± 1.3**
Γ (p.p.m CO_2)	49.6 ± 6.3	45.7 ± 1.52**	41.15 ± 3.9**
q_P	0.54 ± 0.07	0.55 ± 0.06	0.53 ± 0.09
q_N	0.77 ± 0.01	0.76 ± 0.02	0.76 ± 0.02
Fv/Fm	0.80 ± 0.01	0.79 ± 0.01	0.79 ± 0.01
ΦPSII	0.25 ± 0.04	0.25 ± 0.05	0.26 ± 0.06

A: apparent CO_2 assimilation; C_a: CO_2 concentration in the measuring cuvette; Γ, apparent CO_2 compensation point; F_v/F_m: maximum quantum efficiency of photosystem II; ΦPSII: efficiency of PSII photochemistry; q_P: photochemical quenching; q_N: nonphotochemical quenching. Vegetatively propagated plants cultivated in the phytotron or greenhouse (six plants per line) were analyzed for each measurement in two independent experiments. Values represent means ± SD for wild-type (WT) control and transgenic lines (n = 12). No significant difference in photosynthetic performance was observed between plants grown in the phytotron or greenhouse.

*$P < 0.05$; **$P < 0.005$; ±, standard deviation.

higher CO_2/O_2 ratio in the vicinity of RuBisCO. Furthermore, there were no differences in the maximum quantum efficiency of photosystem II, photochemical and nonphotochemical quenching, and the Fv/Fm ratio. Plastid electron transport, membrane energization and energy dissipation therefore remained unchanged in the transgenic lines. Lines 21 and 12 were characterized by a significant ($P < 0.01$) reduction in the apparent CO_2 compensation point (18% and 9.4%, respectively), indicating a higher rate of photosynthesis at low c_i (supplementary Figure S1). These results provided additional evidence to confirm the functionality of the engineered DEFp enzyme, which was able to catalyze the conversion of glycolate to glyoxylate. The reduction in CO_2 compensation point and O_2 inhibition indicated that the resulting glyoxylate was oxidized further to CO_2 within the plastids. Chloroplasts may oxidize glycolate completely to CO_2, producing glyoxylate as an intermediate step (Frederick *et al.*, 1973; Goyal and Tolbert, 1996; Kebeish *et al.*, 2007). Recently, Blume *et al.* (2013) reported that the tobacco chloroplast pyruvate dehydrogenase complex (PDC) can decarboxylate glyoxylate and may therefore participate in the conversion of glycolate to CO_2 in the plastid. The CO_2 released due to the activity of the PDC complex could be directly re-fixed by RuBisCO, generating higher levels of photoassimilates. This hypothesis is supported by the higher carbohydrate content (Figure 3) and biomass production (see below) in DEFp lines. Furthermore, less ammonia would need to be re-assimilated, thus potentially improving nitrogen use efficiency. Preliminary results from our laboratory indicate that DEFp expression in chloroplast of tobacco plants improves their ability to thrive under conditions of nitrogen limitation (unpublished results).

Leaf carbohydrates and other metabolites in DEFp transgenic plants

To determine the impact of DEFp on primary carbon metabolism, we evaluated the ability of transgenic and wild-type plants to accumulate photosynthesis end products using GC-MS to measure the corresponding metabolite pool. We focused on 31 metabolites, but only those selected for physiological importance and/or significant modulation (*t*-test $P < 0.05$) are shown in Figure 3a,b. Metabolite levels relative to the standard ribitol, are presented in the supplementary Table S1. The DEFp transgenic lines showed a significant ($P < 0.05$) increase in the rapidly metabolized monosaccharaides glucose (2.1/5.8-fold in lines 12/21) and fructose (1.5/3.8-fold in lines 12/21), and the major

