High-Level Production of γ-Linolenic Acid in *Brassica juncea* Using a Δ6 Desaturase from *Pythium irregulare*

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γ-Linolenic acid (GLA), a nutritionally important fatty acid in mammals, is synthesized by a Δ6 desaturase. Here, we report identification of *PiD6*, a new cDNA from the oleaginous fungus, *Pythium irregulare*, encoding a 459-amino acid protein that shares sequence similarity to carboxyl-directed desaturases from various species. Expression of *PiD6* in yeast (*Saccharomyces cerevisiae*) revealed that it converts exogenously supplied linoleic acid into GLA, indicating that it encodes a Δ6 fatty acid desaturase. Expression of the desaturase in *Brassica juncea* under the control of the *Brassica napus* napin promoter resulted in production of three Δ6 unsaturated fatty acids (18:2–6, 9; 18:3–6, 9, 12; and 18:4–6, 9, 12, 15) in seeds. Among them, GLA (18:3–6, 9, 12) is the most abundant and accounts for up to 40% of the total seed fatty acids. Lipid class and positional analysis indicated that GLA is almost exclusively incorporated into triacylglycerol (98.5%) with only trace amounts found in the other lipids. Within triacylglycerols, GLA is more abundant at the *sn*-2 position.

In mammals and some plants and fungi, a Δ6 fatty acid desaturase is responsible for conversion of linoleic acid (LA; 18:2–9, 12) to γ-linolenic acid (GLA; 18:3–6, 9, 12) and of α-linolenic acid (ALA, 18:3–9, 12, 15) to stearidonic acid (SDA, 18:4–6, 9, 12, 15). GLA is a nutritionally important fatty acid in mammals. It is involved in membrane structure in various cells and in the biosynthesis of very long-chain polyunsaturated fatty acids, both in humans and animals (Gunstone, 1992; Huang and Milles, 1996). Dietary supplementation with GLA has been shown to reduce the risks of, or have positive effect on, those disorders that are associated with a low level of this fatty acid (Horrobin, 1990, 1992; Huang and Milles, 1996). As a result, there is considerable interest in the large-scale production of GLA in oilseed crops (Huang and Ziboh, 2001).

Fatty acid desaturation involves the regiospecific introduction of carbon-carbon double bonds into aliphatic acyl chains. According to localization and cofactor requirements, desaturases can be classified into two major groups. The soluble group, such as the plant plastid Δ9 desaturases, uses acyl carrier protein thioesters as substrates, and NADPH:ferredoxin oxidoreductase and ferredoxin as electron donors. The membrane-bound desaturases, such as the plant microsomal Δ12 desaturase, use fatty acids esterified to complex lipid as the substrate, and NADH: cytochrome (cyt) b_5 oxidoreductase and cyt b_5 as electron donors (Heinz, 1993; Shanklin and Cahoon, 1998).

Compared with the soluble desaturases, microsomal desaturases are more varied and evolutionarily diversified, and can be further divided into subgroups. The carboxyl-directed desaturases, also known as the front-end desaturases (Napier et al., 1997), "measure" distance from the carboxy terminus of a fatty acid and introduce a double bond between the existing double bond and the carboxy terminus of the fatty acyl chain (Girke et al., 1998). Examples of this type of desaturase are Δ4 (Qiu et al., 2001a), Δ5 (Knutzon et al., 1998; Michaelson et al., 1998a), and Δ6 (Sayanova et al., 1997; Napier et al., 1998; Cho et al., 1999) desaturases. The "methyl-directed desaturases," on the other hand, measure distance from the methyl terminus of a fatty acid and introduce a double bond between the existing double bond and the methyl terminus of the fatty acyl chain. The examples of this type of desaturases are ω-3 desaturases from oilseed and *Caenorhabditis elegans* (Meesapyodsuk et al., 2000). Each type of desaturase possesses characteristic consensus protein sequence motifs. For instance, the methyl-directed desaturases contain three conserved His-rich motifs, whereas the carboxyl-directed desaturases usually contain, besides the three His-rich motifs, an extra heme-binding motif in an N-terminal cyt b_5-like extension. Deletion of this extra domain or site mutagenesis of key amino acids

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.001495.

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in the motif resulted in disruption of the enzymatic function (Sayanova et al., 1999b; Qiu et al., 2002).

Polyunsaturated fatty acids are long-chain fatty acids containing two or more double bonds in their acyl chains. Biosynthesis of polyunsaturated fatty acids involves both methyl-directed and carboxyl-directed desaturases. The primary product of fatty acid biosynthesis in oilseed crops is the 18-carbon monounsaturate, oleic acid (18:1–9). Sequential desaturation of oleic acid and its elongated products at both ends by methyl- and carboxyl-directed desaturases results in various polyunsaturated fatty acids.

