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Conjugated fatty acids accumulate to high levels in phospholipids of metabolically engineered soybean and *Arabidopsis* seeds

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Abstract

Expression of Δ^{12}-oleic acid desaturase-related fatty acid conjugases from *Calendula officinalis*, *Momordica charantia*, and *Vernicia fordii* in seeds of soybean (*Glycine max*) or an *Arabidopsis thaliana fad3/fae1* mutant was accompanied by the accumulation of the conjugated fatty acids calendic acid or α-eleostearic acid to amounts as high as 20% of the total fatty acids. Conjugated fatty acids, which are synthesized from phosphatidylcholine (PC)-linked substrates, accumulated in PC and phosphatidylethanolamine, and relative amounts of these fatty acids were higher in PC than in triacylglycerol (TAG) in the transgenic seeds. The highest relative amounts of conjugated fatty acids were detected in PC from seeds of soybean and *A. thaliana* that expressed the *C. officinalis* and *M. charantia* conjugases, where they accounted for nearly 25% of the fatty acids of this lipid class. In these seeds, >85% of the conjugated fatty acids in PC were detected in the *sn*-2 position, and these fatty acids were also enriched in the *sn*-2 position of TAG. In marked contrast to the transgenic seeds, conjugated fatty acids composed <1.5% of the fatty acids in PC from seeds of five unrelated species that naturally synthesize a variety of conjugated fatty acid isomers, including seeds that accumulate conjugated fatty acids to >80% of the total fatty acids. These results suggest that soybean and *A. thaliana* seeds are deficient in their metabolic capacity to selectively catalyze the flux of conjugated fatty acids from their site of synthesis on PC to storage in TAG.

Keywords: *Arabidiopsis thaliana*; Soybean; *Glycine max*; Leguminosae; Conjugated fatty acid; Calendic acid; α-Eleostearic acid; Unusual fatty acid; Phosphatidylcholine; Triacylglycerol; Oilseed engineering; Metabolic engineering; Fatty acid conjugase; FAD2

1. Introduction

The double bonds of polyunsaturated fatty acids in plants are typically separated by one or more methylene groups. This is exemplified by the methylene-interrupted *cis*-Δ^9, Δ^{12}, and Δ^{15} double bonds of α-linolenic acid ($18{:}3\Delta^{9cis,12cis,15cis}$). By contrast, seed oils of a limited number of plant species are enriched in fatty acids that contain conjugated or non-methylene-interrupted double bonds. Such fatty acids are referred to as "conjugated" fatty acids. The conjugated fatty acids that occur in the seed oils of certain plants include calendic acid ($18{:}3\Delta^{8trans,10trans,12cis}$), α-eleostearic acid ($18{:}3\Delta^{9cis,11trans,13trans}$), catalpic acid ($18{:}3\Delta^{9trans,11trans,13cis}$), punicic acid ($18{:}3\Delta^{9cis,11trans,13cis}$), parinaric acid ($18{:}4\Delta^{9cis,11trans,13trans,15cis}$), and dimorphecolic acid (9-OH-$18{:}2\Delta^{10trans,12trans}$) (Badami and Patil, 1981; Smith, 1971). Selected species from at least eight

Abbreviations: DAG, diacylglycerol; FAME, fatty acid methyl ester; MAG, monoacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

Fatty acid nomenclature: $X{:}Y\Delta^{zcis}$, *X*, total number of carbon atoms in the fatty acid; *Y*, number of double bonds in the fatty acid; *z*, position of the double bond relative to carboxyl end of the fatty acid. Double bonds can be in the *cis* or *trans* configuration, as indicated.

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different plant families are known to produce seeds enriched in conjugated fatty acids (Badami and Patil, 1981; Smith, 1971). These families include the Asteraceae, Euphorbiaceae, Cucurbitaceae, Rosaceae, Lythraceae, Balsaminaceae, Chrysobalanaceae, and Bignoniaceae.

Conjugated fatty acids are of commercial and biotechnological interest because of their chemical and physiological properties. Oils enriched in conjugated fatty acids are more prone to oxidation relative to oils that contain polyunsaturated fatty acids with methylene-interrupted double bonds (Sonntag, 1979). This is a desirable property for vegetable oils that are used as drying agents in inks, paints, and varnishes. In addition, conjugated fatty acids have also been shown to reduce fat accumulation in livestock, which results in enhanced meat quality (Cahoon et al., 2000; Thiel-Cooper et al., 2001; Lee et al., 2002; Sun et al., 2004).