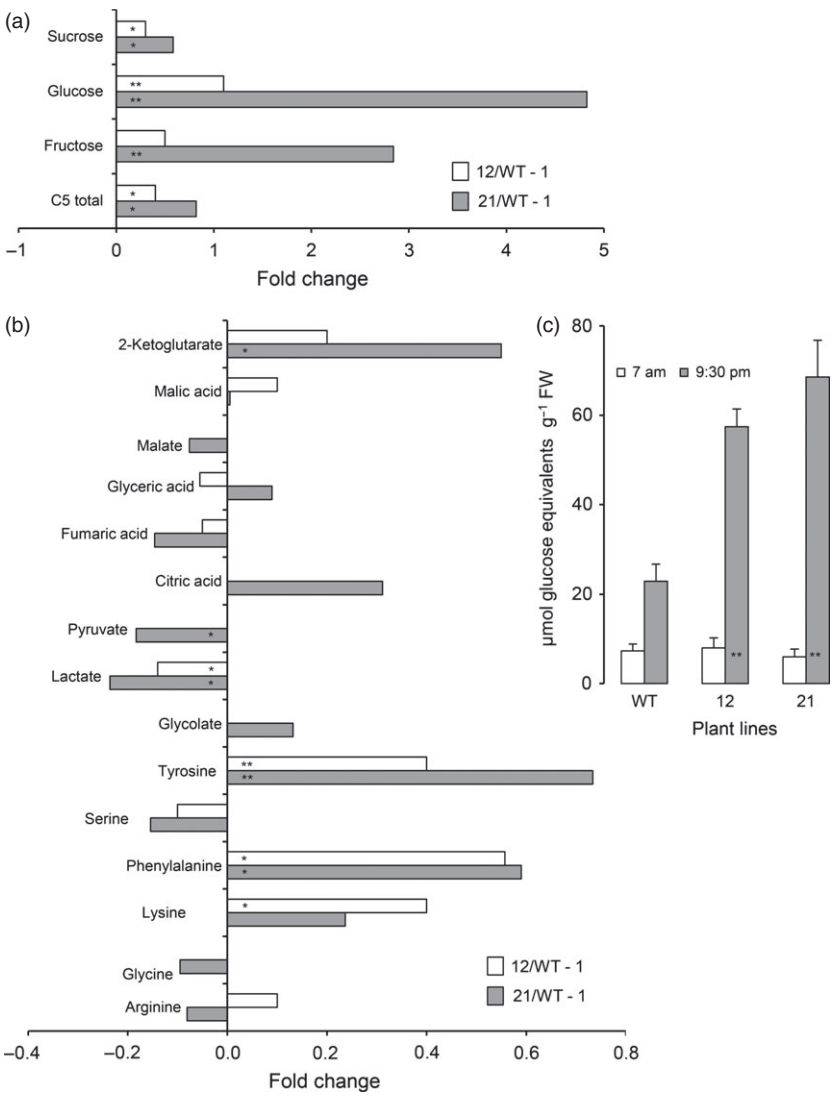

Figure 3 Metabolite levels in the leaves of transgenic and wild-type potato plants. (a) Comparative carbohydrate analysis. (b) Comparative amino and organic acid analysis. Samples were collected 5 h after illumination from five well-expanded leaves representing six plants per transgenic line (and four wild-type controls). The samples from each plant were pooled and analyzed by GC-MS. The ratio between mean relative response from the transgenic lines (12 = white bars, 21 = grey bars) ($n = 6$), and wild-type plants ($n = 4$) are plotted. Values <0 indicate lower metabolite levels in the transgenic lines compared to the control, whereas values >0 represent an increase. The significance of the changes was evaluated using Student's *t*-test. (c) Starch content of leaves of DEFp and wild-type plants. Starch content was analyzed at the beginning (7 : 00 am) and end (21 : 30 pm) of the illumination period. Data represent means ± standard deviation from three independent pools extracted from five well-expanded leaves of each line ($n = 3$). Statistical differences are indicated: * = $P < 0.05$, ** = $P < 0.005$.

exported disaccharide sucrose (1.3/1.6-fold in lines 12/21). The most dramatic increase was observed in line 21, with the highest DEFp levels and highest GlcDH activity. In addition, the amounts of C5 carbohydrates in these lines were also significantly higher (1.4-fold and 1.8-fold, respectively).

Figure 3b shows the relative levels of additional metabolites. Lactate levels declined in both transgenic lines, whereas pyruvate and maleate levels declined only in line 21. Both DEFp lines accumulated significantly higher amounts of 2-ketoglutarate, tyrosine and lysine than wild-type plants, but there were no significant differences in the levels of glycolate, glyceric and citric acids. The glycine to serine ratio also declined in the transgenic lines compared to controls, suggesting a reduction in the level of photorespiration metabolites by diversion to the peroxisomes and mitochondria, although the tendency was not statistically significant (data not shown).