Pythium irregulare is an oleaginous fungus that is unusual in that it contains a large amount of arachidonic acid and eicosapentaenoic acid. Based on the information that microsomal carboxyl-directed desaturases have similar primary structure, we undertook a PCR approach with two degenerate primers targeting the heme-binding and the third His-rich motifs to clone genes that are involved in biosynthesis of the fatty acids in *P. irregulare*. One unique cDNA, *PiD6*, was identified (GenBank accession no. AF419296); it encodes a 459-amino acid protein that shares sequence similarity to carboxyl-directed desaturases from various species (Sayanova et al., 1997; Girke et al., 1998; Napier et al., 1998; Aki et al., 1999; Cho et al., 1999; Huang et al., 1999). Expression of *PiD6* in yeast (*Saccharomyces cerevisiae*) revealed that it converts exogenously supplied LA into GLA, indicating that it encodes a Δ6 fatty acid desaturase. Expression of the desaturase in *Brassica juncea* under the control of the oilseed napin promoter resulted in production of three Δ6 unsaturated fatty acids (18:2–6, 9; 18:3–6, 9, 12; and 18:4–6, 9, 12, 15) in seeds. Among them, GLA is the most abundant and accounts for up to 40% of the total seed fatty acids of transgenic plants; SDA (18:4–6, 9, 12, 15) follows in a range of 3% to 10%. GLA is almost exclusively incorporated into triacylglycerols (TAGs) with a preference for the *sn*-2 position.

RESULTS

Identification of PiD6 from *P. irregulare*

To identify genes encoding Δ6 desaturases involved in the biosynthesis of polyunsaturated fatty acids in *P. irregulare*, a PCR-based cloning strategy was adopted. Two degenerate primers are designed to target sequences corresponding to the heme-binding motif of the cyt b_5-like domain and the third His-rich motif in the microsomal desaturases. Using this strategy we identified a cDNA fragment from *P. irregulare* that encoded a partial amino acid sequence containing a cyt b_5-like domain in the N terminus and having sequence similarity to carboxyl-directed desaturases from other various species.

To isolate the full-length cDNA clone, the insert was used as a probe to screen a cDNA library of *P. irregulare*, which resulted in the identification of several cDNA clones. Sequencing identified a full-length cDNA, *PiD6*. The open reading frame (ORF) of *PiD6* is 1,380 bp and codes for 459 amino acids.

Sequence analysis indicates that the PiD6 protein shares high sequence similarity to the front-end desaturases and related enzymes from various sources, such as a Δ-8 sphingolipid desaturase from oilseed (Sperling et al., 1998), a bifunctional Δ6 acetylenase/desaturase from *Ceratodon purpureus* (Sperling et al., 2000), a Δ8 desaturase from Euglena (*Euglena gracilis*; Wallis and Browse, 1999), a Δ5 desaturase from *C. elegans* (Michaelson et al., 1998b), and Δ6 desaturases from *C. elegans* (Napier et al., 1998), borage (*Borago officinalis*; Sayanova et al., 1997), *Mortierella alpina* (Michaelson et al., 1998a), and *Physcomitrella patens* (Girke et al., 1998). Alignment of those sequences indicated that the identity occurs mainly in heme-binding motif of the cyt b_5-like region and the three conserved His box areas (Fig. 1). It was noted that in the third His box of PiD6, Glu is replaced by Asp. Phylogenetic analysis of these sequences indicated that PiD6 clusters with the main group of the Δ6 desaturases from fungus and moss, which distinguish themselves from the Δ6 desaturases from animal and higher plants and from the desaturase-related enzymes (data not shown). These results suggest that PiD6 may be a Δ6 desaturase involved in the biosynthesis of GLA and SDA in *P. irregulare.*

Expression of PiD6 in Yeast

The yeast host strain Invsc1 was transformed with a plasmid containing the full-length ORF of *PiD6* under the control of the Gal-inducible promoter *GAL1*. When the yeast transformant was induced by Gal in a medium containing LA, a prominent extra peak accounting for about 6% of the total fatty acids was observed in the chromatogram of the fatty acid methyl esters (FAMEs) accumulating in the transformants compared with the control (Fig. 2). A comparison of the chromatogram with that of FAME standards revealed that the peak has a retention time identical to the GLA standard. To confirm the regiochemistry of the products, the diethylamine derivatives of the fatty acid from the expressing strain were analyzed by gas chromatography (GC)-mass spectroscopy (MS). The spectrum was identical to the reference standard (data not shown). These results demonstrate that PiD6 is a Δ6 desaturase that converts LA to GLA in yeast. When the yeast transformant was induced by Gal in a medium without LA, two small peaks (approximately 1% of total) were detected in the transgenic FAME fraction that were determined to be 16:2(6, 9) and 18:2(6, 9), respectively (Fig. 3).

