Nonsynthetic Hemoglobin-2 Leads to an Elevated Energy State and to a Combined Increase in Polyunsaturated Fatty Acids and Total Oil Content When Overexpressed in Developing Seeds of Transgenic Arabidopsis Plants

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Nonsynthetic hemoglobins are ubiquitously expressed in plants and divided into two different classes based on gene expression pattern and oxygen-binding properties. Most of the published research has been on the function of class 1 hemoglobins. To investigate the role of class 2 hemoglobins, transgenic Arabidopsis (Arabidopsis thaliana) plants were generated overexpressing Arabidopsis hemoglobin-2 (AHb2) under the control of a seed-specific promoter. Overexpression of AHb2 led to a 40% increase in the total fatty acid content of developing and mature seeds in three subsequent generations. This was mainly due to an increase in the polyunsaturated C18:2 (ω-6) linoleic and C18:3 (ω-3) α-linolenic acids. Moreover, AHb2 overexpression led to an increase in the C18:2/C18:1 and C18:3/C18:2 ratios as well as in the C18:3 content in mol % of total fatty acids and in the unsaturation/saturation index of total seed lipids. The increase in fatty acid content was mainly due to a stimulation of the rate of triacylglycerol synthesis, which was attributable to a 3-fold higher energy state and a 2-fold higher sucrose content of the seeds. Under low external oxygen, AHb2 overexpression maintained an up to 5-fold higher energy state and prevented fermentation. This is consistent with AHb2 overexpression results in improved oxygen availability within developing seeds. In contrast to this, overexpression of class 1 hemoglobin did not lead to any significant increase in the metabolic performance of the seeds. These results provide evidence for a specific function of class 2 hemoglobin in seed oil production and in promoting the accumulation of polyunsaturated fatty acids by facilitating oxygen supply in developing seeds.

Hemoglobins are an ancient class of oxygen-binding proteins that are ubiquitous in nature and expressed in organisms as different as animals, bacteria, fungi, and plants (Kundu et al., 2003; Vinogradov et al., 2006). The first plant hemoglobins to be discovered were legheemoglobins, which are highly abundant proteins in root nodules of legume plants infected with symbiotic nitrogen-fixing bacteria (Kubo, 1939; Appleby et al., 1988). They have been shown to be indispensable for symbiotic nitrogen fixation, with a role in facilitating oxygen diffusion to nitrogen-fixing bacteria, and to protect bacterial nitrogenase from inactivation (Ott et al., 2005). In contrast to this, nonsynthetic plant hemoglobins have been found in various plant tissues and plant species, especially in crop plants (Taylor et al., 1994; Andersson et al., 1996). Different classes of nonsynthetic plant hemoglobins have been identified and divided into class 1 (Hb1) and class 2 (Hb2) based on phylogenetic characteristics, gene expression patterns, and oxygen-binding properties (Trevaskis et al., 1997; Hunt et al., 2001; Hoy and Hargrove, 2008).

Class 1 and class 2 hemoglobins differ markedly in their oxygen-binding affinities, with the respective $K_m$ being 1 to 2 nM for Hb1 and 150 nM for Hb2 (Hoy and Hargrove, 2008; Dordas, 2009). The extremely high affinity of Hb1 for oxygen makes it unlikely that this protein is acting as an oxygen sensor, oxygen carrier, oxygen storage, or electron transport molecule (Hill, 1998). Expression of Hb1 is very low under normal conditions, but it is strongly induced by hypoxic stress. Studies using Arabidopsis (Arabidopsis thaliana; Perazzolli et al., 2004), maize (Zea mays; Dordas et al., 2003, 2004), and alfalfa (Medicago sativa) lines (Igamberdiev et al., 2004) overexpressing Hb1 indicate a role of class 1 hemoglobins in scavenging of nitric oxide (NO) that is produced under severe hypoxia. Under these stress conditions, overexpression of Hb1 leads to a decrease in NO, maintenance of the cellular energy status and growth, as well as increased survival of the plants (Sowa et al., 1998; Hunt et al., 2002; Dordas et al., 2004; Perazzolli et al., 2004).