In many plant species the immediate products of photosynthetic carbon assimilation in the light are divided between sucrose (immediately available for growth) and starch, which accumulates in the leaf through the day and is degraded to produce sucrose at night (Geiger *et al.*, 2000; Gibon *et al.*, 2004; Lu *et al.*, 2005). We analyzed the content of starch (the major storage compound) in the leaves at two different time points: the beginning and end of the illumination period (Figure 3c). The leaves of all lines contained low levels of starch early in the morning but significant increases (2.5-fold/$P < 0.005$ in line 12 and threefold/$P < 0.005$ in line 21) were observed by the end of the day in transgenic lines compared to wild-type control. The greater increase in the transgenic lines is reflected the more efficient assimilation of CO_2. Ferreira *et al.* (2010) showed that only 70% of the starch synthesized in potato leaves during the day is broken down during the dark phase to provide nocturnal sucrose to sink tissues, whereas 100% is broken down in *Arabidopsis* (Gibon *et al.*, 2004; Schneider *et al.*, 2002). However, the results presented in Figure 3c show almost complete starch degradation during the night in the best-performing DEFp lines, indicating that the transitory starch is either converted to sucrose and exported from the leaf to the sink organs, or used for dark respiration and biomass accumulation in the leaf (Graf and Smith, 2011).

The increase in starch content did not occur at the expense of other carbohydrates, because DEFp lines 12 and 21 (with the highest GlcDH activity and photosynthesis rates) also produced higher levels of glucose, fructose and sucrose (Figure 3a). Photosynthetic carbon assimilation was sufficient to support not only growth, but also the accumulation of storage compounds in the leaf, which are then mobilized to provide carbon for growth during the night.

Phenotypic effects of DEFp expression

We also investigated whether the enhanced photosynthetic performance of the transgenic plants resulted in phenotypic differences and higher biomass accumulation. Indeed, tubers harvested from lines 21 and 12 with the highest levels of GlcDH activity, and the most significant reduction in CO_2 compensation point and O_2 inhibition, showed accelerated sprouting (1 week earlier than normal) and developed 25% more ($P < 0.05$; $n = 10$) shoots per plant than control lines. The transgenic lines also produced more (9 ± 2; $P < 0.05$; $n = 10$) leaves and broader stems than the control plants (Figure 4a). Studies on the manipulation of photosynthetic carbon fixation suggest that plants evolved mechanisms to ensure co-ordination of leaf development and metabolism (reviewed by Raines and Paul, 2006). Our results support the idea of Raines and Paul (2006) that changes in photosynthetic carbon assimilation, resulting in increased availability of carbohydrates not only have an impact on plant yield, but also play a role in modulating the developmental programme of the plant.

Although the mechanisms governing release from dormancy and the initiation of sprouting are not completely understood (Suttle, 1996), starch degradation and sucrose biosynthesis are tightly linked to tuber sprouting (Farré *et al.*, 2001). Furthermore, sucrose levels are responsible for the regulation of metabolic