We have previously demonstrated that the conjugated double bonds of α-eleostearic and parinaric acids are synthesized by divergent forms of the Δ^{12}-oleic acid desaturase (FAD2) (Cahoon et al., 1999; Cahoon et al., 2000; Dyer et al., 2002). These enzymes, which have been designated "fatty acid conjugases", catalyze the conversion of the Δ^{12} double bond of linoleic acid (18:2$\Delta^{9cis,12cis}$) or α-linolenic acid to Δ^{11} and Δ^{13} double bonds (Liu et al., 1997; Cahoon et al., 1999). Divergent FAD2s were subsequently shown to catalyze the formation of conjugated double bonds in calendic, punicic, and dimorphecolic acids (Cahoon et al., 2001; Qiu et al., 2001; Hornung et al., 2002; Cahoon et al., 2003; Iwabuchi et al., 2003; Cahoon and Kinney, 2004). In the cases of calendic and dimorphecolic acids, conjugated double bond synthesis arises from the modification of the Δ^{9}-double bond, rather than the Δ^{12}-double bond, of linoleic acid (Crombie and Holloway, 1985). The fatty acid conjugase from *Calendula officinalis* seeds that functions in calendic acid synthesis has been shown to sequentially remove a hydrogen atom from the Δ^{8} and Δ^{11} carbons that flank the Δ^{9} double bond of linoleic acid (Reed et al., 2002). This results in the conversion of the *cis*-Δ^{9} double bond into conjugated *trans*-Δ^{8} and *trans*-Δ^{10} double bonds. This mechanism has been referred to as "1,4-desaturation" (Reed et al., 2002). It is likely that a similar mechanism involving the removal of a hydrogen atom from the Δ^{11} and Δ^{14} carbons that flank the Δ^{12} double bond of linoleic or linolenic acid is associated with the synthesis of the conjugated double bonds of α-eleostearic, parinaric, and punicic acids. Based on radiolabeling studies conducted with developing *Momordica charantia* seeds, fatty acid conjugases appear to use fatty acids linked principally to phosphatidylcholine (PC) as substrates (Liu et al., 1997). This is similar to what has been shown for the typical FAD2 that generates the Δ^{12} double bond of linoleic acid (Slack et al., 1978; Stymne and Appelqvist, 1978) as well as divergent FAD2s associated with the synthesis of hydroxy and epoxy fatty acids (Bafor et al., 1991; Liu et al., 1998). Based on what is known for the metabolism of other unusual fatty acids, one or more additional steps are likely required for the selective removal of conjugated fatty acids from PC for storage in triacylglycerol (TAG) in seeds (Voelker and Kinney, 2001).

Biotechnological efforts have been directed toward the production of conjugated fatty acids in seeds of transgenic crops because of the high value of these fatty acids for the coatings and livestock feed industries. The transfer of conjugated fatty acid biosynthetic pathways to established crops is an attractive approach because plants that naturally produce oils enriched in conjugated fatty acids are not well-suited for large-scale agronomic production in temperate climates. We have previously described the transgenic production of α-eleostearic and calendic acids to levels of 17% and 19%, respectively, of the total fatty acids in soybean somatic embryos (Cahoon et al., 1999; Cahoon et al., 2001). In addition, Iwabuchi et al. (2003) reported the production of punicic acid in transgenic *Arabidopsis* seeds to amounts of up to 3.5% of the total fatty acids. These amounts of conjugated fatty acids are considerably lower than what is found in seeds of plants that naturally produce conjugated fatty acids. For example, *Vernicia fordii* and *Punica granatum* seeds accumulate α-eleostearic and punicic acids, respectively, to >80% of the total fatty acids (Badami and Patil, 1981). In addition, we have observed that soybean seeds engineered to produce α-eleostearic acid display severely reduced rates of germination and wrinkled morphology (Cahoon, unpublished observation). Such alterations in the physiology of seeds represent a major hurdle that must be addressed for the commercial production of conjugated fatty aids in genetically enhanced oilseeds.

The studies described here were conducted as a step towards identifying metabolic constraints that limit conjugated fatty acid accumulation in transgenic seeds and that contribute to the reduced agronomic performance of these seeds. We show that soybean and *Arabidopsis* seeds engineered to produce calendic and α-eleostearic acids accumulate these fatty acids in membrane phospholipids as well as in the storage lipid triacylglycerol (TAG). In marked contrast, seeds that naturally produce conjugated fatty acids were found to limit the accumulation of these acyl moieties almost exclusively to TAG. The implications of these results to the metabolic engineering of conjugated fatty acid and other unusual fatty acid biosynthetic pathways in seeds of transgenic plants are discussed.

2. Results

2.1. Fatty acid composition of the total lipid extract and major lipid classes from soybean and Arabidopsis seeds engineered to express fatty acid conjugases

Experiments were conducted using mature seeds from soybean and *Arabidopsis* plants that were engineered to

produce conjugated fatty acids. For these studies, a fatty acid conjugase cDNA from *C. officinalis* was used to generate calendic acid ($18{:}3\Delta^{8trans,10trans,12cis}$) (Cahoon et al., 2001), and fatty acid conjugase cDNAs from *M. charantia* (Cahoon et al., 1999) and *V. fordii* (Dyer et al., 2002) were used to generate α-eleostearic acid ($18{:}3\Delta^{9cis,11trans,13trans}$). Strong seed-specific promoters from genes for the soybean α′-subunit of β-conglycinin or the *Phaseolus vulgaris* phaseolin were used to mediate expression of conjugase cDNAs. *Arabidopsis* studies were conducted using a *fad3/fae1* mutant (Smith et al., 2003), which has a seed oil composition that is low in α-linolenic acid and very long-chain fatty acids and high in linoleic acid, the substrate for the fatty acid conjugases. Initial expression studies performed in a wild-type background of *Arabidopsis* yielded seeds with low germination rates and wrinkled morphology, especially in α-eleostearic acid-accumulating seeds. In addition, amounts of conjugated fatty acids accumulated in seeds of wild-type *Arabidopsis* were <5% of the total fatty acids (data not shown). By contrast, we were able to obtain viable seeds that were capable of accumulating calendic and α-eleostearic acids to >10% of the total fatty acids by use of the *Arabidopsis fad3/fae1* mutant. The soybean and *Arabidopsis* lines used for these experiments were selected from >20 independent transformation events as the top performing lines based on their levels of conjugated fatty acid accumulation in seeds.