Relatively little is known concerning the function of Hb2 in plants. In Arabidopsis, AHb2 expression is resilient to hypoxic stress but shows induction in...
response to low temperatures, indicating a possible yet undefined role under cold stress (Trevaskis et al., 1997). AHb2 is also induced in response to the plant hormone cytokinin (Hunt et al., 2001), which has been shown to be mediated by a cytokinin-regulated transcription factor (Ross et al., 2004). Analysis of the expression pattern in plants reveals preferential expression of Hb2 in young developing leaves, vascular bundles, flowers, developing seeds, and immature fruits (Hunt et al., 2002; Wang et al., 2003). Hb2 is also expressed during somatic embryogenesis in chicory (Cichorium intybus; Hendriks et al., 1998). This indicates a possible role of Hb2 in young and developing plant tissues that usually have a high metabolic activity and energy demand. Arabidopsis plants overexpressing AHb2 under the control of a constitutive promoter were mainly analyzed with respect to severe oxygen deprivation. Under these conditions, AHb2 overexpression led to increased survival of the plants, similar to AHb1 overexpression, suggesting that both AHb1 and AHb2 may have overlapping functions, such as scavenging of NO (Hebelstrup et al., 2006). However, a role of AHb2 in scavenging of NO has not been demonstrated yet. Since the oxygen-binding characteristics of AHb2 are comparable to leghemoglobin, with an affinity to oxygen that resembles the $K_m$ of cytochrome oxidase, a specific function of Hb2 in facilitating oxygen diffusion cannot be excluded. Therefore, the underlying mechanisms of AHb2-induced responses are still unresolved and may differ from AHb1.

In the following approach, transgenic Arabidopsis plants were generated that overexpress endogenous class 1 or class 2 hemoglobins specifically in developing seeds under the control of a seed-specific promoter. The results show that overexpressing AHb2, but not AHb1, led to an increase in the lipid content of the seeds that was mainly due to an increase in the level of polyunsaturated fatty acids. This was accompanied by an increase in the adenylate energy state and the Suc content of the seeds. These results indicate a specific role of class 2 hemoglobins in promoting the accumulation of polyunsaturated fatty acids, which have important functions in the response of the plant to cold stress and in human health. This may be attributable to a possible role of class 2 hemoglobins in improving oxygen availability in developing seeds.

RESULTS

Generation of Transgenic Arabidopsis Plants

Overexpressing Either AHb1 or AHb2 under the Control of a Seed-Specific Promoter

AHb1 or AHb2 cDNA was cloned into a plant binary vector downstream of the USP (for unknown seed protein) promoter, identified to be seed specific in Arabidopsis plants (Ba¨umlein et al., 1991). The resulting construct was transformed via Agrobacterium tumefaciens into Arabidopsis var C24 plants. Using hygromycin-containing selective medium, three independent transgenic lines showing a significant and reproducible increase in transcript and protein level of either AHb1 (Fig. 1, A and B) or AHb2 (Fig. 1, G and H) were propagated up to the T3 generation for further analyses.

Overexpression of AHb2 Leads to an Increase in the Total Fatty Acid Content of Developing and Mature Seeds

The transgenic lines overexpressing AHb1 or AHb2 showed no visible phenotype when grown under normal greenhouse conditions. To investigate the influence of increased expression of these endogenous hemoglobins on storage product accumulation, transgenic lines were further analyzed for total fatty acid content in developing and mature seeds using gas chromatography analysis of fatty acid methyl esters. Overexpression of AHb2 led to a 40% increase in the fatty acid content of mature seeds, both as a percentage of total seed dry weight (Fig. 1I) and in absolute values per seed (Fig. 2A), while the protein content decreased slightly (Fig. 1J). Compared with the wild type, the fatty acid content of mature seeds of AHb2-overexpressing lines was found to be stably increased in three subsequent generations as well as in developing seeds that were harvested 13 to 14 d after flowering (DAF), when rapid rates of lipid synthesis occur (Table I). There were no significant changes in transient starch accumulation during seed development in AHb2-expressing lines compared with the wild type (Table I).