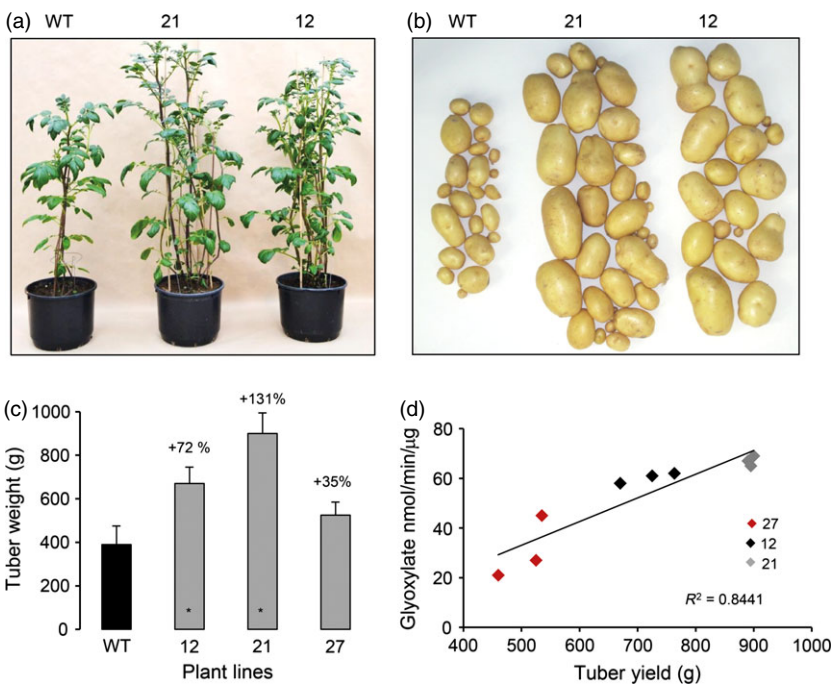

Figure 4 Impact of DEFp expression on potato phenotype and tuber yield. (a) Phenotypic appearance of 8-week-old representative wild-type plant and DEFp lines 21 and 12, planted in soil 6 months after harvest. (b) The phenotype of tubers from one representative wild-type plant, and transgenic lines 12 and 21, 2 days after harvest. (c) Tuber yield per plant in wild-type and DEFp transgenic lines 12, 21 and 27. Data represent means ± standard deviations ($n = 15$). *$P < 0.005$. The increase in tuber yield of the DEFp lines is indicated as a percentage compared to wild-type tubers. (d) Relationship between GlcDH activity (in nmol/min/µg) and tuber yield of transgenic lines 12, 21 and 27. R^2: correlation coefficient. The GlcDH activity of the wild-type control was subtracted from the activity measured in the DEFp lines.

processes during the sink to source transition in potato tubers (Hajirezaei *et al.*, 2003). The metabolic analysis of DEFp-21 tubers showed they contained 1.2-fold higher ($P < 0.05$) levels of sucrose than wild-type tubers, which would affect their sprouting behaviour.

The constitutive production of DEFp led to a significant increase in tuber yield in the transgenic lines (Figure 4b,c), 2.3-fold/$P < 0.005$ in line 21, followed by 1.7-fold/$P < 0.05$ in line 12 and 1.3-fold (not statistically significant but nevertheless a trend in line 27 ($p < 0.1$). The tuber yield in the transgenic lines correlated strongly with GlcDH activity ($R^2 = 0.8441$; Figure 4d). These results were consistent in three different experiments carried out over a period of 2 years.

Similar phenotypic effects were observed in transgenic *Arabidopsis* plants expressing all three subunits of the bacterial glycolate dehydrogenase (Kebeish *et al.*, 2007). The transgenic plants grew faster, produced more shoot and root biomass and contained more soluble sugars, reflecting the increased CO_2 concentration in the vicinity of RuBisCO. Furthermore, the introduction of a complete glycolate catabolic cycle into *Arabidopsis* chloroplasts reduced the photorespiratory flux, increased the photosynthesis rates and improved plant growth (Maier *et al.*, 2012).

The strong correlation between GlcDH activity, photosynthesis rates and tuber yield suggest that the significant increase in photosynthesis rates and the accumulation of end-product metabolites was able to enhance the sink capacity of DEFp transgenic plants. The higher tuber yield did not occur at the expense of leaf starch and primary metabolites (Figure 3). Sucrose is the major form in which fixed carbon is transported to the sink organs. Thus, the rate of sucrose synthesis is likely to be important for sink development and final crop yield, demonstrating the central role of sugars in the coordination of carbon supply and plant growth as previously described by Smith and Stitt (2007). There was no impact on photosynthetic parameters and tuber yield when photosynthetic sucrose biosynthesis was reduced by suppressing cytosolic fructose-1,6-bisphosphate in potato plants (Zrenner *et al.*, 1996). However, our study demonstrated that a 1.7-fold increase in GlcDH activity led to a significant increase in photosynthesis, boosting the accumulation of assimilates, and ultimately biomass and tuber yield. The yield differences reported here are much higher than expected, but transgenic potatoes modified to simultaneously enhance sugar export form the leaf and sugar uptake in the tuber also had a higher starch content and tuber yield (Jonik *et al.*, 2012). This indicates that substantial changes in yield can be achieved by the targeted manipulation of metabolic pathways.

