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Alteration of Fatty Acid Compositions in A Starch-deficient Mutant *Arabidopsis thaliana* (*adg1-1*) during Seed Development

Perubahan Komposisi Asid Lemak semasa Perkembangan Biji dalam Mutan Kurang Kanji Arabidopsis thaliana (adg1-1)

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Abstract

Disruption of the *ADG* gene in *Arabidopsis* results in 6% increase of total fatty acids in mature desiccated seeds. A starchless mutant of *Arabidopsis thaliana*, ADP glucose pyrophosphorylase (*adg1-1*) has been investigated concerning the lack of starch in its leaves. In this work of metabolic analysis of *adg1-1* mutant, there was very little or no starch accumulation throughout seed development. Even though the growth of these mutant plants was delayed, seed development of the mutants did not show much difference from the wild type. In mature desiccated seeds, there was no significant difference in the seed dry weight of the mutant compared to the wild type. The *adg1-1* mutant showed a shift in carbon partitioning to fatty acid (FA) and sucrose accumulation. Major changes have occurred in FA 18.0 (stearic), FA 18.1 (oleic) and FA 18.2 (linoleic). In mature seeds of *adg1-1*, there was a 70% increase in FA 18.2, a 75% increase in FA 18.1 and a 76% reduction in FA 18.0. The results show that the assimilate partitioning within *Arabidopsis* seeds has the potential to be altered by relatively simple genetic manipulations. However, the mechanisms by which these changes occurred remain unknown.

Keywords starchless mutant, ADP glucose pyrophosphorylase (*ADG*), carbon partitioning, seed development, metabolism

Abstrak

Gangguan gen *ADG* dalam *Arabidopsis* memberi peningkatan sebanyak 6% daripada jumlah keseluruhan asid lemak dalam biji kering matang. Satu mutan *Arabidopsis thaliana* yang mengandungi kurang kanji, ADP glukosa pirofosforilase (*adg1-1*) telah dikaji tentang kekurangan kanji pada daunnya. Dalam kajian analisis metabolisme *adg1-1* mutan ini, terdapat sangat sedikit atau tidak ada pengumpulan kanji di sepanjang fasa perkembangan biji. Walaupun pertumbuhan mutan ini agak lambat, perkembangan biji mutan tidak menunjukkan banyak perbezaan daripada jenis liar. Tidak terdapat perbezaan yang signifikan dalam berat biji kering mutan berbanding jenis liar. Mutan *adg1-1* menunjukkan perubahan partisi karbon ke arah pembentukan asid lemak (FA) dan penumpukan sukrosa.

Perubahan besar berlaku pada FA 18.0 (stearat), FA 18.1 (oleik) dan FA 18.2 (linoleat). Dalam biji matang mutan *adg1-1*, berlaku peningkatan sebanyak 70% pada FA 18.2, 75% peningkatan FA 18.1 dan penurunan sebanyak 76% FA 18.0. Keputusan kajian menunjukkan bahawa *partisi asimilat* dalam biji *Arabidopsis* mempunyai potensi untuk diubah secara mudah melalui manipulasi genetik. Namun, mekanisme perubahan ini terjadi masih belum dikenalpasti.

Kata kunci mutan kekurangan kanji, ADP glukosa pirofosforilase (*ADG*), partisi karbon, perkembangan biji, metabolisme

Introduction

The use of knock-out mutants and transgenic plants are relatively novel approaches in plant biology and molecular research. Mutants provide the opportunity to study carbon metabolism in seed development, including the regulation of key enzymes and the roles of genes in the synthesis and breakdown of novel and existing products in seeds.

Recently, a great deal of research has been done on *Arabidopsis* as a model plant system. Nonetheless, studies of *Arabidopsis* seed development have been very limited due to the difficulty in handling its very small seeds.

Mutants are available that lack key metabolic enzymes, usually resulting in the reduction one of storage reserves and often accompanied with an increase in other storage product (Chung *et al*., 1999). Sometimes, these phenomena are followed by the production of wrinkled phenotypes (Bhattacharyya *et al*., 1993; Focks & Benning, 1998).