Expression of PiD6 in *B. juncea*

To produce Δ6-desaturated fatty acids in seeds of *B. juncea*, we transformed the *B. juncea* with the con-

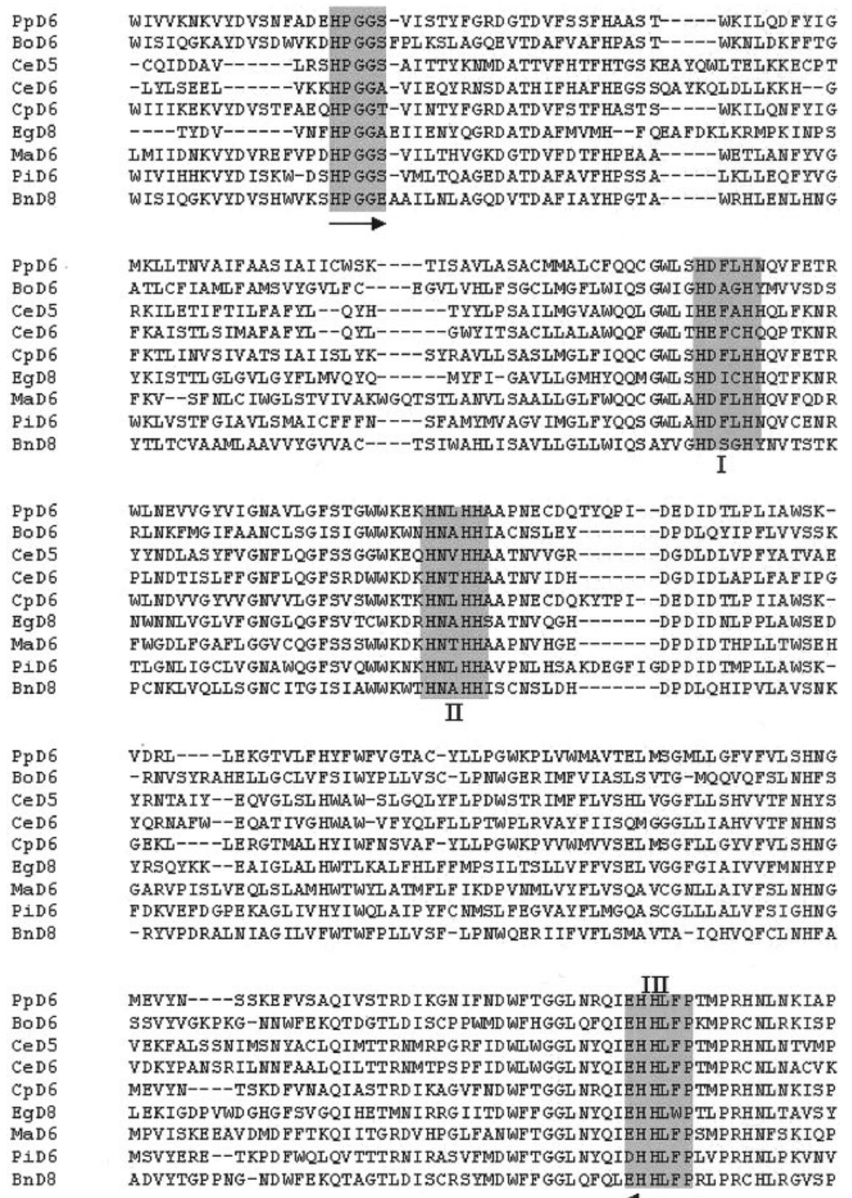

Figure 1. Alignment of PiD6 (ranging from 15 to 426 amino acids) and its orthologs by the Clustal method (Higgins and Sharp, 1989). The conservative motifs such as the cyt b_5 heme binding and the three His boxes are highlighted. The two arrows indicate the binding locations of the two degenerate primers. BnD8, A Δ-8 sphingolipid desaturase from oilseed (Sperling et al., 1998); CpD6, a bifunctional Δ6 acetylenase/desaturase from *C. purpureus* (Sperling et al., 2000); EgD8, a Δ8 desaturase from Euglena (Wallis and Browse, 1999); CeD5, a Δ5 desaturase from *C. elegans* (Michaelson et al., 1998b); CeD6, a Δ6 desaturase from *C. elegans* (Napier et al., 1998); BoD6, a Δ6 desaturase from borage (Sayanova et al., 1997); MoD6, a Δ6 desaturase from *M. alpina* (Michaelson et al., 1998a); PpD6, a Δ6 desaturase from *P. patens* (Girke et al., 1998); PiD6, this report.

struct that contains PiD6 cDNA under the control of a heterologous seed-specific promoter (oilseed napin promoter). Eleven independent transgenic plants were obtained. Transformants were verified by PCR using two internal primers targeting the Δ6 desaturase. Fatty acid analysis of the transgenic seeds indicated that there were three new fatty acids in the gas chromatogram of most transgenics compared with the wild-type control (Fig. 4). The three fatty acids were identified as 18:2(6, 9); 18:3(6, 9, 12); and 18:4(6, 9, 12, 15). This result indicated that *B. juncea* could functionally express PiD6 from *P. irregulare*, introducing a double bond at position 6 of endogenous substrates 18:1(9); 18:2(9, 12); and 18:3(9, 12, 15), resulting in production of the corresponding Δ6 fatty acids in the transgenic seeds.

