Potato tuber expression of Arabidopsis WRINKLED1 increase triacylglycerol and membrane lipids while affecting central carbohydrate metabolism

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Received 2 November 2015; revised 27 January 2016; accepted 12 February 2016.
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Keywords: Solanum tuberosum, tuber, WRINKLED1, lipid, triacylglycerol, starch, metabolite.

Summary
Tuber and root crops virtually exclusively accumulate storage products in the form of carbohydrates. An exception is yellow nutsedge (Cyperus esculentus) in which tubers have the capacity to store starch and triacylglycerols (TAG) in roughly equal amounts. This suggests that a tuber crop can efficiently handle accumulation of energy dense oil. From a nutritional as well as economic aspect, it would be of interest to utilize the high yield capacity of tuber or root crops for oil accumulation similar to yellow nutsedge. The transcription factor WRINKLED1 from Arabidopsis thaliana, which in seed embryos induce fatty acid synthesis, has been shown to be a major factor for oil accumulation. WRINKLED1 was expressed in potato (Solanum tuberosum) tubers to explore whether this factor could impact tuber metabolism. This study shows that a WRINKLED1 transcription factor could induce triacylglycerol accumulation in tubers of transformed potato plants grown in field (up to 12 nmol TAG/mg dry weight, 1% of dry weight) together with a large increase in polar membrane lipids. The changes in metabolism further affected starch accumulation and composition concomitant with massive increases in sugar content.

Introduction
Plants synthesize and deposit storage products as energy to be utilized after a shorter or longer time frame. Long-term storage is primarily in organs responsible for new generations as seeds or tubers and roots. These storage compounds vary among species but are usually present in the form of starch, polyfructose, sucrose, oil and protein. Oil is the most energy dense form of storage and mainly found in seeds and the mesocarp of some fruits. Plant species with underground stem tubers and roots generally store carbohydrates in different forms in such sink organs. An exception to this is yellow nutsedge (Cyperus esculentus) that besides carbohydrates, store triacylglycerols (TAG) up to around 15% of fresh weight (Linsen et al., 1989). Additionally, it stores almost 20% starch of fresh weight in its tuber parenchyma tissue which, on a combined quantitative basis, is very uncommon among plants. This unique example shows that underground storage organs have the capacity to accumulate significant amounts of TAG.

A transcription factor which expression has been found to be closely connected to oil accumulation is WRINKLED1 from Arabidopsis thaliana (Focks and Benning, 1998). It belongs to the AP2/EREB domain family and triggers the expression of genes involved in glycolysis and fatty acid synthesis (Cernac and Benning, 2004; Focks and Benning, 1998). Expression of maize homologs complementing the WRINKLED1 mutation in Arabidopsis and heterologous expression in leaf tissue of Arabidopsis have shown WRINKLED1 to have the capacity for complementation of deficiencies across species as well as to ectopically induce oil accumulation and heterologously enhance oil content in seeds (An and Suh, 2015; Pouvreau et al., 2011; Sanjaya et al., 2011; Yang et al., 2015). Recently, also protein structural properties have been investigated to elucidate and enhance the function of the WRINKLED1 transcription factor (Ma et al., 2015). Of several known transcription factors of importance for oil accumulation in seed embryos, only a WRINKLED1 homolog was highly up-regulated in mesocarp of oil palm (Elaeis guineensis) compared to date palm (Phoenix dactylifera) (Bourgis et al., 2011). This could indicate a different regulating mechanism dominated by WRINKLED1 in other oil accumulating tissues than seed embryo.

Tubers are sink organs with a massive sucrose import, which is similar to the situation in developing seeds and fruits. As oil accumulation is such a rare event in tubers, but evidently can coexist with starch deposition, we were interested to assess whether tubers that only store starch can accommodate TAG accumulation and whether the WRINKLED1 transcription factor can cause such a metabolic change in an underground storage tissue as stem tubers of potato.

It is not only of scientific interest to investigate how a crop not evolved for a dual storage accumulation accommodates additional up-regulated pathways but could also be of importance
from a crop development perspective. Root and tuber crops generally have very high yield although a large part of the harvest is water and only 25–30% is dry matter (www.fao.org/es/faodef/fdef02e.htm). This could be compared with yellow nutsedge that has up to 60% dry matter (Turesson et al., 2010). Adding TAG deposition to typical carbohydrate crops would increase the nutritional density of staple crops that currently provide an exclusive carbohydrate diet. Potato is not only an important food crop but also the main crop used for starch production in northern Europe (www.starch.eu). If TAG is accumulated to substantial amounts in potatoes, it would also be valuable to recover the oil portion besides processing the starch.

In this study, we found WRINKLED1 expression to dramatically change metabolism and induce TAG accumulation in tubers. Polar and membrane lipids were found to be greatly increased in transgenic potato tubers concomitant with TAG accumulation.

Results

Gene construct and transformation for AtWRI1 expression in potato tubers

A synthetically produced gene corresponding to the WRINKLED1 coding region of Arabidopsis thaliana (At3 g54320) (AtWRI1) was cloned after a GBSS promoter (Solanum tuberosum) which has been characterized as tuber specific (Visser et al., 1991). Transformation was performed on potato variety Kuras, and putative transgenic shoots were selected on regeneration medium containing kanamycin. Based on PCR analysis, 45% of analysed shoots showed T-DNA integration (results not shown) and individual shoots were selected for propagation of in vitro microtubers. Lipids were extracted from microtubers of ten individual transgenic lines and analysed for changes in content of neutral lipids in comparison with Kuras in vitro microtubers. Several lines displayed an increase in triacylglycerol (TAG) content, of which lines 8001, 8003, 8016 and 8022 were selected for more detailed studies (Figure S1). TAG content of Kuras microtubers was found to be 0.1 mg/g dw while a content of more than 20 mg/g dw (2% TAG of dry weight) could be measured for individual transgenic microtubers (Figure S2). Consistent for TAG from selected transgenic microtubers was a change in fatty acid (FA) composition with an increase in 18:2 and 18:1 at the expense of 18:3 (Figure S3). All the selected transgenic lines contained multiple inserts of the T-DNA (Figure S4).