In *Arabidopsis* starch-deficient mutants, *pgm1* and *adg1*, which lack one of the most important enzymes in starch pathways, phosphoglucomutase and ADP-glucose pyrophosphorylase respectively, showed a very low level of starch in their leaves (Caspar *et al*., 1985; Lin *et al*., 1988). Mutation in starch metabolism also occurred in other plants. In rice for example, s*hr* mutation resulted in reduction in starch content to about 60% of the wild type level due to reduced activity of ADP-glucose pyrophosphorylase (Kawagoe *et al.*, 2005). A decline in starch as well as the proportion of amylopectin also occurred in wrinkled rr pea (*Pisum sativum* L.) mutation with an increase in protein content (Bhattacharrya *et al.*, 1993). In barley seed, mutant Risj 16 reduced starch content as a result from lacking a small cytosolic subunit of AGPase (Johnson *et al.*, 2003). These reductions in storage compounds also resulted in weight decrease and as well as a decline in seed yield.

The objectives of this research were to analyse the metabolic components of primary metabolisms in developing seeds of *Arabidopsis thaliana* mutant, *adg1-1* by determining the levels and profiles of the relevant metabolites.

Methodology

Seed sampling

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) and a starchless mutant, *adg1-1*, were obtained from the Nottingham Stock Centre (United Kingdom, UK). Seeds were sown in trays (17 cm x 20 cm) containing moist Homebase multipurpose compost (Homebase

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Limited, Surrey, UK) and vernalised at 4 °C for two days, before being transferred to a Gallenkamp Industrial (Leicester, UK) growth cabinet at 20-22 °C with a 10 h light period of photosynthetic photon flux density (PPFD) 250 µmol m^{-2} s^{-1} (supplied by daylight fluorescent tubes). After 10 days, seedlings were transferred into 3 cm diameter pots containing 80-100 mL of multipurpose compost and maintained under the same conditions.

When the plants started flowering, flowers that had just opened widely were tagged using different colours of thread. The siliques at different stages of development, from three days after flowering until the seeds matured, were collected in 1.5 mL safety-cap Eppendorf tubes (Sarstedt, Germany) and immediately frozen in liquid nitrogen. The siliques were then opened under a dissecting microscope (Model SDZ-PL, Kyowa, Japan) and 20-40 of corresponding seeds were pooled in an Eppendorf and immediately frozen in liquid nitrogen. Samples were stored in liquid nitrogen or at -80ºC freezer until they were required. In most analyses, frozen samples were ground before adding the extraction buffer except in lipid analysis in which samples were ground at normal temperature.

Seed development

Pools of 40 seeds taken at different times after flowering were weighed on an AG204 Mettler Toledo (Switzerland) balance just after harvest (fresh weight) and after drying at 50ºC, for 48 hours (dry weight). At least three replicate samples were taken for each measurement.

Biochemical measurements

Glucose, fructose and sucrose measurements were performed using a 96 well microplate (Corning Incorporated, New York, USA) based on the enzymatic reaction where the production of NADPH was followed photometrically at a wavelength of 340 nm (Stitt *et al*., 1989; Sturgeon, 1990). The pellet left from the sugar extraction was used for starch determination (Baud *et al.*, 2002).

Total protein was determined using Bio-Rad protein assay reagent based on a modified version of the procedure described by Bradford (1976). Proteins were assayed using 96 well microplate (Corning Incorporated, New York, USA) and bovine serum albumin (BSA) was used as a standard.

Total fatty acids and the compositional analyses were done by gas chromatography using the method modified by Baud *et al.* (2002).

Verification of ADP-glucose pyrophosphorylase mutant

Verification of the ADP-glucose pyrophosphorylase mutant (*adg1-1*) was carried out using western blotting. For SDS-PAGE, two types of gel were used; a stacking gel containing 120 mM Tris-HCl (pH 6.8), 4.9% (w/v) acrylamide, 0.1% (w/v) N'N'-methylenebisacrylamide, 1% (w/v) SDS, 0.5% ammonium persulphate (APS) and 0.1% (w/v) NNN'N'-tetramethylethylenediamine (TEMED) and a resolving gel containing 370 mM Tris-HCl (pH 8.8), 9.6% (w/v) acrylamide, 0.3% (w/v) N'N'-methylenebisacrylamide,

1% (w/v) SDS, 0.5% APS and 0.1% (v/v) TEMED were used. Blots were probed with a polyclonal antiserum raised against the protein (ADP-glucose pyrophosphorylase (*sh2* and *bt2*)) diluted 1 in 1000 in blocking solution (25ºC, 2 h). Blots were then washed and incubated with an anti-rabbit IgG peroxidase conjugated secondary antibody (Sigma-Aldrich Company Limited, Poole, UK) diluted 1 in 2500 in blocking solution (25ºC, 1 h). Immunoreactive polypeptides were visualised using an enhanced chemiluminescence kit (Amersham Life Sciences Limited, Buckinghamshire, UK) and the image captured on Kodak BioMax MR film (Kodak, New York, USA) using an exposure cassette with intensifying screens (Kodak, New York, USA).