Among the three new fatty acids produced in transgenic seeds, GLA is the most abundant one, the level of which is in the range of 25% to 40% of the total fatty acids in transgenic seeds. The next most abundant component is SDA, which accounts for 2% to 10% of the total fatty acids in several transgenic lines (Fig. 5).

The fatty acid compositions of transgenic seeds are shown in Figure 6. It is clear that the high-level production of Δ6-desaturated fatty acids occurs at the cost of two major fatty acids, LA and linolenic acid. Proportions of oleic and stearic acids in transgenics are slightly but not significantly reduced compared with those in the wild-type control. The content of LA in the transgenics was dramatically reduced. In the untransformed wild type, LA accounts for more

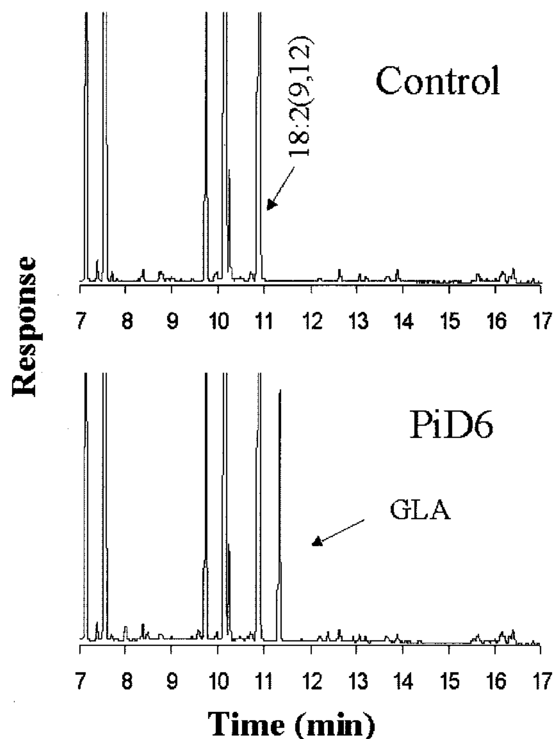

Figure 2. GC analysis of FAMEs from yeast (strain Invsc1) expressing PiD6 cultured with exogenous LA.

than 40% of the total fatty acids in seeds. In transgenics, the level was reduced to less than 10%.

As compared with the reduction of LA in transgenics, the decrease in linolenic acid in transgenics is less dramatic but still significant. In the untransformed wild type, linolenic acid accounts for more than 10% of the total fatty acids in seeds, whereas in transgenics, the level was reduced to less than 5%.

The results are understandable. The two dramatically reduced fatty acids in transgenic seeds are the substrates of the Δ6 desaturase, and the reduction is the cost for producing two corresponding Δ6-desaturated fatty acids.

Distribution of GLA in Lipid Classes and Position of TAG

Analysis of the lipid classes of transgenic seed showed that the majority of the GLA and other new Δ6 products were incorporated almost quantitatively and exclusively into the TAGs with trace amounts found in the some of the other neutral lipids (Table I). This increase in the total lipid unsaturation can be seen in the comparison of the molecular weights of the TAGs in the transgenic and non-transgenic oil. It has been previously shown that matrix-assisted laser desorption/ionization (MALDI)-MS can be used to qualitatively and quantitatively analyze lipid components in oil seeds (Asbury et al., 1999). Figure 7 shows the shift of the molecular weights of the major TAGs down by 2 to 4 D, which indicates an additional one or two double bonds in the TAGs. The most prominent peaks in the wild-type oil correspond to five to six carbon-carbon double bonds, whereas in the transgenic oil, this shifts to seven to eight and, in contrast to wild-type oil, the transgenic oil shows significant peaks (>7% of total TAGs; data not shown) corresponding to nine and 10 C-C double bonds per molecule.

Treatment of the TAG fraction with lipase and subsequent analysis of the fatty acids present in the *sn*-2 monoacylglycerols (MAG) produced (Fig. 8) shows that, as expected, GLA is more abundant in the *sn*-2 position of the TAG than the average for the *sn*-1 and *sn*-3 positions (Stymne and Stobart, 1987). FAME analysis of the MAG showed that 52.6% (wt %) was GLA. When compared with the 38.4% in total TAGs, this indicates an average of 31.3% of the fatty acids at each of *sn*-1 and *sn*-3 positions (Christie, 1982). GC analysis of the released fatty acids in the reaction showed 34% GLA, which compares closely with the calculated value of 31.3%.