Calendic acid was detected at levels of 20% of the total fatty acids in soybean seeds that express the *C. officinalis* conjugase (Table 1). Expression of the same transgene in *Arabidopsis* seeds yielded slightly lower levels of calendic acid accumulation (Table 2). In addition, *Arabidopsis* seeds from plants transformed with the *M. charantia* and *V. fordii* conjugases accumulated α-eleostearic acid to amounts of 13% and 6%, respectively, of the total fatty acids (Table 2). The production of conjugated fatty acids was accompanied by changes in the relative content of other fatty acids. Most notably, oleic acid levels nearly doubled in *Arabidopsis* seeds that produce α-eleostearic acid relative to seeds from non-transformed plants. A twofold increase in oleic acid content was also observed in soybean seeds engineered to produce calendic acid (Table 1). A small increase in amounts of oleic acid was detected in *Arabidopsis* seeds that accumulate calendic acid (Table 2). Decreases in palmitic acid content were also observed in soybean and *Arabidopsis* seeds that produce conjugated fatty acids (Tables 1 and 2).

Examination of the fatty acid content of specific lipid classes of seeds revealed that conjugated fatty acids accumulate to substantial levels in the major phospholipids PC and phosphatidylethanolamine (PE). Most notably, the conjugated fatty acid content of PC was higher than that of TAG in all of the transgenic lines. In the case of soybean and *Arabidopsis* seeds that express the *C. officinalis* and *M. charantia* enzymes, conjugated fatty acids composed > 20% of the fatty acids of PC. In addition, the decrease in palmitic acid content observed in the total lipids was more evident in PC and PE. For example, the palmitic acid content of PC was more than fivefold lower in soybean seeds expressing the *C. officinalis* conjugase than in seeds from non-transformed plants (Table 1). Furthermore, the conjugated fatty acid content of DAG was intermediate between that of TAG and PC in seeds of soybean and *Arabidopsis* plants transformed with the *C. officinalis* and *M. charantia* fatty acid conjugases (Tables 1 and 2).

Immature soybean seeds that express the *C. officinalis* conjugase were also examined to determine whether high levels of conjugated fatty acids are present in PC during seed development. Soybean seeds at early to mid-maturity

Table 1
Fatty acid composition of the total lipid, triacylglycerol (TAG), diacylglycerol (DAG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) from mature seeds of non-transformed soybean plants or soybean plants transformed with a seed-specific expression transgene for the *Calendula officinalis* fatty acid conjugase

Lipid class	16:0	18:0	18:1	18:2	18:3	Calendic
			wt% of total fatty acids			
Non-transformed						
Total	9.8 ± 0.1	2.8 ± 0.6	10.9 ± 0.1	65.1 ± 0.7	10.0 ± 1.6	ND
TAG	8.5 ± 0.4	1.8 ± 0.3	10.4 ± 1.5	68.4 ± 1.3	10.9 ± 0.9	ND
DAG	13.0 ± 0.2	4.1 ± 0.1	5.6 ± 0.5	71.2 ± 0.8	6.0 ± 0.1	ND
PC	15.2 ± 0.9	4.1 ± 0.1	4.8 ± 0.7	67.3 ± 0.5	8.6 ± 1.1	ND
PE	23.2 ± 1.8	2.9 ± 0.1	4.1 ± 0.5	63.4 ± 1.4	6.4 ± 0.2	ND
+C. officinalis conjugase						
Total	5.2 ± 0.2	2.9 ± 0.6	23.1 ± 0.5	39.1 ± 1.1	6.9 ± 0.4	22.0 ± 0.9
TAG	4.9 ± 0.3	1.6 ± 0.8	23.1 ± 1.2	42.5 ± 1.2	6.8 ± 0.1	20.4 ± 0.8
DAG	3.3 ± 0.2	1.4 ± 0.1	27.1 ± 3.9	41.0 ± 2.8	4.6 ± 0.4	22.6 ± 2.4
PC	2.8 ± 0.3	1.1 ± 0.2	26.8 ± 2.2	38.9 ± 2.3	4.1 ± 0.4	26.2 ± 2.0
PE	5.4 ± 1.1	1.6 ± 0.6	30.8 ± 3.5	44.9 ± 3.7	4.2 ± 0.5	13.2 ± 1.9

The values shown are the means ± s.d. for measurements of lipids from three to five single seeds. ND, not detected. 16:0, palmitic acid; 18:0, stearic acid; 18:2, linoleic acid; 18:3, α-linolenic acid. 18:1 consists principally of oleic acid ($18{:}1\Delta^{9}$), but also contains *cis*-vaccenic acid ($18{:}1\Delta^{11}$), which may account for 1–3% of the total fatty acids of a given lipid class.