AtWRI1 induces oil accumulation in soil grown tubers

Cuttings of Kuras and lines 8001, 8003, 8016 and 8022 were transferred to soil in the greenhouse. Growth and development of transgenic lines were indistinguishable from control with the exception for line 8022 which appeared erect, thin in growth and with small curly leaves. Greenhouse cultivation lasted for 4 months, and sampling was performed after 2 (S2), 3 (S3) and 4 months (S4). After 4 months, the haulm was cut and watering stopped 2 weeks prior to harvesting. All transgenic lines displayed altered tuber morphology. The parental variety Kuras has rounded and slightly flattened tuber morphology, while tubers of the transgenic lines generally were slightly elongated, had deeper eyes and were rich in secondary tuber developments (Figure 1). Transgenic lines, with the exception of line 8022, on average produced more tubers than wild type but of less average weight (Figure 2). Total tuber mass production per plant showed a slight average increase with the exception for line 8022 (Figure 2). Line 8022 was excluded from further studies and generalized conclusions as it displayed aberrant growth. Dry matter content was on average less for transgenic lines compared to control but increased for both control and transgenic lines between S3 and S4 (Figure 2).

TAG had increased up to 20-fold, 3 mg/g dw over control tubers at the time of sampling after 4 months (Figure 3a). However, an intermediate sampling (S3), made after 3 months to represent a metabolic status of active development, showed that the TAG content decreased between S3 and S4 (results not shown). TAG composition analysis revealed a large decrease in 18:3 levels among FA contained in TAG (Figure 3b). Fatty acids associated with polar lipids increased in transgenic lines with a similar absolute amount as for TAG but with no obvious alterations in fatty acid composition (Figure 3c,d).

Expression of AtWRI1 directly or indirectly affects carbohydrate metabolism including starch

Metabolites and starch were analysed for the S3 stage, which represents tubers in an active accumulation phase. Transgenic

Figure 1 Phenotype of greenhouse grown tubers, (a) Kuras, (b) 8001, (c) 8003, (d) 8016. Solid arrows indicate secondary tuber developments and dashed arrows indicate deeper eyes of transgenic tubers.
lines showed a 10–18% decreased starch content of dry weight compared to the parental variety at this stage (Table 1).

Metabolites were extracted and investigated for differential concentration in transgenic lines compared to parental genotype. Sugars as sucrose, glucose and fructose were greatly increased in transgenic tubers (Table 1). Most prominent was the increase in glucose which amounted to up to 100-fold higher (18.4% of dw) than in the parental genotype. Fructose was increased up to 37-fold (0.5% of dw). TCA cycle intermediates were also affected with a fourfold increase of malic acid or decrease of citric and isocitric acid, respectively (Table 1). Amino acids as alanine and valine were found increased while asparagine was decreased (Dataset S1). The backbone for TAG assembly, glycerol-3-P, was also increased in the transgenic lines (Table 1).

Transcriptome analysis reveals differential gene expression of transgenic tubers

Of interest to investigate were pathways leading from sucrose to precursors for fatty acid synthesis and subsequent fatty acid modification and assembly into complex lipids. Line 8016 was selected for comparative analysis at three different time points with the parental control. Sequenced reads corresponding to AtWRI1 could be detected at all three stages of development in line 8016 while very low levels of AtWRI1 were detected in the parental control (results not shown). Several genes involved in late steps of plastid glycolysis were found to be up-regulated in transgenic tubers (Table 2). Genes for plastid localized glycolysis encoding phosphoglyceromutase, enolase and pyruvate kinase were up-regulated (12-, 7- and 6-fold, respectively, at S3). Also transcripts encoding plastidic NADP-malic enzyme were up-regulated (10-fold at S3), which could provide an alternate route to supplying pyruvate. In line with these transcripts, encoding plastid dicarboxylate transporters, potentially of importance for malate transport, were up-regulated. All transcripts encoding subunits of plastidic pyruvate dehydrogenase were up-regulated (up to 27-fold at S3) indicating a push towards acetyl-CoA production. Cytosolic ATP-citrate lyase transcripts were also found up-regulated (up to 22-fold at S3) (Table 3) which could provide additional supply in the direction of pyruvate via oxaloacetate and malate but also acetyl-CoA as substrate for complex lipids.

Figure 2  Greenhouse tubers from Kuras and transformed lines after stage S4 (a–c) and stage S3 compared to S4 (d). (a) Number of tubers per plant, (b) Tuber mass per plant, (c) Mass per tuber, (d) Dry matter content at stage S3 and S4. Letters distinguish significant different means according to Tukey’s test at level $P < 0.05$.

Figure 3  Analysis of triacylglycerols (TAG) and polar lipids (PL) of greenhouse grown tubers, (a) TAG content of tubers of Kuras and transgenic lines, (b) Fatty acid composition of TAG from Kuras and transgenic lines, (c) PL content of tubers of Kuras and transgenic lines, (d) Fatty acid composition of PL from Kuras and transgenic lines. Three biological replicates ±SD.
the mevalonate pathway. A transcript annotated as a phosphoenolpyruvate (PEP) transporter (PPT) was up-regulated (sevenfold at S3) indicating an enforcement of plastid PEP import and subsequent plastid enzymatic reactions leading to an increased pyruvate supply for fatty acid synthesis (Table 2). The major potato tuber plastid transporter for carbohydrate import, glucose-6-phosphate/phosphate translocator (GPT), showed essentially unchanged transcriptional expression level while another contig representing a minor form was up-regulated although to a low range in comparison with other genes.

Structural studies show presence of oil droplets and changes of starch granules

The cell structure of the tubers was studied under light microscope and transmission electron microscope (TEM) to examine and compare storage cells of the wild-type and transgenic tubers. Transgenic material displayed smaller and irregularly shaped starch granules compared to the wild type (Figure 4a,c). Oil droplets were detected along the cell walls, which could not be found in the wild type (Figure 4b,d). Furthermore, impact on membranes due to increased amounts of membrane lipids was noted. TEM micrographs revealed clear membrane invaginations (abbreviated as CWT) in the transgenic line (Figure 5).