In contrast to the results with AHb2, overexpression of AHb1 did not lead to any significant changes in the fatty acid content of the seeds (Fig. 1C). Compared with the wild type, AHb1-overexpressing seeds also did not show significant changes in the protein content, except line 13, where the protein content was increased (Fig. 1D).

Overexpression of AHb2 Leads to an Increase in the Specific Triacylglycerol Marker C20:1 Eicosenoic Acid and in the Flux of Suc to Triacylglycerol in Arabidopsis Seeds

In Arabidopsis seeds, almost all of the fatty acids are esterified in the form of triacylglycerols (TAGs; Baud et al., 2002; Li et al., 2006). To investigate whether the AHb2-induced increase in total fatty acid content was attributable to an increase in TAG, the level of C20:1 eicosenoic acid was measured. Eicosenoic acid is a very-long-chain fatty acid that is solely confined to TAG and excluded from polar or membrane lipids in Arabidopsis plants (Lemieux et al., 1990). Therefore, it serves as a specific marker for the formation of storage TAG in Arabidopsis seeds (Lemieux et al., 1990). Changes in the level of eicosenoic acid have been frequently found to correlate with changes in TAG or oil content (Taylor et al., 1992; Germain et al., 2001; Martin et al., 2002; Baud et al., 2003; Rylott et al., 2003; Mu et al., 2008). Overexpression of AHb2 led to a significant 20% to 60% increase in the level of C20:1
eicosenoic acid in mature seeds (Fig. 2B). This indicates that the AHb2-induced increase in total fatty acids is mainly due to an increase in TAG (oil) content.

To confirm these results by investigating the rate of TAG synthesis more directly, developing seeds (13–14 DAF) were incubated with $^{14}$C]Suc for 2 h to measure label incorporation into TAG. In seeds of the wild type, the flux of Suc into TAG was approximately 280 nmol Suc h$^{-1}$ g$^{-1}$ dry weight (Fig. 3), which is in the same range as measured previously for Arabidopsis seeds (Focks and Benning, 1998). Compared with the wild type, there was a significant 30% to 40% increase in the rate of TAG synthesis in the lines overexpressing AHb2 (Fig. 3).

**Overexpression of AHb2 Leads to an Increase in Polyunsaturated ω-6 Linoleic and ω-3 α-Linolenic Acid Levels and a General Increase in Fatty Acid Desaturation in Mature Seeds**

The increase in total fatty acids in response to overexpression of AHb2 was mainly due to an increase...
in the polyunsaturated C18:2 (\(\omega-6\)) linoleic and C18:3 (\(\omega-3\)) \(\alpha\)-linolenic acids, the two most abundant fatty acids in Arabidopsis seeds, deriving from C18:1 oleic acid (Fig. 2, compare A with C–E). AHb2 overexpression led to an increase in the C18:2/C18:1 (Fig. 2F) and C18:3/C18:2 (Fig. 2G) ratios as well as in the C18:3 content in mol % of total fatty acids (Fig. 2H). These changes in fatty acid desaturation were also reflected by a general increase in the unsaturation/saturation index (USI) of the seed lipids (ratio between the total number of double bonds and the total content of saturates; Fig. 2I).

### Table I. Overexpression of AHb2 leads to a specific increase in the seed lipid content of transgenic Arabidopsis lines in three subsequent generations

Total fatty acid content was determined in mature seeds in T1, T2, and T3 generations and in developing seeds (14 DAF). In the latter, starch content was also measured. Values are means ± se; \(n = 6\) independent measurements. Asterisks represent values significantly different from the wild type (based on Student’s t test with \(P < 0.05\)).