Table 2
Fatty acid composition of the total lipid, triacylglycerol (TAG), diacylglycerol (DAG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) of lipid extracts from mature *Arabidopsis thaliana* (*fad3/fae1* mutant) seeds that were collected from non-transformed plants or plants transformed with seed-specific transgenes for the *Calendula officinalis*, *Momordica charantia*, or *Vernicia fordii* fatty acid conjugases

Lipid class	16:0	18:0	18:1	18:2	18:3	Calendic	α-Eleostearic
				wt% of total fatty acids			
Non-transformed							
Total	8.5 ± 0.2	4.2 ± 0.6	28.8 ± 0.1	55.1 ± 0.2	2.0 ± 0.1	ND	ND
TAG	8.3 ± 0.1	2.4 ± 0.5	28.0 ± 0.9	59.0 ± 1.7	1.8 ± 0.1	ND	ND
DAG	7.0 ± 0.3	1.9 ± 0.0	27.5 ± 1.5	61.4 ± 1.3	2.1 ± 0.2	ND	ND
PC	12.1 ± 0.6	1.3 ± 0.3	24.3 ± 1.0	57.5 ± 1.4	4.4 ± 0.4	ND	ND
PE	15.2 ± 0.6	1.2 ± 0.2	17.6 ± 2.0	63.8 ± 2.0	2.2 ± 0.1	ND	ND
+C. officinalis conjugase							
Total	6.6 ± 0.2	3.7 ± 0.1	36.0 ± 0.1	35.8 ± 0.4	1.3 ± 0.0	15.0 ± 0.4	ND
TAG	7.1 ± 0.2	3.7 ± 0.0	35.0 ± 1.4	37.1 ± 1.4	1.5 ± 0.2	14.3 ± 1.6	ND
DAG	3.7 ± 0.4	1.4 ± 0.4	35.1 ± 3.1	37.4 ± 2.5	2.0 ± 0.2	20.4 ± 1.2	ND
PC	5.5 ± 0.9	1.3 ± 0.2	33.2 ± 4.1	32.2 ± 3.1	3.6 ± 0.5	24.2 ± 1.9	ND
PE	6.5 ± 0.6	1.8 ± 1.0	30.1 ± 4.4	37.5 ± 1.9	3.1 ± 0.3	21.1 ± 1.6	ND
+M. charantia conjugase							
Total	5.4 ± 0.2	5.4 ± 0.1	54.1 ± 1.7	19.5 ± 0.9	0.6 ± 0.1	ND	13.0 ± 0.5
TAG	5.3 ± 0.3	5.4 ± 0.2	55.3 ± 2.0	19.6 ± 1.5	0.6 ± 0.1	ND	11.8 ± 0.4
DAG	4.7 ± 0.2	2.6 ± 0.3	50.7 ± 0.6	24.2 ± 1.3	0.9 ± 0.1	ND	16.9 ± 1.6
PC	4.8 ± 0.1	1.2 ± 0.4	46.3 ± 0.9	22.5 ± 0.1	1.9 ± 0.1	ND	23.4 ± 0.6
PE	9.4 ± 0.3	1.2 ± 0.0	44.1 ± 0.8	35.3 ± 0.2	0.9 ± 0.1	ND	9.2 ± 0.5
+V. fordii conjugase							
Total	7.0 ± 0.2	3.7 ± 0.1	46.0 ± 1.4	34.4 ± 1.3	1.2 ± 0.3	ND	6.2 ± 0.4
TAG	6.9 ± 0.3	1.8 ± 0.3	41.4 ± 3.3	42.6 ± 3.2	1.6 ± 0.4	ND	5.4 ± 0.4
DAG	4.7 ± 0.2	1.7 ± 0.6	62.5 ± 0.5	23.8 ± 0.6	1.7 ± 0.4	ND	5.6 ± 0.6
PC	8.1 ± 0.5	1.5 ± 0.2	51.6 ± 2.0	24.6 ± 1.2	3.9 ± 1.0	ND	10.1 ± 1.4
PE	10.3 ± 1.6	1.0 ± 0.3	45.3 ± 1.1	37.1 ± 0.9	1.7 ± 0.1	ND	4.5 ± 0.6

The values shown are the means ± s.d. from analyses of seeds collected from three to four separate pots of plants. ND, not detected. 18:1 consists principally of oleic acid (18:1Δ^9), but also contains *cis*-vaccenic acid (18:1Δ^{11}), which may account for 1–3% of the total fatty acids of a given lipid class.

were used for these analyses. As shown in Fig. 1, relative amounts of calendic acid in PC were at least equal or slightly higher than those in TAG.