Expression of AtWRI1 in field grown tubers affects tuber yield, size and phenotype

In a field trial, Kuras, 8001, 8003, 8016 and 8022, were grown in four rows of 13–15 plants per row. Lines 8001, 8003 and 8016 could not be distinguished from the parental variety. No differences in emergence, plant morphology, haulm height and size, maturation, wilting or susceptibility to diseases could be observed. The tuber fresh weight yield at maturity was found to be higher for two of the transgenic lines, 8001 and 8016 with 13 and 27 percentage increase, respectively compared to the parental variety, while line 8003 had 28 percentage points lower yield (Table 4).

For all transgenic lines, the average tuber size decreased and the fraction of tubers smaller than 55 mm increased from 35 to approximately 60% by weight of the harvested tubers for line 8001 and 8016 and more than 90% by weight for line 8003 (Table 4).}

Harvested tubers from the transgenic lines showed similar phenotypic alterations as for greenhouse grown tubers with elongated shape and deeper eyes, but the characters were more pronounced (Figure 5).

Dry matter content of smaller and larger fraction of tubers from transgenic lines was similar or slightly lower compared to that of Kuras (Figure 6).
<table>
<thead>
<tr>
<th>Contig</th>
<th>Function</th>
<th>Location</th>
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<tbody>
<tr>
<td>C2973_g1</td>
<td>Plastid transporter</td>
<td>S2: 492.69, S3: 740.72, S4: 365.80&lt;br&gt;S2: 477.90, S3: 874.80, S4: 870.01&lt;br&gt;S2: 196.69, S3: 84.77, S4: 181.64&lt;br&gt;S2: 4.66, S3: 4.66, S4: 4.66&lt;br&gt;S2: 2.38, S3: 2.38, S4: 2.38</td>
</tr>
<tr>
<td>C2851_g1</td>
<td>Glucose-6-phosphate/phosphate translocator</td>
<td>S2: 0.04, S3: 0.23, S4: 0.09&lt;br&gt;S2: 0.09, S3: 7.54, S4: 15.50&lt;br&gt;S2: 1.32, S3: 3.27, S4: 7.19&lt;br&gt;S2: 7.15, S3: 15.32, S4: 29.51&lt;br&gt;S2: 3.42, S3: 3.42, S4: 3.42</td>
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<tr>
<td>C2778_g1</td>
<td>Glucose transporter</td>
<td>S2: 36.32, S3: 18.40, S4: 10.85&lt;br&gt;S2: 47.97, S3: 85.38, S4: 50.57&lt;br&gt;S2: 3.27, S3: 7.03, S4: 2.75&lt;br&gt;S2: 1.27, S3: 2.54, S4: 0.97&lt;br&gt;S2: 1.32, S3: 1.32, S4: 1.32</td>
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<tr>
<td>C2849_g1</td>
<td>Phosphoenolpyruvate transporter</td>
<td>S2: 23.74, S3: 14.67, S4: 9.84&lt;br&gt;S2: 65.31, S3: 11.49, S4: 4.16&lt;br&gt;S2: 0.24, S3: 0.43, S4: 0.67&lt;br&gt;S2: 2.24, S3: 3.77, S4: 5.37&lt;br&gt;S2: 2.24, S3: 2.24, S4: 2.24</td>
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<tr>
<td>C1682_g1</td>
<td>Transketolase</td>
<td>S2: 172.22, S3: 225.37, S4: 263.82&lt;br&gt;S2: 1850.35, S3: 1491.42, S4: 1735.27&lt;br&gt;S2: 10.68, S3: 6.62, S4: 6.62&lt;br&gt;S2: 0.15, S3: 0.15, S4: 0.15&lt;br&gt;S2: 3.88, S3: 3.88, S4: 3.88</td>
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<tr>
<td>C2849_g1</td>
<td>Phosphoglycerate kinase</td>
<td>S2: 1.45, S3: 1.54, S4: 1.37&lt;br&gt;S2: 7.65, S3: 7.92, S4: 5.60&lt;br&gt;S2: 0.27, S3: 0.27, S4: 0.27&lt;br&gt;S2: 3.47, S3: 3.47, S4: 3.47&lt;br&gt;S2: 4.17, S3: 4.17, S4: 4.17</td>
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<td>C2512_g1</td>
<td>Enolase</td>
<td>S2: 75.68, S3: 58.54, S4: 35.77&lt;br&gt;S2: 317.76, S3: 423.82, S4: 329.56&lt;br&gt;S2: 4.20, S3: 4.20, S4: 4.20&lt;br&gt;S2: 2.19, S3: 2.19, S4: 2.19&lt;br&gt;S2: 9.21, S3: 9.21, S4: 9.21</td>
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<td>C2628_g1</td>
<td>Phosphoglucoisomerase</td>
<td>S2: 21.35, S3: 10.21, S4: 6.19&lt;br&gt;S2: 29.70, S3: 47.10, S4: 31.84&lt;br&gt;S2: 1.39, S3: 1.39, S4: 1.39&lt;br&gt;S2: 4.61, S3: 4.61, S4: 4.61&lt;br&gt;S2: 5.15, S3: 5.15, S4: 5.15</td>
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<td>C2849_g1</td>
<td>Hexokinase</td>
<td>S2: 16.31, S3: 13.42, S4: 11.01&lt;br&gt;S2: 41.59, S3: 64.06, S4: 42.76&lt;br&gt;S2: 2.55, S3: 2.55, S4: 2.55&lt;br&gt;S2: 5.70, S3: 5.70, S4: 5.70&lt;br&gt;S2: 8.02, S3: 8.02, S4: 8.02</td>
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<td>C2628_g1</td>
<td>Malate dehydrogenase</td>
<td>S2: 0.88, S3: 1.01, S4: 0.84&lt;br&gt;S2: 6.21, S3: 4.23, S4: 4.07&lt;br&gt;S2: 7.06, S2: 7.06, S3: 7.06&lt;br&gt;S2: 4.07, S3: 4.07, S4: 4.07&lt;br&gt;S2: 4.07, S3: 4.07, S4: 4.07</td>
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Table 3. Transcripts found differentially expressed and annotated as belonging to given specific pathways in line 8016 compared to Kuras control. Contig names are given with annotated function and closest Arabidopsis thaliana homolog. Expression is given as fragments per kilobase of exon per million fragments mapped (FPKM). Differential expression is given as fold difference using the quote between 8016 and Kuras FPKM with significant differences highlighted in bold. S2, S3 and S4 represent samplings 2-, 3- and 4-month postplanting.