<table>
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<th>Variable</th>
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<td>mg g(^{-1}) dry wt</td>
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<td>430 ± 20*</td>
<td>488 ± 79*</td>
<td>502 ± 34*</td>
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</tr>
<tr>
<td>T2 generation</td>
<td>383 ± 13</td>
<td>451 ± 48*</td>
<td>499 ± 21*</td>
<td>507 ± 30*</td>
<td></td>
</tr>
<tr>
<td>T3 generation</td>
<td>331 ± 26</td>
<td>380 ± 18</td>
<td>435 ± 17*</td>
<td>464 ± 25*</td>
<td></td>
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<tr>
<td>Lipid and starch content in developing seeds</td>
<td>128 ± 11</td>
<td>245 ± 22*</td>
<td>224 ± 13*</td>
<td>208 ± 34*</td>
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<tr>
<td>Starch content</td>
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<td>8.6 ± 0.2</td>
<td>7 ± 0.2</td>
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Overexpression of AHb2 Leads to an Increase in the Adenylate Energy Status and in the Suc Content of Developing Seeds

In developing oilseeds, Suc derived from photosynthetic leaves is unloaded from the phloem and used as a carbon source for fatty acid synthesis, while energy is
Overexpression of AHb2 Leads to an Increased Energy State and Avoidance of Fermentation When Developing Seeds Are Exposed to Low External Oxygen

Seeds of various species have been found to develop in an internal environment with about 2% to 4% (v/v) oxygen, compared with 21% (v/v) oxygen outside (Porterfield et al., 1999; Gibon et al., 2002; Rolletschek et al., 2002, 2005, 2007; Geigenberger, 2003; Vigeolà et al., 2003, van Dongen et al., 2004). The positive effect of AHb2 on the energy state of the seeds, therefore, may be attributable to a specific role of AHb2 related to the low internal oxygen concentrations within the seeds. To investigate a possible role of AHb2 under low oxygen, external oxygen around the siliques was decreased from 21% to 4% (v/v) for 2 h before aedynate energy state, glycolytic intermediates, and the fermentation products lactate and Gly3P were analyzed in the seeds. In seeds of the wild type, there was a drop in the ATP-ADP ratio from 2 to 0.4 (Fig. 4A) and a decrease in the glycolytic intermediates Glc6P (Fig. 4B) and 3PGA (Fig. 4C), while the fermentation products Gly3P (Fig. 4D) and lactate (Fig. 4E) accumulated under these conditions. This provides evidence that the decrease in external oxygen led to severe limitations in oxygen availability within the seeds, resulting in a shift to anaerobic metabolism. In contrast to this, AHb2 overexpression maintained an up to 5-fold higher ATP-ADP ratio (Fig. 4A) and prevented an increase in the fermentation products Gly3P (Fig. 4D) and lactate (Fig. 4E) as well as a decrease in glycolytic intermediates (Fig. 4, B and C) under these conditions. These data are consistent with AHb2 overexpression results in improved oxygen availability within developing seeds.

DISCUSSION

Nonsymbiotic hemoglobins are widely distributed in plants and divided into two different classes based on gene expression pattern and oxygen-binding properties. Most of the published work has been on the function of class 1 hemoglobins. This study shows a previously unknown function of Arabidopsis class 2 hemoglobin in seed oil production. Overexpression of AHb2 in Arabidopsis seeds resulted in a significant increase in the seed lipid content (Fig. 1; Table I), which was mainly due to an increase in the polyunsaturated (ω-6) C18:2 linoleic and (ω-3) C18:3 α-linolenic acids (Fig. 2). There were both an increase in C20:1 eicosenoic acid, which is a specific marker for TAG (Fig. 2B), and an increase in the rate of TAG biosynthesis (Fig. 3), providing evidence that the increase in total fatty acids in response to AHb2 expression is mainly attributable to an increase in oil content. This was accompanied by an elevated carbon and energy status of the seeds, both under normal conditions (Fig. 1) and in response to low external oxygen (Fig. 4).