Results

Mutant verifications

The presence of ADGase was confirmed by western blotting using antibodies raised against the small subunit of ADGase at 51 kDa and the large subunit at 54 kDa from wheat. A clear band was detected at 51 kDa in the wild type (Col-0) and wheat endosperm extracts, when using the antibody against ADGase small subunits (Fig. 1A) and at 54 kDa with antibody against ADGase large subunits (Fig. 1B). As Rubisco protein has almost the same molecular mass (55 kDa), a western blot was done to distinguish between the two proteins (Fig. 1C).

Studies in *Arabidopsis* ADGase have shown that the wild type ADGase comprises two small and two large subunits (Lin *et al.*, 1988). Both small and large subunits were present in the western blot of wild type *Arabidopsis* leaf extract (Lin *et al*., 1988). However, an unclear band was detected in the large subunit western blotting (Fig. 1B), suggesting that proteolytic degradation of ADGase might have occurred which caused the appearance of a band at approximately 54 kDa. Proteolysis can be prevented by using PMSF and

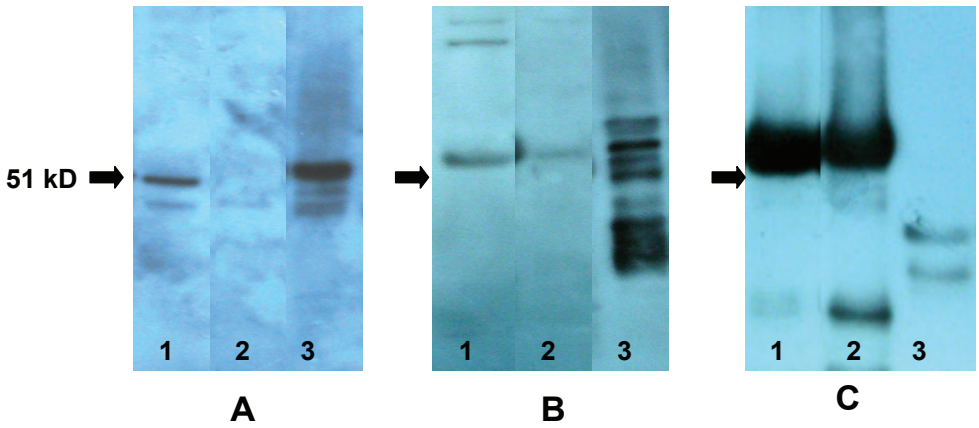

Figure 1 Western-blot analysis of ADGase from *Arabidopsis* wild type Col-0 leaf (lane 1), mutant *adg1-1* leaf (lane 2) and young wheat endosperm (lane 3) extracts containing 80-200 µg protein. Figure 1(A), using antibody raised against ADGase small subunit protein; Figure 1(B), using antibody against ADGase large subunit protein; and Figure 1(C), using antibody against Rubisco protein.

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chymostatin during extraction (Plaxton & Preiss, 1987). The *adg1-1* mutant is a monogenic recessive mutant on the ADGase small subunit (Wang *et al*., 1998). It has the large subunit genes, which can be transcribed and translated in mutant plants. However, without the small subunit, large subunits could not assemble and remain functionless. Therefore, the results showed that both subunits were absent in the western blot of *adg1-1* mutant (Fig. 1A & 1B), agreeing with the previous findings (Lin *et al*., 1988; Wang *et al*., 1998).

Seed Growth and Development

Determination of mass measurement was done throughout the seed development; starting from three days after flowering (DAF) until the seed matured and desiccated. Both the wild type and *adg1-1* mutant show the same pattern throughout the seed development; seed dry weight increased gradually reaching maximum weight when the seeds desiccated (Fig. 2). Mean dry weight of mature desiccated seed of Col-0 is 21.6 ± 0.9µg and *adg1-1* is 21.7 ± 1.0µg. There is no significant difference in dry weight of mature desiccated seeds between them (Students' t-test; $p>0.05$).