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<tr>
<th>Contig Location</th>
<th>Function</th>
<th>Contig</th>
<th>S2 (FPKM)</th>
<th>S3 (FPKM)</th>
<th>S4 (FPKM)</th>
<th>8016/Kuras</th>
<th>Location</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
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<td>8016 (FPKM)</td>
<td>8016/Kuras</td>
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<td>8016/Kuras</td>
<td></td>
<td></td>
<td>S2</td>
<td>S3</td>
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<td>S2</td>
<td>S3</td>
<td>S4</td>
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<tr>
<td>8016</td>
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<tr>
<td>Pyruvate Dehydrogenase, Ela component</td>
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<td>30.81</td>
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<td>NAD-dependent Glycerol-3-Phosphate Dehydrogenase Cytosol</td>
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<td>131.04</td>
<td>8.86</td>
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TAG and polar lipid levels and compositions are affected in field grown tubers

TAG was found to accumulate in both smaller and larger tubers of field grown plants. Line 8003 had the highest TAG content with a 38-fold increase in fraction of smaller tubers and a 125-fold (0.8% of dw) increase in fraction with larger tubers (Figure 7). Diacylglycerol (DAG) levels were also higher in tubers of line 8003 with an eightfold and 10-fold increase in respective tuber fractions over control tubers (Figure 7).

TAG composition was altered in the transgenic lines, and the most prominent change was related to higher TAG content. Fraction of 18:2 was increased from approximately 40% to around 64% of total FA in TAG, while the fraction of 18:3 was decreased. A very similar pattern could be observed for DAG composition with increases for 18:2 and decreases for 18:3 FA.

Polar lipids were found to be increased in transgenic lines, and their fatty acid composition showed the same trend in the shift as for TAG although not as dramatically (Figure 8).

Increase in tuber lipids is associated with altered starch content and composition in field grown tubers

Starch content and starch structure in tubers of the transgenic lines showed alterations in both accumulation and composition. Increased levels of tuber lipids led to a progressive decrease in starch content in almost all tuber fractions. The exception was line 8001, which showed an unaffected starch content. Starch content in both 8003 and 8016 small tuber fractions was 81% compared to wild type and only 68 and 74% compared to parental genotype in the large tuber fraction, respectively (Figure 9). The higher tuber yield in both 8001 and 8016 compensated for the low starch content, and the total starch yield per plant ended up at the same magnitude as the parental genotype (Figure 9). All transgenic lines had a higher ratio of amylopectin in the starch; however, the increase was not directly correlated to the oil content of the tubers (Figure 9). Compared to Kuras, the starch granules of 8016 were smaller and more irregular in shape (Figure 10).

Starch-bound neutral lipids increased in the transgenic lines

Potato starch has a very low amount of lipids bound to the starch. In the transgenic lines, the total content of neutral lipids bound to starch was drastically higher, ranging between 2 and 24-fold increase compared to parental genotype starch (Table 5). However, the lipid content varied a lot between different tubers from the same line. The average starch-bound lipid content of lines 8003 and 8016 was in the same range as commercially available maize starch, for which levels of 0.4% have been published (Vasanthan and Hoover, 1992).

Discussion

Root and tuber crops often have a high yield per area of land and are of great importance both as food and feed and as sources for industrial raw materials. It could be attractive to supplement carbohydrate-rich diets with fats in the harvested root or tuber crop. In addition, it could be of interest to combine starch and oil extraction which for potatoes involves a centrifugation phase where oil would go to the top and could thus easily be retrieved.

The question we wanted to answer was whether oil accumulation could be induced in potato tubers and what impact this
would have on general tuber metabolism. It could be that yellow
nutedge has unique features enabling a high oil deposition in
tubers, although there seem to be a big variation among different
genotypes in this accumulation capacity (Matthiesen and Stoller,
1978). In a previous attempt to increase oil accumulation in
potato, a single metabolic gene, acetyl-CoA carboxylase from
Arabidopsis thaliana, was expressed in amyloplasts of potato
tubers with a fivefold increase in accumulated TAG as a result
(Klaus et al., 2004). In the present study, a transcription factor
was expressed with a potential of influencing a wider range of
genes that could positively influence glycolysis and fatty acid
synthesis.

A potato GBSS promoter was used in our study to ensure
expression in tubers and a low expression in other tissues such as
leaves (Visser et al., 1991). WRINKLED1 has been shown to
negatively influence plant development if expressed in photosyn-
thetic tissues (Cernac and Benning, 2004).

In greenhouse, transgenic lines had a higher number of tubers
that in general were of smaller size with wider, deeper eye
developments and frequent budding of additional tubers on the
primary one. It is not directly evident what the cause for higher
number of tubers and tuber size reduction might be, although it
could be speculated that increased CO₂ production or the
additional metabolic and increased need of oxygen supply was
causing the effect with reduced volume but maintained total
tuber mass.

Greenhouse grown tubers from the S3 sampling were more
closely investigated as this was tubers under development and
would say more about the interaction between different
metabolic pathways. The TAG composition was greatly changed

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**Figure 4** Light micrographs of Kuras (a,b) and
transgenic potato line 8016 (c,d). (a,c) Overview
staining with MAS (Triple staining Methylene blue
—Azur A—Safranin O) and (b, d) Oil specific
staining, Sudan black. Arrows indicate oil droplets,
s—starch granule, v—vacuole, cw—cell wall,
n—nuclei. Scale bars 10 μm.