To our knowledge, this is the first report of a transgenic approach leading to an increase in both lipid quantity and α-linolenic acid content of oil seeds. Due to the great economic importance of vegetable oils and their expanded use as industrial and nutritional feedstock, there has been considerable interest in en-
engineering plants for high oil quantity and quality. Previous attempts to improve oil quantity have been performed with limited success and concentrated mainly on manipulating enzymes involved in the pathways of fatty acid synthesis (Thelen and Ohlrogge, 2002, Hills, 2004), glycerol-3-phosphate provision (Vigeolas et al., 2007), and TAG assembly (Zou et al., 1997; Jako et al., 2001; Taylor et al., 2002; Zheng et al., 2008). This study shows that increased expression of AHb2, a protein that is not integral to these pathways, leads to a 40% increase in the fatty acid content of the seeds. This is most likely due to the strong increase in seed energy state in response to AHb2 expression (Fig. 1). Previous studies have shown that the energy status is colimiting for the rates of Suc import (Vigeolas et al., 2003; van Dongen et al., 2004) and lipid biosynthesis in developing seeds of different species (Vigeolas et al., 2003; Rolletschek et al., 2005, 2007). Moreover, external conditions, such as light, can have a considerable impact on oil accumulation in seeds by affecting energy and redox parameters (Ruuska et al., 2004; Goffman et al., 2005; Li et al., 2006). Starch synthesis has been found to be more resilient to changes in the seed energy status, since it requires less ATP than lipid synthesis (Neuhaus and Emes, 2000; Vigeolas et al., 2003). This provides a possible explanation why transient starch synthesis was not significantly affected in AHb2-overexpressing seeds (Table I).

Attempts to improve fatty acid composition of seed oil were aimed to increase the production of specific fatty acids with increased industrial or nutritional values (Cahoon et al., 2007; Napier, 2007; Napier and Graham, 2010). One important goal to increase the nutritional value of vegetable oil in food products is to increase the content of polyunsaturated fatty acids. \(\omega-3\) polyunsaturated \(\alpha\)-linolenic acid is considered an essential fatty acid because it is required for health, but it cannot be synthesized by humans. Vegetable oil is one of the major dietary sources of \(\alpha\)-linolenic acid, and a large body of scientific research suggests that increasing the relative abundance of dietary \(\omega-3\) fatty acids has a number of clinical benefits for human health through the prevention and management of many diseases (Simopoulos, 1991; Crawford et al., 2000). Previous approaches were able to increase the \(\alpha\)-linolenic acid content of oil in relative terms by ectopic expression of fatty acid desaturases in seeds, but this was often accompanied by severe yield penalties (Kinney et al., 2002; Anai et al., 2003). Other approaches involved the engineering of pathways generating very-long-chain \(\omega-3\) polyunsaturated fatty acids, such as eicosapentanoic acid and docosahexaenoic acid in oilseed crops, by introducing genes from diverse organisms that produce these fatty acids (Napier, 2007; Napier and Graham, 2010). While these attempts have met with some degree of success, they did not lead to the production of economically sufficient amounts of these novel fatty acids (Cahoon et al., 2007). Overexpression of AHb2 provides a possible route to achieve a combined increase in \(\alpha\)-linolenic acid and total oil content in plants. More studies are needed to investigate whether these results can be transferred from Arabidopsis to economically important oilseeds grown under high-light conditions in the field.