DISCUSSION

In this report, we describe isolation of a gene encoding Δ6 desaturase from *P. irregulare*. Expression of

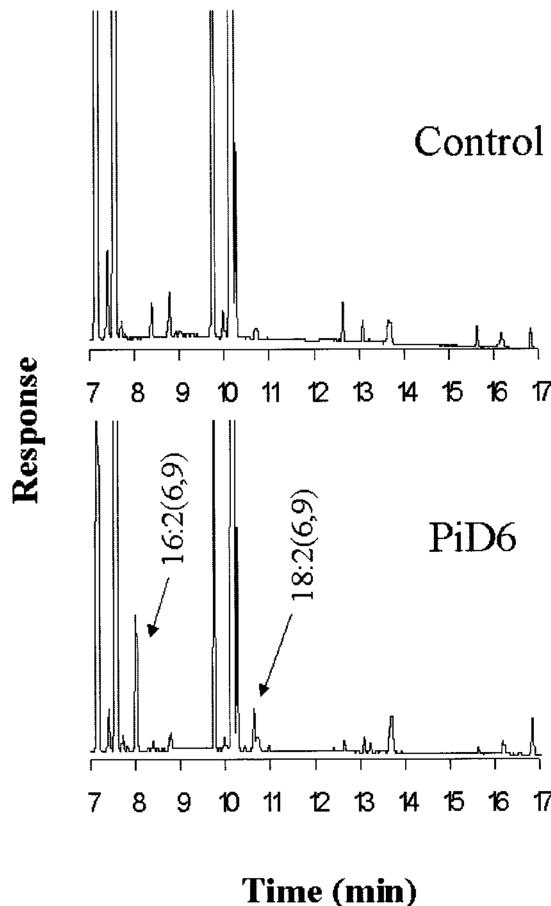

Figure 3. GC analysis of FAMEs from yeast (strain Invsc1) expressing PiD6 cultured without exogenous fatty acids.

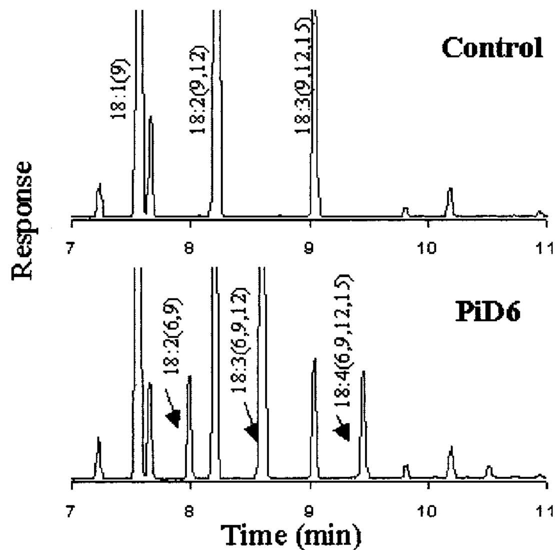

Figure 4. GC analysis of seed FAMEs from *B. juncea* expressing PiD6. Three new peaks indicate three Δ6-desaturated fatty acids in transgenic seeds.

the gene in *B. juncea* resulted in production of a high level of Δ6-desaturated fatty acids, especially GLA in the seeds. At present, the predominant sources of GLA are oils from plants such as evening primrose (*Oenothera biennis*), borage, and black currant (*Ribes nigrum*), and from microorganisms such as *Mortierella* spp., *Mucor* spp., and cyanobacteria (Phillips and Huang, 1996). However, these GLA sources are not ideal for dietary supplementation due to large fluctuations in availability and extraction costs.

Transgenic plants have long been considered as an efficient way to produce biological compounds. The cost effectiveness of production in genetically modified plants has attracted tremendous scientific and industrial effort directed toward production of various compounds (Ohlrogge and Browse, 1998; Somerville and Bonetta, 2001). Thus, use of oilseed crops to produce the Δ6-desaturated fatty acids is economically attractive. Oilseed crops have high yield and oil content compared with the traditional GLA-producing species. For instance, with common cultivation practice in southern Saskatchewan, Canada, the yield of borage is around 70 kg of seeds per acre, the oil content is in a range of 30% to 33%, and GLA content is around 23% of the total fatty acids in seeds. The yield of *B. juncea* is around 600 kg of seeds per acre, and the oil content is in a range of 40% to 45% (B. McCann and D. Potts, personal communication). In the transgenic seeds (see Fig. 5), the GLA content is around 25% to 40%. The efficiency of GLA production in transgenic *B. juncea* mustard is obviously advantageous over the traditional borage system.

Because of the potential efficiency of transgenic production of GLA in crops, there have been several previous attempts to express Δ6 desaturases in plants (Knutzon et al., 1999; Sayanova et al., 1999a; Qiu et al., 2002). Expression of a borage Δ6 desaturase under the control of 35S promoter in tobacco and flax resulted in a high-level accumulation of GLA in vegetative tissue (more than 20% of the total fatty acids), but the level in seeds was low (less than 2% of the total fatty acids; Sayanova et al., 1999a; Qiu et al., 2002). Expression of a borage Δ6 desaturase under the control of napin promoter in *B. juncea* resulted in a moderate production of GLA (3%–9%) in mature seeds (Qiu et al., 2002). However, expression of a Δ6 desaturase along with a Δ12 desaturase from *M. alpina* under the control of the napin promoter resulted in production of up to 43% of GLA in seeds of *B. napus* (Knutzon et al., 1999; Palombo et al., 2000; Liu et al., 2001). The high-level production of GLA accompanied accumulation of only a small amount of SDA, no 18:2(6, 9) in the seeds. Three quarters of the GLA produced was located at *sn*-1 and *sn*-3 positions of the TAG.