**Figure 5** Transmission electron micrographs of
potato tubers. (a) Kuras, scale bar 1 μm, (b) 8016
scale bar 1 μm, (c) 8016 scale bar 500 nm.
s—starch granule, cw—cell wall, cwt—cell wall
thickness, o—oil droplet, m—mitochondria, p—peroxisome.
in transgenic tubers with an increased fraction of 18:2 primarily at the expense of 18:3. Transcripts corresponding to stearoyl desaturase and oleoyl desaturase were up-regulated 14-fold and eightfold, respectively, at this stage while those related to linoleoyl desaturase were essentially unchanged and furthermore expressed at very low level (Table 3, Dataset S4). This would indicate that the linoleoyl desaturation could not keep up with the increased flow of fatty acids towards TAG resulting in an increased 18:2/18:3 ratio. A prominent feature of lipid analysis from greenhouse tubers was that polar lipids increased. Polar lipids in potato tubers are mainly part of membranes and the major classes being phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Galliard, 1968). TAG is most likely produced in all tissues but generally on a very low level where most of the fatty acid synthesis in nonlipid storing tissue is sequestered to other products such as membrane lipids. WRINKLED1 expression has been associated with large increases in TAG deposition (Baud et al., 2009; Bourgis et al., 2011; Cernac and Benning, 2004; Tranbarger et al., 2011). Under normal conditions, in a tissue not storing TAG, fatty acids from TAG are exposed to acyl editing and exchange with membrane lipid fractions while in oil accumulating tissue, it is to a large extent shielded from this cycling via oil body proteins organizing TAG into lipid droplets. In oil accumulating tissues, polar and membrane lipid content is rapidly passed by TAG content. The polar and membrane fatty acids found in Kuras tubers were 24-fold in excess of TAG fatty acids but only twofold in excess in tubers of transformed lines. This indicates that although fatty acids in TAG were increased 30-fold in transgenic potato tubers, a still much larger proportion of fatty acids stayed in polar lipids and it could be speculated that either the flux into TAG was not strong enough or budding of TAG in proper lipid bodies was not sufficiently developed which could lead to a large fatty acid exchange with polar lipids. Supply of DAG for MGDG and DGDG synthesis seemed to follow the eukaryotic pathway in potato. This means that the regulation between TAG, DAG, PC, MGDG and DGDG takes place outside the plastid where all interacting lipid species, at least theoretically, have an equal priority to increased fatty acid synthesis. It can be argued that this is a reason why the TAG must be shielded from interacting enzymes and potential shuffling between TAG and membrane lipids in nonaccumulating tissue. From transcriptomic analysis, it could be seen that genes homologous to oleosins actually did increase substantially in expression, although still to a rather low

<table>
<thead>
<tr>
<th>Tuber yield (kg/plant)</th>
<th>&lt;42 mm</th>
<th>42-55 mm</th>
<th>55-65 mm</th>
<th>&gt;65 mm</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kuras</td>
<td>0.04</td>
<td>0.19</td>
<td>0.47</td>
<td>0.44</td>
<td>1.14</td>
</tr>
<tr>
<td>8001</td>
<td>0.21</td>
<td>0.54</td>
<td>0.41</td>
<td>0.21</td>
<td>1.36</td>
</tr>
<tr>
<td>8003</td>
<td>0.35</td>
<td>0.40</td>
<td>0.11</td>
<td>0.01</td>
<td>0.87</td>
</tr>
<tr>
<td>8016</td>
<td>0.35</td>
<td>0.39</td>
<td>0.42</td>
<td>0.18</td>
<td>1.54</td>
</tr>
<tr>
<td>8022</td>
<td>0.18</td>
<td>0.09</td>
<td>0.01</td>
<td>0.00</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Figure 6: Dry matter content of field grown tubers size fractionated into <55 mm and >55 mm. Three biological replicates ±SD. Letters distinguish significant different means according to Tukey’s test at level $P < 0.05$.

Figure 7: Analysis of TAG and DAG from field grown tubers, (a) TAG content of tuber of Kuras and transgenic lines, (b) DAG content of tubers of Kuras and transgenic lines, (c) TAG composition of Kuras and transgenic lines, (d) DAG composition of Kuras and transgenic lines. Three biological replicates ±SD.
level (Table 3). Interestingly, expression of oleosins has previously not been considered as an effect of WRINKLED1, but rather transcription factors upstream as LEC2 (Santos Mendoza et al., 2005). From other aspects, increases of transcript types that have previously been associated with WRINKLED1 were observed, which in particular applies to genes encoding enzymes involved in late glycolysis steps and fatty acid synthesis (Baud and Graham, 2006; Cernac and Benning, 2004; Ruuska et al., 2002), and found much more highly expressed in the transgenic tubers (Tables 2 and 3). In particular, it was evident that transcripts associated with steps in glycolysis beyond those needed for starch synthesis were found up-regulated. This is important as potato tubers also are sink tissues just like seeds under development, where WRINKLED1 normally is operating and there are then enzymatic steps of common importance independent of which storage compound that is accumulated. Among up-regulated transcripts were also genes indicating metabolic activity not normally associated with WRINKLED1 but possibly a secondary effect leading towards a higher pyruvate supply. Examples are transcripts encoding enzymes in the pentose phosphate pathway (PPP) and phosphoribulosokinase (PRK), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which together could pull metabolites from plastid hexose phosphates associated with starch synthesis (Figure 11 and Dataset S2). This observation indicated development of a Rubisco shunt which has previously been investigated in relation to CO2 capturing and metabolite flux (Figure 11 and Dataset S2). This observation indicated development of a Rubisco shunt which has previously been investigated in relation to CO2 capturing and metabolite flux (Figure 11 and Dataset S2). These are likely not direct targets of WRINKLED1 but most probably secondary effects. Interestingly, a potato homolog to one of the known master regulators LEC1 was found up-regulated in the investigated transgenic line. LEC1 is known to among others, induce oleosin encoding genes (Lotan et al., 1998; Mu et al., 2008). These, for oil accumulation, important transcripts have not been found up-regulated upon ectopic WRINKLED1 expression even when a great number of WRINKLED1 homologs were assessed in leaf tissue (Grimberg et al., 2015).