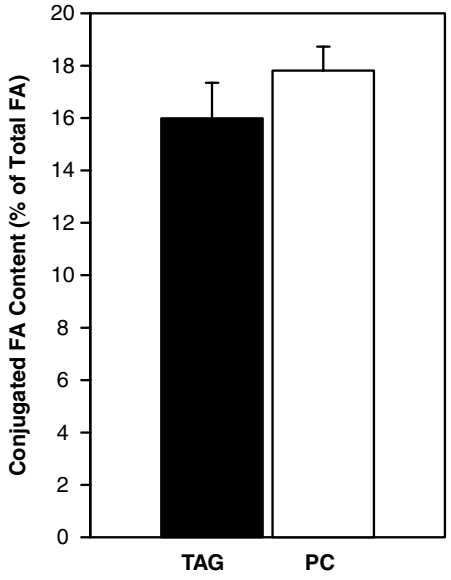

Fig. 1. Relative content of conjugated fatty acids in PC and TAG from developing soybean seeds (early to mid-development) engineered to express a fatty acid conjugase from *Calendula officinalis*. The values represented by the bars are the means ± s.d. from analyses of three independent samples.

2.2. Stereospecific distribution of conjugated fatty acids in PC and TAG from seeds of soybean and Arabidopsis engineered to express fatty acid conjugases

Stereospecific analysis of PC from soybean seeds that express the *C. officinalis* conjugase and *Arabidopsis* seeds that express the *M. charantia* conjugase revealed that conjugated fatty acids accumulate principally in the *sn*-2 position of this lipid (Fig. 2). In the engineered soybean seeds, >90% of the total calendic acid in PC was present in the *sn*-2 position. Similarly, >85% of the total α-eleostearic acid in PC of the transgenic *Arabidopsis* seeds was detected in the *sn*-2 position.

Amounts of conjugated fatty acids were also found to be disproportionately distributed at the *sn*-2 position of TAG from seeds of the transgenic plants (Fig. 3). In this regard, 60–70% of the conjugated fatty acids in TAG were detected in the *sn*-2 position. By contrast, ca. 30% of the oleic acid and 35–40% of the linoleic acid in TAG from these seeds was found in the *sn*-2 position of TAG, which suggested that these fatty acids are more evenly distributed in TAG.

For comparison, the fatty acid composition of the *sn*-2 position of TAG from *C. officinalis* and *M. charantia* seeds was determined (Table 3). Like TAG from transgenic soybean and *Arabidopsis* seeds, disproportionate amounts of conjugated fatty acids were found in the *sn*-2 position of TAG from these seeds, and these fatty acids were, in fact,

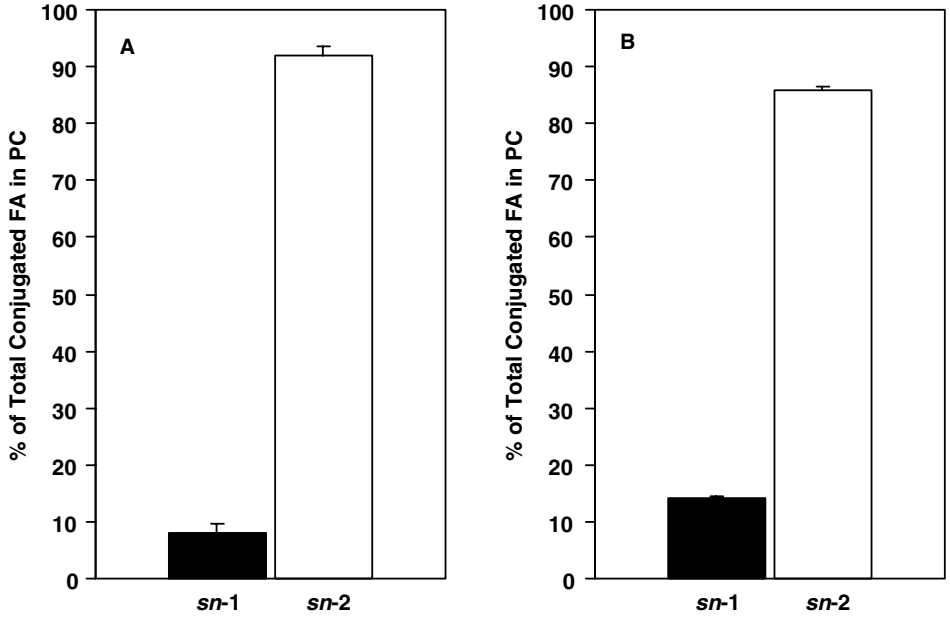

Fig. 2. Stereospecific distribution of conjugated fatty acids in PC from soybean seeds engineered to express a fatty acid conjugase from *Calendula officinalis* (A) and *Arabidopsis thaliana* seeds engineered to express a fatty acid conjugase from *Momordica charantia* (B). The values indicated by the bars are the means ± s.d. from three independent stereospecific analyses of PC isolated from the transgenic soybean and *Arabidopsis* seeds.

the predominate fatty acids in the *sn*-2 position. In this regard, calendic acid was found to compose 80% of the total fatty acids in the *sn*-2 position of TAG from *C. officinalis* seeds, and α-eleostearic acid was detected in amounts of >90% of the fatty acids in the *sn*-2 position of TAG from *M. charantia* seeds (Table 3).