Conclusion

We have established a powerful approach to increase the biomass of potato plants by improving photosynthetic carbon fixation using a glycolate dehydrogenase polyprotein. The constitutive expression of DEFp in potato chloroplasts boosted photosynthetic efficiency and carbohydrate metabolism. Changes in the photosynthetic capacity of the plants were directly reflected in the phenotype, i.e. more leaves, a thicker stem and a 2.3-fold increase in tuber yield. Molecular and biochemical analysis revealed a strong correlation between GlcDH activity, the photosynthetic performance and overall yield. This is the first study describing such a substantial photorespiratory bypass effect in a crop species. As previously suggested, it may be possible to improve the yield further by boosting the sink strength (Jonik *et al.*, 2012; Zhang *et al.*, 2008). Further work is required to understand the mode of action of DEFp, the extent of the metabolic changes, and the feasibility of this strategy to improve yields in other crop species.

Experimental procedures

Engineering the recombinant GlcDH polyprotein

The multi-subunit fusion cassette DEFp contained the *E. coli* genes *glc*D, *glc*E and *glc*F in tandem (*E. coli* K12 genome sequence: gi49175990) and was codon optimized for *Brassica napus* and synthesized by Entelechon GmbH (Bad Abbach, Germany). A genetic algorithm was also used to optimize the synthetic genes simultaneously for a large set of competing parameters, such as mRNA secondary structure, cryptic splice sites, codon and motif repeats, and homogenous GC content. The synthesized DEFp-cDNA was transferred to the EcoRI and XbaI restriction sites of pUC18 to generate plasmid pUC-DEFp.

Plasmid DNA, bacteria and plants

The plasmids pET22b(+) (Novagen, Darmstadt, Germany) and pTrc99a (Pharmacia, Freiburg, Germany) were used for protein expression in bacteria, and pTRA was used in plants (Sack *et al.*, 2007). *Escherichia coli* strain DH5α was used for general cloning and ER2566 (New England Biolabs, Frankfurt, Germany) was used for protein expression. Strains JA155 (*ara*D Δ*lac rps*L *flb*B *deo*C *pts*F *rbs*R glcD::cat), JA156 (*ara*D Δ*lac rps*L *flb*B *deo*C *pts*F *rbs*R glcE::cat) and JA157 (*ara*D Δ*lac rps*L *flb*B *deo*C *pts*F *rbs*R glcF::cat) were used for complementation experiments (Pellicer *et al.*, 1996). *Agrobacterium tumefaciens* strain GV3101 (pMP90RK, Gm^R, Km^R, Rif^R) was used for plant transformation (Konz and Schell, 1986). Leaf discs from 4–5-week-old wild-type potato plants (*Solanum tuberosum cv.* Bintje) were transformed by infection with *Agrobacterium tumefaciens* carrying binary vector pTRA-DEFp (Dietze *et al.*, 1995). Potato plants were cultivated in the phytotron and greenhouse in DE73 standard soil in 13-L pots with a 16-h natural daylight photoperiod and 21/18 °C day/night temperature. In phytotrons, metal-halide lamps at light intensity of 100–110 μE were used. Unless otherwise specified, the data presented here are average of greenhouse conditions. Tubers were harvested when the plants entered senescence. Plants were propagated by cuttings and vegetative multiplication of tubers. Tubers of the same weight (~30 g) were chosen for plant propagation.

Construction of the bacterial and plant expression cassettes

DEF_p was subcloned from pUC18-DEFp into the bacterial expression vector pET22b(+) downstream of the *pel*B leader peptide using the EcoRI and NotI restriction sites, generating vector pET-DEFp. This was facilitated by shuttling the DEFp cassette into pTRA upstream of the *Cauliflower mosaic virus* (CaMV) 35S terminator sequence at the EcoRI/XbaI restriction sites. The constitutive CaMV 35S promoter (Kay *et al.*, 1987), the 5′-untranslated region of the chalcone synthase gene and the chloroplast targeting peptide from the potato *rbc*S1 gene (gi21562) were amplified by PCR using vector pTRAkc-rbcs1-cTP as the template (Kebeish *et al.*, 2007). The amplified 35S-rbcs-cTP PCR product was subcloned into the shuttle vector using AscI/AatII restriction sites introduced by PCR, yielding the final plant expression vector pTRA-35S-rbcs-cTP: DEFp.