![Figure 4. Metabolic changes in wild-type and AHb2-overexpressing seeds exposed to low external oxygen concentrations in planta. To investigate the effect of AHb2 on the response of developing seeds to low oxygen, wild-type (black bars) and transgenic (gray bars) siliques attached to the plant were exposed to either 4% or 21% (control) oxygen for 2 h, before they were harvested, rapidly frozen in liquid nitrogen, and seeds dissected to measure ATP-ADP ratio (A), Glc6P (B), 3PGA (C), Gly3P (D), and lactate (E) levels. Values are means ± SE (n = 6 different sets of plants). Changes that are significantly different from the wild type (based on Student’s t test with \(P < 0.05\)) are labeled with asterisks. DW, Dry weight.](image-url)
An increase in polyunsaturated fatty acids has also been reported to play a crucial role in the chilling tolerance of plants. Specifically, an increased production of C18:3 \( \alpha \)-linolenic acid has been found to be connected with cold acclimation and the protection of plant cells against cold damage (Kodama et al., 1994). Class 2 hemoglobins, therefore, may play an important role in the adaptive response of plants to cold stress by promoting the synthesis of C18:3 \( \alpha \)-linolenic acid. This may explain why expression of class 2 hemoglobins is induced by low temperature rather than low oxygen (Trevaskis et al., 1997). In addition to this, it has been reported that an increase in the unsaturation level of C18 fatty acids promotes climacteric fruit ripening by affecting the synthesis of jasmonates (Gutiérrez et al., 2005). This provides a possible explanation for the preferential expression of class 2 hemoglobins in immature fruits (Wang et al., 2003). More studies are needed to investigate the importance of Hb2 under chilling stress and during fruit ripening.

To our knowledge, this is also the first report showing that the biosynthetic performance of plants can be improved by overexpressing endogenous hemoglobins. Previous studies in this context were mainly focused on the heterologous expression of the bacterial *Vitreoscilla* hemoglobin in plants. This led to positive effects on growth and metabolism (Kallio et al., 2001, 2008; Frey and Kallio, 2003, 2005; Jokipii-Lukkari et al., 2009) as well as on the production of different secondary metabolites in tobacco (*Nicotiana tabacum*; Holmberg et al., 1997), aspen (*Populus* spp.; Häggman et al., 2003), and Arabidopsis (Wang et al., 2009). Although the underlying mechanisms are not fully resolved yet, *Vitreoscilla* hemoglobin is mainly considered as an oxygen-binding and -delivering protein with a possible function in facilitating oxygen supply (Jokipii-Lukkari et al., 2009).

In addition to its biotechnological potential, this study provides evidence that class 1 and class 2 hemoglobins have different functions in plants. Previous investigations were mainly focused on class 1 hemoglobins and have led to the conclusion that the main function of these hemoglobins is to metabolize NO formed during severe hypoxia (for review, see Hebelstrup et al., 2007). Under these conditions, Hb1 is strongly induced and serves as part of a NO dioxygenase system, using traces of oxygen to convert NO to nitrate. This not only leads to scavenging of NO but also to the establishment of a connection between NO turnover and the maintenance of redox and energy levels under anaerobic conditions (Sowa et al., 1998; Dordas et al., 2003; Igamberdiev et al., 2004, 2006; Stoimenova et al., 2007). The removal of NO allows nitrite and NAD(P)H-driven ATP synthesis to proceed in anaerobic mitochondria without inhibition of cytochrome oxidase by NO. Overall, this will explain why increased Hb1 expression leads to improved survival under severe hypoxic stress (Hunt et al., 2001).

Here, we show that overexpressing AHb2 has a different effect on seed energy state, Suc level, and fatty acid accumulation than AHb1 (Fig. 1). Under normal growth conditions, overexpression of AHb2 led to an increase in the biosynthetic performance of developing seeds that was not evident for AHb1. This indicates a specific function of AHb2 expression to promote energy-dependent storage metabolism in developing seeds, most probably by improving oxygen supply for mitochondrial respiration. This contrasts with Hb1 having a role to improve plant survival under anaerobic stress conditions by scavenging of NO. The different physiological functions of AHb1 and AHb2 are most likely due to structural differences between the two proteins. First, there are differences in ligand migration properties allowing AHb1 to bind NO and limit NO binding in AHb2. This makes it unlikely that AHb2 plays a role in NO oxidation similar to AHb1 (Bruno et al., 2007; Nienhaus et al., 2010). Second, there are strong differences in the oxygen-binding properties of the two proteins, with AHb2 having a 2 orders of magnitude lower affinity to oxygen than AHb1 (Hoy and Hargrove, 2008). The oxygen affinity of AHb2 is similar to cytochrome oxidase, making it likely that AHb2 may have a specific function in facilitating oxygen supply to mitochondrial respiration. The resulting increase in the energy state will improve the biosynthetic performance of the seeds, which explains the increase in the Suc and fatty acid contents in response to AHb2, but not AHb1, expression (Fig. 1). Internal oxygen concentrations in developing seeds have been frequently reported to be limiting for respiration and biosynthetic performance (Rolletschek et al., 2003, 2005, 2007; Vigeolas et al., 2003; van Dongen et al., 2004).