2.3. Relative content of conjugated fatty acids in PC and TAG from seeds of unrelated species that naturally produce conjugated fatty acids

The relative amounts of conjugated fatty acids in TAG and PC were examined in seeds of plants that naturally synthesize and accumulate high levels of these fatty acids in their seed oils (Fig. 4A). For these analyses, seeds were chosen from species representing five different families: Asteraceae, Cucurbitaceae, Lythraceae, Balsaminaceae, and Bignoniaceae. The species used in these studies and the major conjugated fatty of their seed oils are: (1) *C. officinalis*, calendic acid; (2) *M. charantia*, α-eleostearic acid; (3) *P. granatum*, punicic acid ($18{:}3\Delta^{9cis,11trans,13cis}$); (4) *Impatiens balsamina*, parinaric acid ($18{:}4\Delta^{9cis,11trans,13trans,15cis}$); and (5) *Catalpa speciosa*, catalpic acid, ($18{:}3\Delta^{9trans,11trans,13cis}$). In strong contrast to what was observed in the transgenic soybean and *Arabidopsis* seeds (Fig. 4B), conjugated fatty acids were present in high amounts in TAG, but were nearly absent from PC in seeds of each of these species (Fig. 4A). The highest relative amounts of conjugated fatty acids in PC were detected in extracts from *C. speciosa* seeds. In PC from these seeds, conjugated fatty acids composed 1.5% of the total fatty acids. In seeds from all other species, conjugated fatty acids accounted for <1% of the total fatty acids in PC. The greatest disparity in conjugated fatty acid content between TAG and PC was observed in seeds from *P. granatum*. In these seeds, the conjugated fatty acid punicic acid composed >80% of the fatty acids in TAG, but only 0.8% of the fatty acids in PC (Fig. 4A).

Examination of lipids from developing *C. officinalis* seeds also revealed the presence of calendic acid in amounts of <1% of the total fatty acids of PC (data not shown). Similarly, Liu et al. (1997) reported that α-eleostearic acid accounted for 1.7% of the fatty acids of PC, but >60% of the fatty acids in TAG in developing *M. charantia* seeds. Such results indicate that the ability to selectively exclude the accumulation of conjugated fatty acids in PC is observed not only in mature seeds, but occurs throughout seed development in plants that naturally produce high levels of these unusual fatty acids in their seeds.

3. Discussion

In this report, we show that moderate amounts of conjugated fatty acids can be produced in seeds of soybean and *Arabidopsis* by transgenic expression of fatty acid conjugases. Most notably, calendic acid levels of 20% of the total fatty acids were obtained in soybean seeds by expression of the Δ^9-specific conjugase from *C. officinalis*. This amount of calendic acid is approximately 40% of that found in *C. officinalis* seeds (Crombie and Holloway, 1985). The most striking observation from these studies was the accumulation of conjugated fatty acids to levels >20% of the fatty acids in PC in metabolically engineered soybean and *Arabidopsis* seeds. By contrast, we observed that conjugated fatty acids typically account for <1% of