Surprisingly concomitant with an increase in TAG accumulation, an almost 100-fold increase of glucose to more than 18% of dry weight was found. This was accompanied by a large increase of also sucrose and fructose but to more moderate absolute levels and could indicate an increased mobilization of starch. However, in the transcriptional changes caused by WRINKLED1 expression, there were no obvious clues which could explain this increase in sugars although many enzymes participating in carbohydrate and starch metabolism are subject to post-translational modifications as phosphorylation and redox modifications, which regulate the activity of and interaction between enzymes (Geigenberger, 2011).

The potato lines were subjected to a field trial where tubers were planted and harvested following a general time schedule for starch potatoes. Tubers were divided into two fractions of being either <55 mm in size or larger than 55 mm in size. Trends were similar to that of greenhouse tubers in that total tuber yield in malate and expression of genes encoding ATP-citrate lyase and malic enzyme (Figure 11). In summary, the transcriptional analysis of potato tubers expressing WRINKLED1 displayed features going beyond what is normally associated with WRINKLED1 induction. These are likely not direct targets of WRINKLED1 but most probably secondary effects. Interestingly, a potato homolog to one of the known master regulators LEC1 was found up-regulated in the investigated transgenic line. LEC1 is known to among others, induce oleosin encoding genes (Lotan et al., 1998; Mu et al., 2008). These, for oil accumulation, important transcripts have not been found up-regulated upon ectopic WRINKLED1 expression even when a great number of WRINKLED1 homologs were assessed in leaf tissue (Grimberg et al., 2015).

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increased and tuber sizes decreased in comparison with control. Tuber phenotype was altered as for greenhouse tubers but with a tendency for more elongated tubers with wider and deeper eyes. Alterations in tuber phenotype have previously been observed in connection with modification of the starch metabolism resulting in a decrease in starch content and an increase in soluble sugars (Hofvander et al., 2004; Riewe et al., 2008; Tjaden et al., 1998). The tubers in these published studies did show an elongated shape and a tendency to bud-off additional tubers.

Table 5 Starch-bound lipids in field grown tubers. N = 3

<table>
<thead>
<tr>
<th>Tubers</th>
<th>Starch-bound lipids (% per mg starch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubers &lt; 55 mm</td>
<td>0.018 ± 0.007 Kuras 0.007 0.017 /C6 0.004 8001 0.100 ± 0.030 0.030 0.038 /C6 0.019 8003 0.436 ± 0.436 0.344 ± 0.076 0.076 8016 0.339 ± 0.339 0.281 ± 0.083</td>
</tr>
<tr>
<td>Tubers &gt; 55 mm</td>
<td></td>
</tr>
</tbody>
</table>

Table 5 shows the starch-bound lipids in field grown tubers. The starch-bound lipids were measured as a percentage of mg starch. The values are presented for tubers smaller than 55 mm and tubers larger than 55 mm. The values are given as mean ± standard deviation. The tubers were classified based on their size, and the starch-bound lipids were measured and compared between the two groups.

Figure 10 Light micrographs of starch granules of parental variety Kuras stained with iodine (a-c) and transgenic line 8016 (d-f). Scale bars 200 μm (a,d), 100 μm (b,e) and 50 μm (c,f).

An open question is if the observed increased level of starch-bound lipids could cause any of these effects. This study showed that accumulation of triacylglycerols with an altered composition in potato tubers could be induced by the Arabidopsis thaliana transcription factor WRINKLED1. It was clear from this study that a starch storing tuber crop as potato does not have a dormant capacity for oil accumulation in the form of TAG at least not by up-regulating basic factors for fatty acid synthesis. One factor of importance that could be identified was that unlike in storage organs which primarily store TAG, there was a concomitant increase in membrane lipids. This deposition of additional fatty acids could also disturb tuber development. Commonly, TAG produced in oil-storing tissue is organized by oil body proteins which lead to structures of a defined size that also are shielded and protected from other metabolic activities. From structural studies of the transgenic tubers, there was an indication of oil in droplets which would lead to the conclusion that proteins for organization into oil bodies are present in the tubers. This is similar to the generation of starch granules during starch accumulation which also is a way to pack the deposition product and shield it from other metabolic activities. Thus, as further studies, it would be of great interest to overexpress genetic factors which could protect the accumulated TAG and reduce interactions with membrane lipids via PC or increase the pull towards TAG via diacylglycerol acyltransferase (DGAT) as has been carried out in transgenic tobacco (Vanhercke et al., 2014). To fully utilize the storage product accumulation capacity of potato and potato tubers, another crucial topic for further studies is which mechanism or mechanisms that triggered or caused the huge accumulation of sugars as the concentration increase in itself is very likely to interfere with efficient sink mechanisms and storage product accumulation.
**Figure 11** Metabolic map of greenhouse grown potato tubers: Quantified metabolites are displayed in rectangular boxes. Metabolites were analysed by GC-MS. Kurus control tubers (grey bar) and transgenic lines 8001, 8003 and 8016 (black bars) were sampled at stage S3. Values are means of three biological replicates analysed each in two technical replicates. Error bars, standard deviations of the three biological replicates. Transcripts are displayed in square boxes. Transcript levels of Kurus control tubers (white bar) and the transgenic line 8016 (black bar) were sampled at stage S3. Transcript levels were calculated as fragments per kilobase of exon per million fragments mapped (FPKM). Values are means of three biological replicates. Error bars, standard deviation (For individual transcript levels used see Table S3). Abbreviations Enzymes: P, plastidial; M, mitochondrial; ZODGH, 2-oxoglutarate dehydrogenase; ACAT, Acetyl-CoA C-acyltransferase; AGPase, ADP-glucose pyrophosphorylase; CS, citrate synthase; DGAT, diacylglycerol o-acetyltransferase; DT, dicarboxylate transporter; G3P Deh., glycerol-3-phosphate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate 2-0-acetyltransferase; GPT, glucose-6-phosphate phosphate translocator; HMG, 3-Hydroxy-3-methylglutaryl-CoA reductase; HMGs, hydroxymethylglutaryl-CoA synthase; IDH, isocitrate dehydrogenase; LPAT, 1-Acyl-sn-glycerol-3-phosphate acyltransferase; malic enzyme, NADP-dependent malic enzyme; MDH, malate dehydrogenase; PEP, phosphoenolpyruvate carboxylase; PFSP, pyrophosphate-fructose-6-P phosphotransferase; PFK, phosphofructokinase; PGI, phosphoglucone isomerase; PGK, 3-Phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; PPT, phosphoenolpyruvate phosphate translocator; PRK, phosphoribulokinase; pyruvate DH, pyruvate dehydrogenase; RibSP epimerase, ribulose-5-phosphate-3-epimerase; RibSP isomerase, ribose 5-phosphate isomerase; SCL, succinyl-CoA ligase; SDH, succinate dehydrogenase; SuSy, sucrose synthase; TA, transaldolase; TK, transketolase; TPI, triosephosphate isomerase; UGPase, UDP-glucose pyrophosphorylase. Abbreviations Metabolites: 1,3BPG, 1,3-bisphosphoglycerate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; DAG, diacylglycerol; DHAP, dihydroxacetone phosphate; GA3P, glyceraldehyde 3-phosphate; MAG, monoacylglycerol; PEP, phosphoenolpyruvate/phosphate translocator; PRK, phosphoribulokinase; pyruvate DH, pyruvate dehydrogenase; RibSP epimerase, ribulose-5-phosphate-3-epimerase; RibSP isomerase, ribose 5-phosphate isomerase; SCL, succinyl-CoA ligase; SDH, succinate dehydrogenase; SuSy, sucrose synthase; TA, transaldolase; TK, transketolase; TPI, triosephosphate isomerase; UGPase, UDP-glucose pyrophosphorylase.