The increase in polyunsaturated fatty acids is also consistent with a role of AHb2 to improve oxygen availability in the seeds. AHb2 overexpression led to a combined increase in C18:2 linoleic and C18:3 linolenic acids, both in absolute terms and relative to C18:1 oleic acid (Fig. 2), indicating activation of the membrane-bound fatty acid desaturases FAD2 and FAD3 located at the endoplasmic reticulum. Unlike other organisms, plants regulate these fatty acid desaturases at the posttranslational rather than the transcriptional level (Sakamoto and Murata, 2002; Lu et al., 2009). It has been shown for sunflower (*Helianthus annuus*) seeds that internal oxygen levels act as key regulators for the activities of fatty acid desaturase enzymes (Martínez-Rivas et al., 2003; Rolletschek et al., 2007). Increased oxygen supply raised the energy status of the seeds and produced a dramatic increase in FAD2 activity as well as linoleic acid content. Moreover, low temperature increased the unsaturation degree of sunflower oil by its effect on dissolved oxygen levels in the developing seeds (Rolletschek et al., 2007). Therefore, it is most likely that overexpression of AHb2 led to an increased production of polyunsaturated fatty acids by facilitating the availability of molecular oxygen as a substrate to fatty acid desaturases. Unfortunately, there is no method available at present that allows...
direct analysis of oxygen concentrations within developing Arabidopsis seeds.

In conclusion, this paper provides evidence for a specific function of class 2 hemoglobins in promoting seed lipid production when overexpressed in developing seeds. It also provides a possible route to improve the production of polyunsaturated fatty acids in oil crops by overexpressing AHB2. Further studies using antisense or RNA interference approaches to knock down AHB2 would be required to establish whether this represents the normal function of AHB2 in seed development rather than a specific function under conditions where its expression is induced (i.e., under cold stress).

MATERIALS AND METHODS

Plant Cultivation, Oxygen Treatments, and Sampling

Arabidopsis (Arabidopsis thaliana) plants were grown in a greenhouse (21°C day and 17°C night, 50% humidity, with a photoperiod of 16-h day/8-h night at a light intensity of 180 μmol photons m⁻² s⁻¹). For incubation experiments with low oxygen, silique-bearing stalks were enclosed in a transparent plastic bag through which air with 21% or 4% (v/v) oxygen was circulated. The premixed gases (Messers Grieseim) used for this treatment contained 350 μL L⁻¹ CO₂, oxygen concentrations as indicated above, and nitrogen. After 2 h, siliques were harvested and immediately frozen in liquid N₂. Seeds were dissected from 13- to 14-d-old lyophilized siliques using a custom-made vacuum sampler as described by Gibon et al. (2002).

Generation and Cultivation of AHB1- and AHB2-Overexpressing Lines

For both AHB1 and AHB2, cDNA was produced by reverse transcription-PCR (SuperScript II; Invitrogen) on total RNA isolated from 6-week-old Arabidopsis plants using the following specific primers: AHB1 f (5'-TTTGGTACCATGGG) and AHB1 r (5'-TTTGGATCCATGGGAAGATTGGGTTTACAGAG-3'). AHB2 f (5'-TTTGGTACCATGGGAAGATTGGGTTTACAGAG-3') and AHB2 r (5'-TTTGGATCCTTGAATCTTGGTACCATGGGAAGATTGGGTTTACAGAG-3'). The cDNA was cloned in a plant binary vector downstream of the seed-specific USP promoter (Bäumlein et al., 1991). The resulting construct was transformed via Agrobacterium tumefaciens into Arabidopsis var. C24 plants. Using hygromycin-containing selective medium, three independent primary USP-AHB1 and four USP-AHB2 transgenic lines were selected and propagated until the third generation, which was used for further analyses.