Our experiments demonstrate that the introduction of a single gene encoding Δ6 desaturase from *P. irregulare* into *B. juncea* results in the accumulation of up to 40% GLA in seeds. GLA is almost exclusively incorporated into TAG (98.5%), and within the TAGs, the GLA is more abundant at the *sn*-2 position. It is not clear why this preference for the *sn*-2 position in *B. juncea* differs from that of *B. napus*. In the *B. juncea* system described, along with GLA, a small amount of 18:2(6, 9) also accumulates. In biotechnological applications in which this diene may be undesirable, it may be possible to reduce or eliminate it by co-expression of a Δ12 desaturase (Liu et al., 2001), thereby, reducing the level of its oleate precursor. Our results suggest that Δ6 desaturases isolated from oleaginous fungi may not be tightly regulated, as are plant counterparts in oilseed crops, or they are simply more active than those of plant sources.

MATERIALS AND METHODS

Strains and Culture Condition

Pythium irregulare 10951 obtained from the American Type Culture Collection (Manassas, VA) was grown at 25°C for 6 d in a liquid medium consisting of 3 g L^{-1} yeast (*Saccharomyces cerevisiae*) extract, 3 g L^{-1} malt extract, 5 g

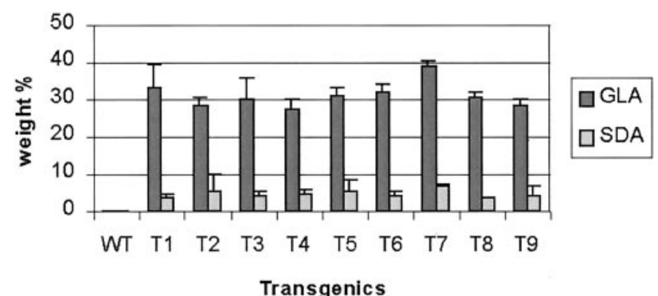

Figure 5. Weight percentage of GLA and SDA accumulating in transgenic seeds of transgenic lines of *Brassica juncea*.

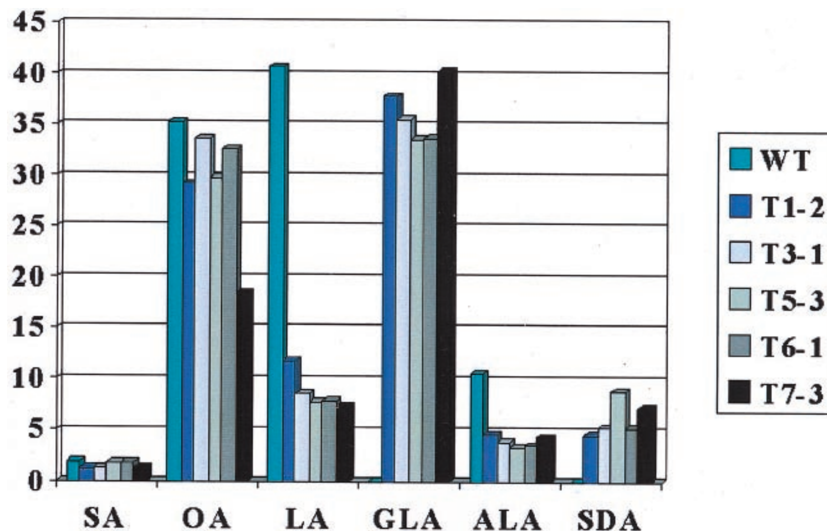

Figure 6. Fatty acid compositions of the seed lipids from five transgenic lines of *B. juncea* expressing PiD6. SA, Stearic acid; OA, oleic acid.

L^{-1} peptone, 10 g L^{-1} Glc, 0.68 g L^{-1} K_2HPO_4, pH 6.0, and 1 N HCl (Stinson et al., 1991). Cells were harvested by filtration and washed with distilled water three times. The dried mass was frozen in liquid nitrogen, ground with mortal and pestle into a fine powder, and stored at −70°C.

DNA Cloning

Single-stranded cDNA was synthesized from total RNA using First-Strand cDNA Synthesis kit (Amersham-Pharmacia Biotech, Uppsala) and was used as a template for PCR amplification with degenerate primers (forward; GCXA/GAXGAXCAC/TCCXGGXGG; and reverse, ATX TG/TXGGA/GAAXAG/AA/GTGA/GTG; Qiu et al., 2001a). PCR amplification was run on a DNA thermal cycler (PerkinElmer Life Sciences, Boston, MA) using a program of 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C for 35 cycles followed by extension for 10 min at 72°C. The amplified products were separated on a 1.0% (w/v) agarose gel, and fragments from 800 to 1,000 bp were isolated and purified using a kit (Qiaex II gel purification, Qiagen USA, Valencia, CA) and were subsequently cloned into the TA cloning vector pCR 2.1 (Invitrogen, Carlsbad, CA). The cloned inserts were then sequenced by PRISM DyeDeoxy Terminator Cycle Sequencing System (PE-Applied Biosystems, Foster City, CA).