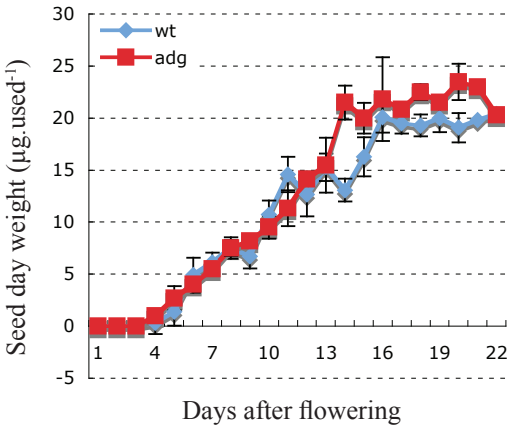

Figure 2 Seed development Col-0 and *adg1-1* based of dry weight measurement. Data are means of samples from at least four replicates (± SE) and each replicate contained 40 seeds.

Development of Seed Compositions

The seeds of Col-0 and *adg1-1* transiently accumulated glucose and fructose during the early stage of seed development, but the accumulated hexoses gradually disappeared as the seeds reached maturity (Fig.3). In contrast, sucrose was at a very low concentration in the early stage of seed development of both Col-0 and *adg1-1*, increased steadily and reached maximal amount in mature seeds. However, *adg1-1* accumulated significantly higher amount of sucrose (97.2 ± 2.3 ng.seed^{-1}) in mature desiccated seed compared with Col-0 (71.2 ± 5.3 ng.seed^{-1}) (Table 1).

The compositions of mature desiccated seeds of Col-0 and *adg1-1* are shown is Table 1. As expected, there was a significant difference of starch content, as *adg1-1* contained no starch at all. There was a significant difference in protein content (p <0.01), hexose (p <0.001), sucrose (p <0.01) and total amino acid (p <0.05) between Col-0 and *adg1-1*. The

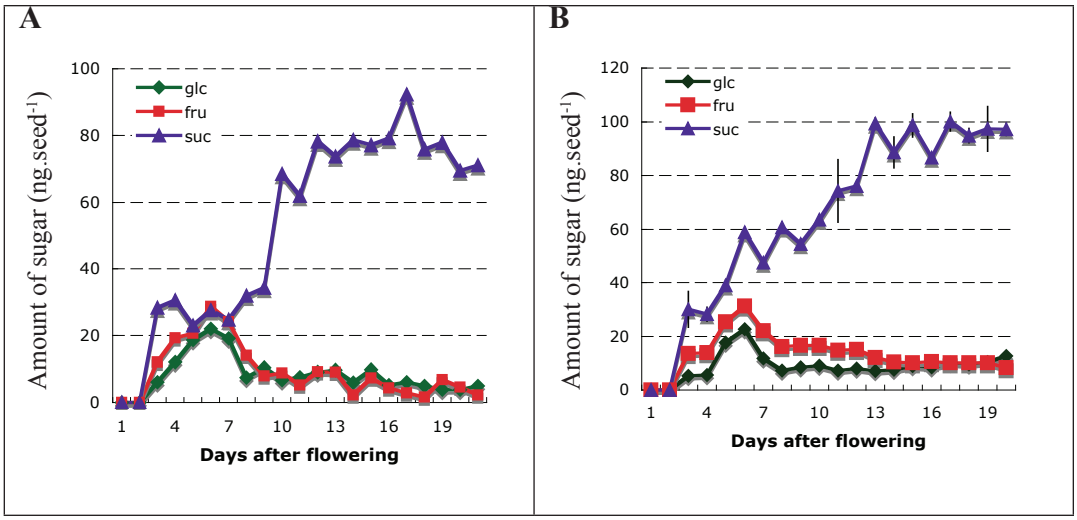

Figure 3 Sugar compositions at different stages of seed development of Col-0 (A) and *adg1-1* (B); glc: glucose, fru: fructose, suc: sucrose. Data are means of samples from at least four replicates (± SE) and each replicate contained 40 seeds.

amount of total fatty acid in mature desiccated seeds of wild type and *adg1-1* were about 9-12 mg.seed^{-1} that accounted for approximately 40-50% of seed dry weight. On a seed dry weight basis, total fatty acids in mature desiccated seeds in *adg1-1* mutant was not significantly different from Col-0 (p >0.01) (Table 1).