Analysis of AHB1 and AHB2 mRNA and Protein Levels

Expression levels of AHB1 and AHB2 mRNA levels were analyzed on northern blot. Therefore, total RNA was extracted using Concert RNA Plant Reagent (Invitrogen), and full-length cDNA encoding AHB1 or AHB2 was used as a probe for hybridization. AHB1 and AHB2 protein expression was tested on western blots after hybridization with homolog-specific antibodies raised on rabbit (Hunt et al., 2002) and detection by peroxidase-conjugated anti-rabbit antibodies (Bio-Rad) on x-ray film and enhanced chemiluminescence sub-stantiation (Hunt et al., 2002) and detection by peroxidase-conjugated anti-rabbit antibodies (Bio-Rad) on x-ray film and enhanced chemiluminescence sub-stantiation. Therefore, total RNA was extracted using Concert RNA Plant Reagent (Invitrogen), and full-length cDNA encoding AHB1 or AHB2 was used as a probe for hybridization. AHB1 and AHB2 protein expression was tested on western blots after hybridization with homolog-specific antibodies raised on rabbit (Hunt et al., 2002) and detection by peroxidase-conjugated anti-rabbit antibodies (Bio-Rad) on x-ray film and enhanced chemiluminescence sub-stantiation.

Analysis of Metabolites, Lipids, and Protein

Seeds were homogenized using a liquid nitrogen-cooled ball mill, then metabolites were extracted using trichloroacetic acid, and the levels of Suc, G6P, 3PGA, glycerol-3-phosphate, ATP, ADP, and lactate were subsequently quantified using enzymic cycling assays as described by Gibon et al. (2002).

Total fatty acids were extracted following the method of Bligh and Dyer (1959), and lipid content and composition were determined by gas chroma-tography of fatty acid methyl esters, using pentadecanoic acid as an internal standard (Benning and Somerville, 1992). The USI of fatty acids is calculated as described by Gutierrez et al. (2005). USI = U / Σi (saturates), with U = Σmi / r, where mi is the number of double bonds and ri is the relative content of each fatty acid. Protein content was determined according to the method described by Bradford (1976) using bovine serum albumin as a standard. Starch content was determined as described by Vigeolas et al. (2003).

Feeding Experiments and Analysis of Label Incorporation into TAG

[U-¹⁴C]Suc was provided to developing seeds (13–14 DAF) by injection into the siliques that remained attached to the plant in the light as described by Gibon et al. (2002). Each silique was injected with 5 μL of [U-¹⁴C]Suc (specific activity of 740 kBq μmol⁻¹) using a 5-μL Hamilton syringe. After 2 h, siliques were harvested, frozen in liquid nitrogen, and lyophilized, and seeds were separated from the silique wall. TAGs were extracted and separated by thin-layer chromatography as described by Focks and Benning (1998). Silica material containing TAG was transferred from the thin-layer chromatography plate into scintillation cocktail, and radioactivity was determined by scintil-lation counting.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_127165 and NM_118877.

ACKNOWLEDGMENTS

We are very grateful to Peter Hunt and R.A. Watts (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) for kindly providing the AHB1 and AHB2 hemoglobin antibodies, to Peter Dörmann for help with gas chromatography analysis of fatty acids, to Fabien Poree for help with cloning experiments, and to Mark Stitt and Joost T. van Dongen (all from Max-Planck-Institute of Molecular Plant Physiology, Golm, Germany) for support and for critical readings of the manuscript. Received September 22, 2010; accepted December 24, 2010; published January 3, 2011.

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