The mRNA was extracted from the total RNA by using Dynabeads oligo(dT)$_{25}$ (Dynal Biotech, Lake Success, NY). The cDNA library was constructed using ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA) and screened according to standard methods (Ausubel et al., 1995).

Table I. *Distribution of GLA and total lipids by lipid class (wt %) in B. juncea expressing PiD6*

ND, Not detected.

	Polar Lipids	Free Fatty Acids	MAGs	Diacylglycerols	TAGs
			%		
GLA	ND	0.28	ND	0.14	98.50
Total fatty acid	0.16	0.67	0.10	0.23	97

Expression of PiD6 in Yeast

The ORF of *Pid6* was amplified by PCR using the high-fidelity *Pfu* polymerase (Stratagene). After initial amplification, normal *Taq* polymerase was added to the reaction, and it was then incubated at 72°C for another 10 min to facilitate the TA cloning (pCR 2.1, Invitrogen). Having confirmed that the PCR products were identical to the original cDNAs by sequencing, the fragments were then released by a *Bam*HI-*Eco*RI double digestion and inserted into the yeast expression vector pYES2 (Invitrogen). The following yeast transformation and growth were as described by our previous work (Qiu et al., 2001b).

Transformation of *B. juncea*

The hypocotyls of 5- to 6-d-old seedlings of *B. juncea* 1424, a zero-erucic acid breeding line, were used as explants for inoculation with *Agrobacterium tumefaciens* GV3130::pMP90 that hosts a binary vector with the full-length PiD6 cDNA under the control of the oilseed (*Brassica napus*) napin promoter (−1,102 to +43; Ericson et al., 1986). The *B. juncea* transformation was according to Radke et al. (1992).

Fatty Acid Analysis

Total fatty acids were extracted from yeast cultures and plant seeds and were analyzed as FAMEs by GC according to Qiu et al. (2001b). For double-bond position analysis, FAMEs were saponified and derivatized with diethylamine (Nilsson and Liljenberg, 1991).

Lipid Class Analysis

Total lipids were extracted by grinding the individual seeds with a polytron homogenizer (Kinematica, Basel) in 2