Table 1 The compositions of mature desiccated seeds

Genotype	Col-0	*Adg1-1*
Mean dry weight (mg.seed^{-1})	21.6 ± 0.9	21.7 ± 1.0
Hexose content (ng.seed^{-1})	7.2 ± 1.1	21.0 ± 0.9***
Sucrose content (ng.seed^{-1})	71.2 ± 5.3	97.2 ± 2.3*
Starch content (ng.seed^{-1})	37.8 ± 3.5	0.0 ± 0.0***
Protein (mg.seed^{-1})	7.5 ± 0.2	6.1 ± 0.2**
Total amino acid (mg.seed^{-1})	3.6 ± 0.2	2.3 ± 0.1*
Total fatty acid (%)	45.0 ± 1.6	51.1 ± 2.4

The results are presented as means (n=3). Differences between Col-0 and *adg1-1* mutant were analysed using Student's t-test. Significance levels are *p <0.05, **p <0.01, ***p <0.001.

Fatty acid compositions during seed development

The number of fatty acid types increased throughout the seed development of Col-0 and about seven major fatty acids were detected in mature desiccated seeds (Fig. 4). Three fatty

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acids (FA 16.0, 18.0 and 18.1) were detected during the early stages of seed development, of which FA 16.0 and 18.0 contributed to more than 90% of total fatty acids. An increase in the number of fatty acid occurred mostly during maturation stages. In mature desiccated seeds of Col-0, eight fatty acids were determined with FA 16.0, 18.0, 18.1 and 19.0 contributing more than 60% of the total fatty acids.

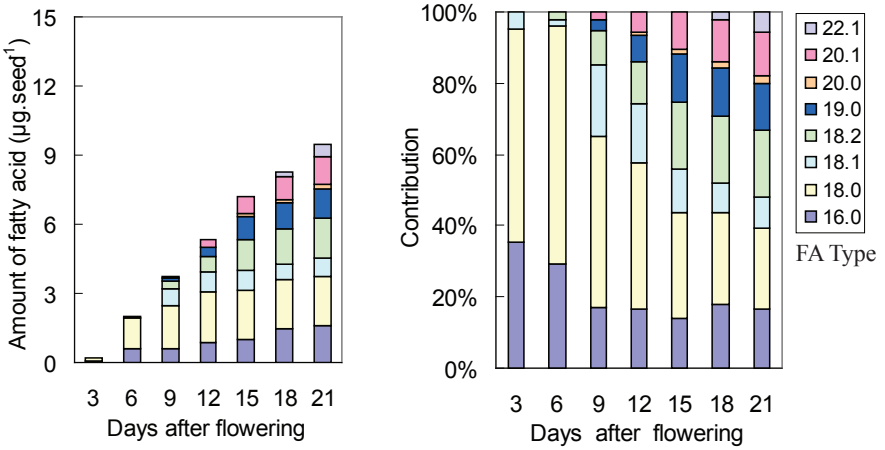

Figure 4 Fatty acid compositions at different stages of seed development of Col-0. Data are means of samples from at least four replicates (± SE) and each replicate contained 40 seeds.

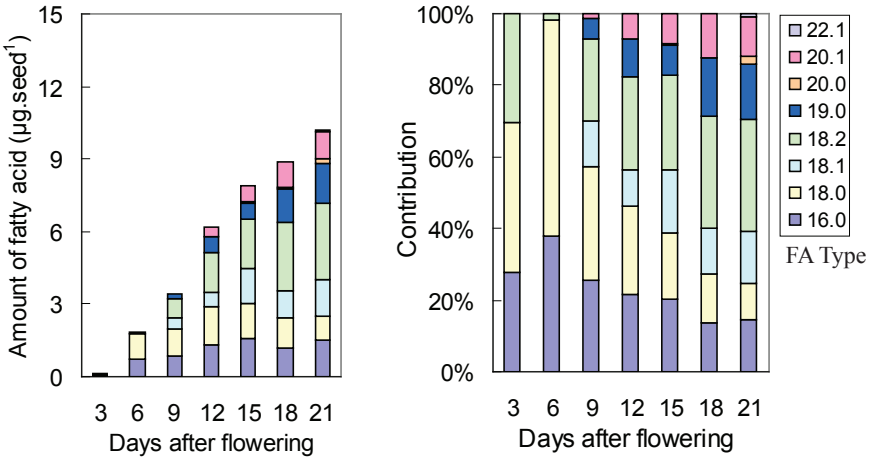

Figure 5 Fatty acid compositions at different stages of seed development of *adg1-1*. Data are means of samples from at least four replicates (± SE) and each replicate contained 40 seeds