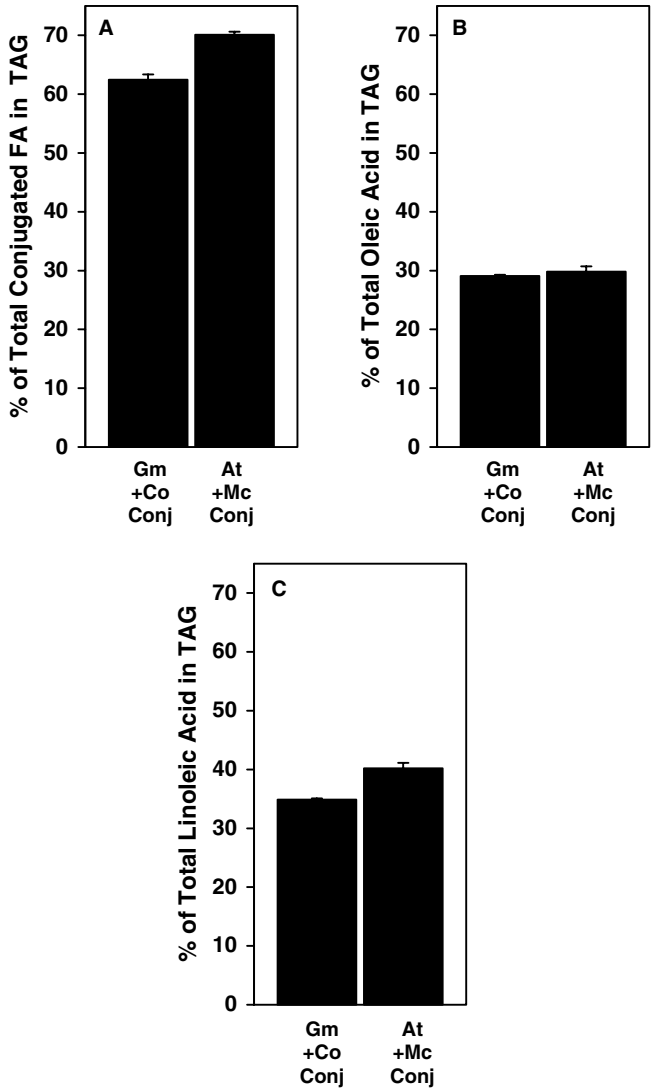

Fig. 3. Percentage of the total conjugated fatty acids (A), oleic acid (B), or linoleic acid (C) in TAG that occurs in the *sn*-2 position. TAG was analyzed from soybean seeds engineered to express a fatty acid conjugase from *Calendula officinalis* (*Gm + Co conj*) and *Arabidopsis thaliana* seeds engineered to express a fatty acid conjugase from *Momordica charantia* (*At + Mc conj*). The values indicated by the bars are the means ± s.d. from three independent analyses of TAG isolated from the transgenic soybean and *Arabidopsis* seeds.

Table 3
Fatty acid composition of the *sn*-2 position of TAG isolated from *C. officinalis* and *M. charantia* seeds

Fatty acid	*C. officinalis*	*M. charantia*
	wt% of total fatty acids in Sn-2 position	
16:0	ND	ND
18:0	ND	ND
18:1	4.9 ± 0.1	1.5 ± 0.1
18:2	12.6 ± 0.2	5.6 ± 0.3
18:3	0.3 ± 0.0	ND
Calendic	82.2 ± 0.3	ND
α-Eleostearic	ND	92.9 ± 0.4

The values shown are the mean of fatty acid compositions from three independent analyses of TAGs isolated from seeds of each species ± s.d. ND, not detected.

the fatty acids in PC from seeds that naturally produce high levels of these unusual fatty acids. In addition, relative amounts of conjugated fatty acids were higher in PC than in TAG in all of the transgenic lines, and this phenotype was also observed in developing soybean seeds engineered to express the *C. officinalis* conjugase. A similar observation has been previously reported for the transgenic production of acetylenic fatty acids in *Arabidopsis* seeds (Thomæus et al., 2001). However, in this study, levels of acetylenic fatty acids in the total lipid extract and in PC were <2% of the total fatty acid.

The accumulation of conjugated fatty acids in PC and other membrane phospholipids undoubtedly affects the physiology and agronomic performance of seeds, and this effect appears to be most pronounced with the production of α-eleostearic acid. For example, we previously reported the transgenic production of α-eleostearic acid in soybean somatic embryos (Cahoon et al., 1999); however, the seeds obtained from the regenerated embryos displayed very low germination rates and had a wrinkled appearance (Cahoon, unpublished observation). Soybean seeds engineered to synthesize calendic acid also displayed reductions in germination rate, but this effect was less severe than that observed with α-eleostearic acid production. In addition, wrinkled seeds and poor germination were also observed in our attempts to produce α-eleostearic acid in wild-type *Arabidopsis* seeds by expression of the *M. charantia* conjugase. Interestingly, these phenotypes were largely absent when a *fad3/fae1* mutant of *Arabidopsis* (Smith et al., 2003) was used as the background for expression of conjugases. Understanding the basis for the differences observed in germination and morphology of seeds from wild-type *Arabidopsis* and the *fad3/fae1* mutant is a focus of our current research.

It is likely that the presence of high levels of conjugated fatty acids in membrane phospholipids impacts their thermotropic properties. Our attempts to measure this effect by differential scanning calorimetry of isolated phospholipids were complicated by the oxidative instability of the conjugated fatty acids and by the propensity of their double bonds to undergo *cis–trans* isomerization. It has been reported, however, that the melting points of calendic and α-eleostearic acids are 40 and 49 °C, respectively (Gunstone et al., 1994). These melting points are less than those of the saturated fatty acids palmitic (m.p. 63 °C) and stearic (m.p. 69 °C) acids but are considerably higher than those of the polyunsaturated fatty acids linoleic (m.p. −5 °C) and α-linolenic (m.p. −11 °C) acids. The nearly 10 °C difference in melting points between calendic acid and α-eleostearic acid may account for the differences in the agronomic fitness of seeds engineered to produce these fatty acids.

Up to 90% of the conjugated fatty acids in PC from the transgenic seeds was detected in the *sn*-2 position. This observation suggests that linoleic acid linked to the *sn*-2 position of PC is the primary substrate for fatty acid conjugases, which is consistent with what has been observed