### Experimental procedures

#### Gene construct
A synthetic gene corresponding to *AtWRINKLED1* (Cernac and Benning, 2004) was ordered (Eurofins/MWG, Ebersberg, Germany) and cloned after a 990 bp *Solanium tuberosum* GBSS promoter fragment (Hofvander et al., 2004) in a pBII19 (Bevan, 1984) derivative binary vector. After controlling the integrity of the plasmid, it was transformed into *Agrobacterium tumefaciens*, strain AGLO (Lazo et al., 1991) via direct transformation (An et al., 1989).

#### Plant material and growth conditions
Leaf tissue of potato cultivar Kurus was transformed as previously described (Andersson et al., 2003), but with 50 mg/L kanamycin as selection agent. Regenerated shoots were analysed for the presence of nptII and the absence of *Agrobacterium* virG sequence using REDEXtract-N-Amp Plant PCR Kit (Sigma-Aldrich, St Louis, MO). Primers: nptII 5'-CTTCGTACCTACCTGTCCTC-3', nptIIr 5'-AGTCCCGCTCAGAA-3', 498 bp npt II PCR product amplified with primers 5'-CTTCGTACCTACCTGTCCTC-3' and 5'-AGTCCCGCTCAGAA-3' and labelled with PCR DIG Probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) was used as probe and hybridized to the membrane o/n. CDP-Star, ready-to-use (Roche Diagnostics GmbH, Mannheim, Germany) was used for detection of the hybridized probe and exposed to an X-ray film.

#### Determination of copy number by Southern blotting
Genomic DNA was isolated using Illustra DNA Extraction Kit Phytoture (GE Healthcare, Buckinghamshire, UK). About 10 μg DNA was digested with EcoRV and separated on a 1% agarose gel and blotted to a Hybond N+ membrane (GE Healthcare). A 498 bp npt II PCR product amplified with primers 5'-CTTCGTACCTACCTGTCCTC-3' and 5'-AGTCCCGCTCAGAA-3' and labelled with PCR DIG Probe synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) was used as probe and hybridized to the membrane o/n. CDP-Star, ready-to-use (Roche Diagnostics GmbH, Mannheim, Germany) was used for detection of the hybridized probe and exposed to an X-ray film.

#### Lipid analyses
Tuber samples were freeze-dried (Coolsafe, Scanvac, Lyngby, Denmark). Total lipids were extracted (Bligh and Dyer, 1959), and the CHCl₃ extracts were subsequently separated (aliquots corresponding to 100 mg fw) using thin layer chromatography on TLC Silica gel 60 (Merck, Darmstadt, Germany). For neutral lipids, 70:30:1 heptane:diethyl ether:H₂O. Fatty acid methyl esters were quantified using gas chromatography as previously described (Leonova et al., 2008).

#### Quantitative and qualitative starch determination
Dry matter was determined after 3 days of freeze drying (Coolsafe, Scanvac, Lyngby, Denmark). Homogenized freeze-dried tuber tissue was used for measurement of total starch content using a Total starch kit (Megazyme, Bray, Co. Wicklow, Ireland). Samples were washed with 80% ethanol to remove glucose present in the sample. For calculation of starch content, a standard curve was used based on a maize starch sample supplied in the kit.

Starch was isolated and purified from fresh tubers (Larsson et al., 1996). Amylese content of isolated starch was measured according to a DMSO and iodine-based colorimetric analysis method (Andersson et al., 2006).

Starch-bound lipids were extracted from purified starch using a water-saturated n-propanol extraction method (Vasanthan and Hoover, 1992).
Extraction, determination and quantification of metabolites

For the metabolite measurements by GC-MS analysis, 20 mg of flash-frozen and lyophilized potato tuber material was ground to a fine powder using a shaking mill and glass beads (5 mm). The polar fraction was extracted and derivatized with 30 μL methoxamine hydrochloride and 60 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as previously described (Bellaire et al., 2014) to transform the metabolites into their methoxymino (MEOX)- and trimethylsilyl (TMS)-derivatives. allo-Inositol was used as an internal standard. The samples were analysed on an Agilent 5973 Network mass selective detector connected to a Agilent 6890 gas chromatograph equipped with a capillary HPS-MS column (30 m × 0.25 mm; 0.25 μm coating thickness; J&W Scientific, Agilent, Santa Clara, CA). Helium was used as carrier gas (1 mL/min). The inlet temperature was set to 230°C, and the temperature gradient applied was 50°C for 2 min, 50–330°C at 5 K/min 330°C for 2 min. Electron energy of 70 eV, an ion source temperature of 230°C and a transfer line temperature of 300°C were used. Spectra were recorded in the range of 71–600 da/e.