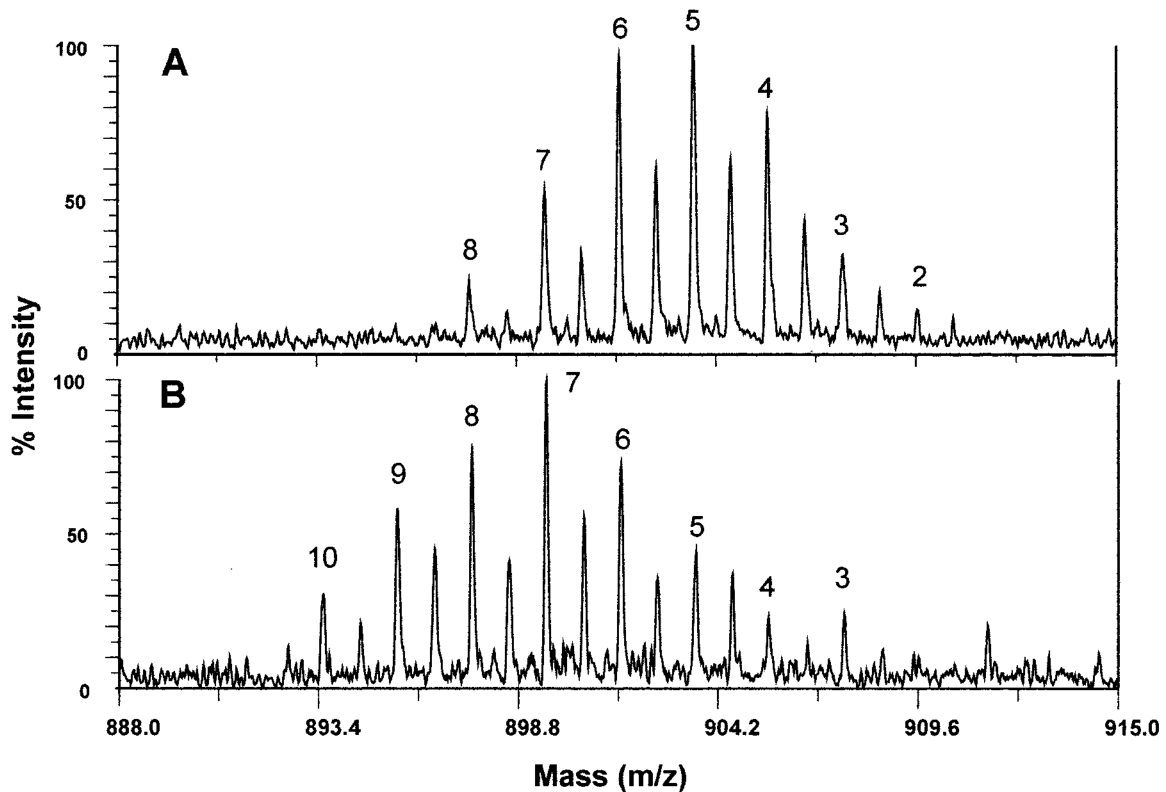

Figure 7. MALDI mass spectrum of *B. juncea* seed oil TAGs containing 18-carbon fatty acids. Peaks represent M–H+Na ions and are labeled to indicate the number of unsaturations present in the molecule. The *B. juncea* expressing PiD6 (B) contains TAGs with higher levels of unsaturation than the corresponding wild-type *B. juncea* (A) TAGs.

mL of a mixture of $CHCl_3$:isopropanol (2:1, v/v) containing internal standards and butylated hydroxytoluene as an antioxidant. The slurry was filtered through a glass fiber filter and eluted with CH_2Cl_2. A small aliquot was taken for total FAME analysis. Lipid extracts from several seeds that were determined to be high in GLA (approximately 40%) and a wild type were spotted in separate lanes along with standard lanes and developed on a silica gel 60 thin-layer chromatography plate (Merck, Rahway, NJ) and developed with a solvent mixture of hexane:diethyl ether: acetic acid (70:30:1, v/v). TAGs, diacylglycerols, MAG, free fatty acids, and polar lipids were isolated separately by scraping the appropriate areas from thin-layer chromatography plates. The lipids were eluted from the silica with 4 mL of CH_2Cl_2, except for polar lipids, which were eluted with acidic Bligh and Dyer (1959) solution. The lipid fractions were dried under N_2 and transmethylated with 2 mL of 1% (v/v) H_2SO_4 in methanol at 50°C for 30 min. The mixture was cooled, 2 mL of H_2O was added, and the FAMEs were extracted into hexane for GC analysis.

TAG Positional Analysis

For TAG positional analysis, lipid extracts high in GLA (as determined by GC of FAME) were pooled and applied to a 1-mL silica gel column and eluted with 3 mL of CH_2Cl_2 to yield the neutral lipid fraction, which was predominantly TAG. A portion of the combined eluate was dried under N_2 and transmethylated to FAMEs with 2 mL of 1% (v/v) H_2SO_4 in CH_3OH for GC analysis. The remainder was combined and subjected to a pancreatic lipase hydrolysis as described by Christie (1982) for positional analysis.

Mass spectra of TAGs were obtained from MALDI-TOF Mass Spectrometer:Voyager DE STR (PE-Applied Biosys-

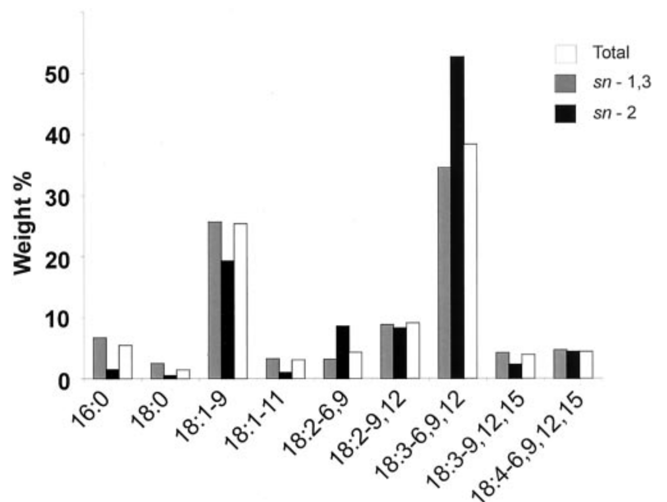

Figure 8. Comparison of the relative wt % of fatty acids for total TAG, *sn*-2 position, and average of the *sn*-1 and *sn*-3 positions in TAGs of *B. juncea* expressing PiD6.

tems) equipped with a nitrogen laser 337-nm, 3-ns pulse with an average of 50 shots per spectrum acquired. Data were acquired in reflectron mode, and the M–H+Na ions were observed for the triglycerides. The matrix used was 0.5 μL of 2,5-dihydroxybenzoic acid (70 mg mL^{-1} in 90:10 [v/v] acetone:water; Sigma, St. Louis) spotted on plate and air-dried. The samples were dissolved in chloroform, and 0.5 μL of sample was placed on top of dried matrix film and air-dried. The instrument was calibrated using a one-point calibration with the M–H+Na ion for triolein (907.7731 D).

ACKNOWLEDGMENTS

We thank Dr. Derek Potts for providing *B. juncea* germplasm, Stephen Ambrose for performing GC-MS analysis, and Doug Olson for performing MALDI-MS analysis.

Received December 14, 2001; returned for revision January 17, 2002; accepted February 11, 2002.

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