Protein expression and complementation analysis

Escherichia coli ER2566 cells (carrying pET-DEFp, pER-DEp, pET-EcGlcDH or empty pET) were cultivated in 200 mL LB medium until the OD_{600} reached 0.5. The pET-EcGlcDH vector contained part of the *E. coli glc* operon encoding the separate glycolate dehydrogenase D, E and F subunits (Bari *et al.*, 2004). Expression was induced by adding β-D-isopropyl-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubating for 2 h at 37 °C. The cells were then washed in 10 mM potassium phosphate (pH 8.0) and resuspended in 1 mL of the same buffer. The cells were lysed by sonication on ice (4 cycles, 4 × 30 s; Sonicator Bandelin Sonopulus GM 70, Berlin Germany) and centrifuged at 30 000 ***g*** for 25 min at 4 °C.

Escherichia coli mutants JA155, JA156 and JA157 deficient for *glc*D, *glc*E and *glc*F subunits of endogenous glycolate dehydrogenase, respectively (Pellicer *et al.*, 1996), were transformed with the vectors pTrc99a-DEFp and pTrc-EcGlcDH (Bari *et al.*, 2004) plasmids and cultivated for 2 days in minimal medium (Miller, 1972) using glycolate as the sole carbon source, supplemented with appropriate antibiotics (25 μg/mL chloramphenicol and 100 μg/mL ampicillin). The cells were diluted into 3 L of fresh medium and grown to an OD_{600} of 0.7–0.9. Protein expression was induced by adding 0.5 mM IPTG and the cells were incubated for a further 3 h at 37 °C followed by the extraction of soluble proteins for the glycolate dehydrogenase assay as described below. The concentration of extracted soluble protein was determined in triplicate by the Bradford assay against bovine serum albumin (BSA) standards.

Total protein extraction and immunoblot analysis

The upper fully expanded leaves from 6-week-old potato plants were ground to a fine powder under liquid nitrogen, and total soluble protein (TSP) was extracted as described by Nölke *et al.* (2008) with two volumes of extraction buffer [50 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM dithiothreitol (DTT), 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% (v/v) Tween-20]. The extracts were centrifuged at 8500 ***g*** for 20 min at 4 °C and used for immunoblot analysis. DEFp was detected with a rabbit anti-His_6 monoclonal antibody (RAb-His; 200 ng/mL) and a horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (GAR^{AP}; 120 ng/mL) (Jackson ImmunoResearch Laboratories, Suffolk, UK). DEFp band intensities were quantified using Aida software (Raytest, Straubenhardt, Germany) against known concentrations of bacterial affinity-purified DEFp as a standard.

Isolation of chloroplasts from potato leaves

Intact chloroplasts were isolated from 6-week-old potato plants as described by Goyal *et al.* (1988). Leaf material (5 g) was ground in 100 mL grinding buffer (50 mM HEPES-KOH pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 1 g/L BSA, 0.2 g/L sodium ascorbate, 0.3 M mannitol, 5 g/L polyvinylpyrrolidone) and all subsequent steps were performed in the dark at 0 °C. Crude protein extract was filtrated through three layers of Miracloth and the solution was centrifuged at 1000 ***g*** for 10 min at 0 °C. The pellets were resuspended in 1 mL SH-buffer (50 mM HEPES-KOH pH 7.5, 0.33 M sorbitol) and 1 mL of the solution was loaded onto 8-mL 35% (v/v) Percoll gradient (35% Percoll, 65% SH-buffer). The gradient was centrifuged at 500 ***g*** for 5 min at 0°C. The chloroplast pellet was washed in 1 mL SH-buffer and chloroplast proteins were extracted in 500 μL extraction buffer (50 mM HEPES-NaOH pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 20% (v/v) glycerol). The purity (> 95%) of the chloroplast fraction was confirmed by catalase and fumarase activity assays (Figure S2) as previously described (Ferri *et al.*, 1978). The chloroplast proteins were directly used in the glycolate dehydrogenase activity assay.