Masses used for quantification of the individual metabolites are depicted in Dataset S1. For absolute quantification, all compounds were quantified against the standard using a calibration curve performed with pure substances based on a total of 12 measurements for each substance.

Structural analysis of tuber tissue

For fixation and plastic embedding, tubers with an approximate weight of 10 g were harvested from pots grown in greenhouse. Tissue samples were taken from six different locations in the tuber, and orientation of the samples in relation to the stolon was noted. The tissue was immediately aldehyde-fixed, post-fixed in osmium tetroxide and embedded in fresh Spurr’s resin (Low viscosity kit; Ted Pella, Redding, CA) (Turesson et al., 2010).

For transmission electron microscopy, ultrathin sections were cut from Spurr-embedded blocks and handled as in Turesson et al. (2010). A JEM-1010 electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 60 kV was used to examine the sections.

For staining of light micrographs, an overview was obtained by using proteins, lipids and starch (Heneen et al., 2008; Turesson et al., 2010; Warmke and Lee, 1976). Sudan Black was used for staining oil and lipids as described earlier (Heneen et al., 2008; O’Brien and McCully, 1981; Turesson et al., 2010). The stained and air-dried sections were mounted with Biomount (British Biocell, Cardiff, UK) and studied in a light microscope (Leica Microsystems, Wetzlar, Germany).

For staining of homogenized tissue, Lugol’s solution (Scharlau, Sentmenat, Spain) and glycerol (1:1) was added to purified starch granules and studied under a light microscope (Leica Microsystems, Wetzlar, Germany).

Transcriptome analysis

Tuber tissue from line 8016 and Kuras was sampled from greenhouse grown plants at three different time points, 2, 3 and 4 months after planting (S2, S3 and S4, respectively), and immediately flash-frozen in liquid nitrogen. Tissue from three tubers grown in individual pots was pooled prior to total RNA extraction using Plant RNA Reagent (Invitrogen, Life technologies Ltd, Waltham, MA) according to the manufacturer’s instructions. This was applied in triplicates as biological replicates for all three stages. Quality and quantity of the RNA samples were measured on a NanoDrop (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific, Waltham, MA) and using electrophoresis on a 1.2% E-gel (Invitrogen, Life Technologies Ltd).

Library construction and sequencing using Illumina HiSeq technology was performed by GATC Biotech (Constance, Germany).

All the prepared samples were sequenced through Illumina sequencing platform HiSeq 2000 as unpaired-end reads with 50-bp read length. Reads were trimmed by removing adapter sequences, and low quality sequences were removed. Assembly was carried out with high-quality reads using Trinity version trinityrnaseq_r2014-07-17 with default parameters (Haas et al., 2013).

To assess the quality of the transcriptome assembly, reads were mapped back to the assembled transcripts using the Bowtie2 aligner (Langmead and Salzberg, 2012). The mapping results were visualized using Integrated Genomics Viewer version 2.3.2 (IGV) (Thorvaldsdottir et al., 2013). The completeness of the transcriptome assembly was estimated with CEGMA version 2.4.013012 software (Parra et al., 2007). CEGMA analysis was performed on default parameters. High-quality-assembled transcriptome was annotated with BLAST2GO (Conesa et al., 2005). BLASTX was used against the NCBI nonredundant (nr) protein database using an e-value cut-off of 1E-5. InterProScan was used to identify the conserved domains/motif for each transcript.

RSEM version 1.2.15 was used for abundance estimation of the assembled transcripts using the default parameters (Li and Dewey, 2011). The relative measure of transcript abundance was TPM (transcripts per million) and FPKM (fragments per kilobase of transcript per million mapped reads). The relative level of all assembled transcript can be found in Dataset S4. EdgeR (Robinson et al., 2010) was used in identification and analysis of differentially expressed genes and transcripts through Trinity version trinityrnaseq_r20140717 on default settings. Data on all transcripts determined as differentially expressed can be found in Dataset S3.

Acknowledgements

We would like to thank Ann-Sofie Fält for careful supervision of transformation, tissue culture and greenhouse plants, Salla Marttila and Kerstin Brismar for technical assistance in TEM work and interpretation and Sten Stymne for support and discussions on the subject matter of this manuscript. We would also like to thank Sabine Freitag for her excellent help in sample preparation for GC-MS measurements.

This project had financial support from SSF (Swedish Foundation for Strategic Research), The Swedish Research Council Formas strategic research program Trees and Crops for the Future (TC4F) and Einar and Inga Nilsson’s foundation.

Conflict of interest

All authors declare no conflict of interest.


Tranbarger, T.J., Dussert, S., Joel, T., Argout, X., Summo, M., Champion, A., Cros, D. et al. (2011) Regulatory mechanisms underlying oil palm fruit


Supporting information

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

Figure S1 TLC separation of neutral lipids extracted from microtubers of Kuras, transgenic lines and potato seed.

Figure S2 Triacylglycerol content of microtubers representing Kuras and transgenic lines 8001 and 8016.

Figure S3 Fatty acid composition of triacylglycerol from Kuras control microtubers, microtubers of transgenic lines 8001 and 8016, potato seed.

Figure S4 Southern blotting of total DNA from Kuras and transgenic potato lines.

Figure S5 Field grown tubers of transgenic line 8016.

Dataset S1 Metabolites shown in Figure 11.

Dataset S2 Transcripts shown in Figure 11.

Dataset S3 Transcripts differentially expressed in transgenic line 8016.

Dataset S4 Expression levels of transcripts in Kuras and transgenic line 8016.