Three major fatty acids (FA 16.0, 18.0 and 18.2) were detected during the early stages of *adg1-1* seed development (Fig. 5). They increased gradually, reaching seven major fatty acids in mature desiccated seeds. FA 18.2 showed a highly significant increase (70%) in *adg1-1* mature desiccated seeds compared with the wild type ($p<0.01$). In contrast, FA 18.0 and FA 18.1 were very significantly reduced (76% and 75.6% respectively) in *adg1-1* compared with the wild type ($p<0.001$) (Table 2).

Table 2 Major fatty acid compositions in mature desiccated seeds of Col-0 and *adg1-1* on dry weight basis (%).

Fatty acid		Col-0	*Adg1-1*
16.0	Palmitic acid	7.7 ± 0.5	6.2 ± 2.0
18.0	Stearic acid	10.8 ± 0.5	2.6 ± 0.4***
18.1	Oleic acid	3.9 ± 0.9	7.0 ± 1.4***
18.2	Linoleic acid	8.7 ± 0.8	14.9 ± 0.8**
19.0	Nonadecylic acid	6.1 ± 0.6	7.3 ± 2.9
20.0	Arachidic acid	1.1 ± 0.1	1.0 ± 0.6
20.1	Eicosenoic acid	5.6 ± 0.8	5.8 ± 2.3
22.1	Erucic acid	2.6 ± 1.3	0.3 ± 0.3

The results are presented as means (n=3). Differences between Col-0 and each mutant were analysed using Student's t-test. Significance levels are *p <0.05, **p <0.01, ***p <0.001.

Discussion

As much of our knowledge in the metabolism and regulation of storage product formation is derived from mutants and transgenic plants, a starchless mutant, ADP glucose pyrophosphorylase (*adg1-1*), of *Arabidopsis thaliana* was chosen for this study to develop a more comprehensive insight in this field. In previous studies, most work carried out on these mutants were focussed on metabolism in the leaves and very few considered the developing seeds. It is the aim of this work to understand how these mutations in primary metabolism affect assimilate partitioning and metabolism during seed development in *Arabidopsis.*

Alteration in metabolites during the seed development of the starchless mutant, *adg1-1*

The starchless mutant has revealed a different metabolite alteration during seed development compared with the wild type, Col-0 which results in significantly different metabolites in mature desiccated seed. In starchless mutants, as starch accumulation did not occur; hexose may be the most important sugar translocated into seeds as its content in mature desiccated seeds of *adg1-1* was found significantly high compared with Col-0 (Table 1). Caspar and co-workers (1985) discovered that hexose comprises 98% of the carbohydrate that accumulated in stem tissues of an *Arabidopsis* starchless mutant.

In the *adg1-1* mutant, starch was below a detectable level in mature desiccated seeds (Table 1). *Adg1-1* lacks ADP-glucose pyrophosphorylase, a key regulatory enzyme in the starch biosynthetic pathway. Therefore, in chloroplasts and amyloplasts of this mutant, there is no enzyme to catalyze the synthesis of ADP-glucose and pyrophosphate from ATP and glucose-1-phosphate. However, there was an accumulation in hexose during the early stages of development as well as sucrose, which builds up towards seed maturation.

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This suggests that hexoses were not derived from starch degradation or that sucrose was synthesised via the degradation of starch. Instead, the accumulation of hexose probably resulted from the import of free hexoses from the unloading processes (Lalonde *et al*., 2003) or via photosynthesis in the green seeds during the early stage of development, which later will be mobilized or accumulated as a storage compound (Ruuska *et al*., 2004).

In addition, the build-up of glucose-1-phosphate may also contribute to the rise of sucrose accumulation through the combined activities of UDPglucose pyrophosphorylase, sucrose phosphate synthase and sucrose phosphatase (Dennis *et al*., 1997; Lea & Leegood, 1999). Moreover, phosphorylation of hexose generated through the sucrose synthase pathway or invertase pathway may have contributed to the accumulation of sucrose in seeds (Edwards & ap Rees, 1986; Tomlinson *et al*., 2004).