Glycolate dehydrogenase assay

Glycolate dehydrogenase activity was determined as described by Lord (1972) using 40 μg of bacterial crude protein extract or chloroplast proteins added to 150 μL of buffer (10 mM potassium phosphate, pH 8.0, 0.025 mM phenazine methosulfate, 10 mM potassium glycolate). At fixed time points (1, 2, 3, 4, 5 and 10 min) individual assays were terminated by adding 30 μL 12 M HCl. After incubation for 10 min, 70 μL 0.1 M phenylhydrazine was added and the mixture was again incubated at room temperature for 10 min. The extinction due to the formation of glyoxylate phenylhydrazone was measured at 324 nm in a quartz 96-well microtiter plate using a Synergy HT-I multiplate reader (BioTec, Bad Friedrichshall, Germany).

Gas-exchange measurements

Fully expanded upper leaves from 7-week-old potato plants were used for gas-exchange measurements in LI-6400 system (Li-Cor), as previously described (Kebeish *et al.*, 2007). The following parameters were used: photon flux density 1 000 mmol/m^2/s, chamber temperature 26 °C, flow rate 150 mmol/s, relative humidity 60–70%. The oxygen inhibition of carbon assimilation (A) was calculated from A at C_a = 400 ppm, and atmospheric oxygen concentrations of 21% and 2%, using the following equation:

$$\text{oxygen-inhibition}\,(\%) = (1 - A_{21}/A_2) \times 100.$$

The CO_2 compensation point (Γ) was determined by measuring the photosynthesis rates at 400, 300, 200, 100, 80, 60 and 40 ppm CO_2. The apparent CO_2 compensation point (Γ) was deduced from A/Ci curves by regression analysis in the linear range of the curve. Measurements were taken from the same plants after 4 h light on two different days. After these measurements, leaf samples were used to determine glycolate dehydrogenase activity and carbohydrate levels.

Sugar and starch quantities in potato leaves

For sugar analysis, 40 mg of leaf material harvested from five well-expanded leaves from 7-week-old plants after 4 h of illumination was flash frozen in liquid nitrogen and ground in 1 mL prechilled chloroform/methanol/water (2.5/1/1 v/v/v). The extract was incubated at 4 °C for 10 min with moderate shaking and centrifuged at 16 000 ***g*** for 2 min at 4 °C before 500 μL of the supernatant was mixed with 250 μL of water. The samples were centrifuged as above, the top layer was collected and dried in a speed vacuum concentrator and the glucose, fructose and sucrose concentrations were determined enzymatically (Stitt *et al.*, 1989). For starch measurements, 50 mg leaf material was collected in two different time points, at the beginning (7:00 am, after 1.5 h illumination) and at the end of the illumination period (21:30). The frozen leaf material was ground in liquid nitrogen and resuspended in 80% (v/v) ethanol. The extract was mixed for 10 min at 80 °C and centrifuged for 20 min. The pellet was resuspended in 80% (v/v) and 50% (v/v) ethanol, respectively, followed by mixing at 80 °C and centrifugation as above. The resulting pellet was washed with 90% (v/v) ethanol, resuspended in 400 μL 0.2 KOH and incubated at 95 °C

for 1 h. Finally, samples were mixed with 70 μL 1 M acetic acid and the starch content was measured enzymatically.

Analysis of metabolites from potato leaves

For metabolite analysis, samples were collected 5 h after illumination from five well-expanded leaves representing six plants per transgenic line and four wild-type controls. The samples from each plant were pooled and the leaf material was homogenized in liquid nitrogen. Metabolites were quantified in 20 mg of homogenized material by GC-MS as described by Lisec *et al.* (2006). Chromatograms were analyzed using CHROMA TOF software (Leco Corporation, Mönchengladbach, Germany) and TAGFINDER (Luedemann *et al.*, 2008).

Statistical analysis

Significance was determined according to Student's *t*-test using Excel software (Microsoft). Two-sided tests were performed for homoscedastic matrices.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 A/Ci-curves measured in transgenic plants.
Figure S2 Fumarase and catalase activity assays of the chloroplast fractions from wild-type and DEFp transgenics used for glycolate dehydrogenase activity analysis.
Table S1 Leaf metabolite contents of wild-type and DEFp transgenic potato plants.