Interestingly, *adg1-1* showed a similar pattern and the same level of fatty acid accumulation with the wild type (Table 2, Fig. 4 & Fig. 5). It is possible that the accumulation of glucose-1-phosphate may provide the precursors for fatty acid biosynthesis via glycolysis (Tomlinson *et al*., 2004). Another precursor resource for fatty acid biosynthesis that needs to be considered is sucrose degradation. Sucrose accumulation in *adg1-1* occurred at a high level during the early stages of seed development that peaked at the maturation stage but declined during the desiccation stage. There is a possibility that the products from sucrose degradation may serve as precursors during the rapid deposition of lipid in the maturation stage. Sucrose can be degraded and utilized for starch synthesis via the sucrose synthase pathway or for oil synthesis via the invertase pathway (Tomlinson *et al*., 2004). As no starch has been accumulated in *adg1-1* mutant, it can be proposed that the contribution of sucrose utilization for fatty acid biosynthesis is appreciably greater.

With the alteration of resources for metabolic precursors, large changes occurred in the fatty acid compositions in mature desiccated seeds of *adg1-1*. There was an increase in FA 18.1 and 18.2, as well as a severe reduction in FA 18.0 (Table 2). Nonetheless, there is also a possibility that a primary metabolic defect may occur during the conversion of carbohydrates into precursors of fatty acid biosynthesis or there may be defects in enzymes of fatty acid biosynthesis pathways, which may lead to the alteration in fatty acid compositions. Changes in fatty acids have been studied extensively in diacylglycerol acyltransferase (DGAT) mutants in *Arabidopsis* (Routaboul *et al*., 1999; Jako *et al*., 2001). Nevertheless, nothing has been reported about the fatty acid alteration in *Arabidopsis ADG* mutants. Therefore, this finding provides a new insight on metabolic changes through mutations.

Importance of starch in seed development

Starch acts as a transitory reserve in seeds and is degraded during the middle stage of seed development to provide energy for seed growth as well as precursors for the synthesis of other compounds (Kang & Rawsthorne, 1994). However, the question arises as to whether this starch is essential for *Arabidopsis* seed development. In the starchless mutant, *adg1-1* showed very little or no starch accumulation throughout seed development (Lin *et al*., 1988). Even though the growth of these mutant plants is delayed, seed development of the mutant does not show much difference from the wild type (Fig.2). In mature desiccated

seeds, there was no significant difference in the mutant's seed dry weight compared to the wild type (Table 1). Therefore, it can be suggested that transitory starch is not essential for seed development.

Interestingly, in this study, a defect in ADGase resulted in a 6% increase in total fatty acids (Table 1) with the major changes occurring in FA 18.0 (stearic), FA 18.1 (oleic) and 18.2 (linoleic) (Table 2). In mature seeds of *adg1-1*, there was a 70% increase in polyunsaturated FA 18.2, a 75% increase in monounsaturated FA 18.1 and a 76% reduction in saturated FA 18.0 (Fig. 3). FA 18.2 is an essential fatty acid for humans (Kinsella *et al*., 1981) and this could well improve the nutritional value of these seeds. As people in this wide world are looking for more healthy food, this finding may improve their alternatives. Oil palm and coconuts are largely grown in tropical countries such as in Malaysia (Dummet *et al*., 1993) and Indonesia (Casson, 1999), and are poor in unsaturated fatty acids. The total lipid content of palm oil, for example, contains about 12% of polyunsaturated and 43% of monounsaturated fatty acids (Wan Nik *et al*., 2005). In coconut oil, both unsaturated fatty acids comprise less than 2% (Reiser *et al*., 1985). This finding suggests the possibility of improving the quality of palm and coconut oil by increasing the polyunsaturated fatty acids through manipulating the *ADG* gene. If the *ADG* could be manipulated, there is a possibility of producing seeds with a higher amount of oil accompanied by an increase in polyunsaturated fatty acids and a reduction in very long chain fatty acids. However the mechanisms by which these changes occurred remain unknown and require further investigation.

Conclusion

In conclusion, this work has shown that the *adg1-1* mutant has adapted some mechanisms to utilize the available photosynthates for the synthesis of the storage reserves. Under the growth conditions applied in this work, alterations in the end products of the seeds of the mutant have occurred especially in the production of fatty acids.

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