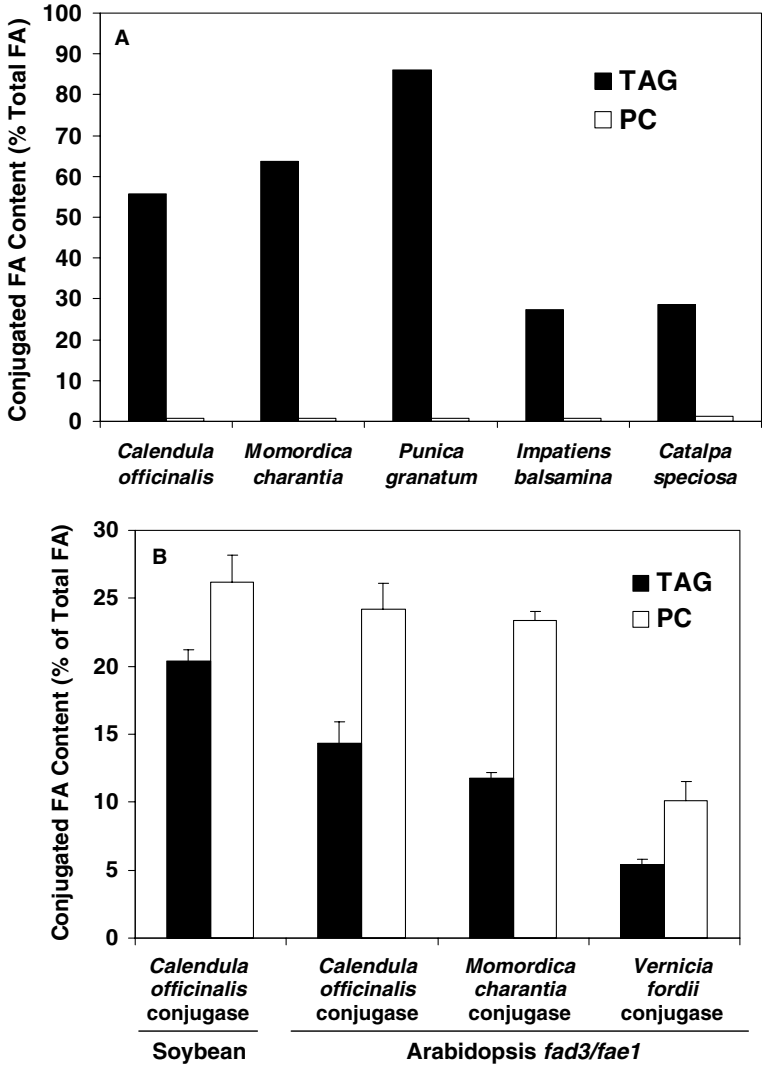

Fig. 4. Relative content of conjugated fatty acids in TAG and PC from seeds of plants that naturally accumulate high levels of conjugated fatty acids (A) and from soybean and *Arabidopsis fad3/fae1* seeds engineered to express fatty acid conjugases (B). Amounts of conjugated fatty acids are expressed as the wt.% of the total fatty acids in TAG or PC. Shown in (A) is the relative content of conjugated fatty acids in TAG and PC from seeds of *Calendula officinalis*, *Momordica charantia*, *Punica granatum*, *Impatiens balsamina*, *Catalpa speciosa*. Shown in (B) is the relative content of conjugated fatty acids in TAG and PC from soybean seeds engineered to express the *C. officinalis* conjugase and from *Arabidopsis fad3/fae1* seeds engineered to express the *C. officinalis* conjugase, the *M. charantia* conjugase and the *Vernicia fordii* conjugase. Values in (B) were derived from Tables 1 and 2.

with FAD2-related hydroxylases and epoxygenases (Bafor et al., 1991; Liu et al., 1998). Conjugated fatty acids in the transgenic soybean and *Arabidopsis* seeds were also enriched in the *sn*-2 position of TAG. This implies that a large portion of the conjugated fatty acid in PC remains at the *sn*-2 position upon incorporation into TAG. One route of metabolism that can explain this observation is that the phosphorylcholine head group of PC is removed to form DAG, which is subsequently incorporated into TAG by diacylglycerol acyltransferase activity. Removal of the headgroup of PC could occur by reverse activity of the CDP-choline:choline phosphotransferase (Slack et al., 1983) or by phospholipase C activity. Alternatively, the conjugated fatty acid may be cleaved from the *sn*-2 position of PC by a phospholipase A_2 or a transacylase (Dahlqvist et al., 2000) and then be re-incorporated onto the *sn*-2 position of a glycerol backbone destined for TAG synthesis by the activity of lysophosphatidic acid acyltransferase. Given that soybean and *Arabidopsis* seeds appear to be deficient in the selective removal of conjugated fatty acids from PC, the conversion of PC to DAG may account for the majority of conjugated fatty acid incorporation into TAG in the transgenic seeds. Similar to the transgenic seeds, conjugated fatty acids accounted for 80–90% of the total fatty acids in the *sn*-2 position of TAG from *C. officinalis* and *M. charantia* seeds (Table 3). This high content of conjugated fatty acids in the *sn*-2 position of TAG from these seeds could arise from either of the metabolic scenarios described above. Of note, radiolabeling studies with developing *M. charantia* seeds have shown that α-eleostearic acid is quickly metabolized (or channeled) from PC following its synthesis on this lipid and subsequently

sequestered in TAG (Liu et al., 1997). These studies, however, did not show whether α-eleostearic acid remains attached to the glycerol backbone from PC upon incorporation into TAG. Regardless of the nature of this channeling, it is obvious from fatty acid compositional analyses of lipids from the transgenic seeds that the mechanism that maintains the selective flux of conjugated fatty acids from PC to TAG in seeds of plants such as *C. officinalis* and *M. charantia* is deficient or missing in soybean and *Arabidopsis* seeds.

Other explanations for the presence of high levels of conjugated fatty acids in phospholipids of the engineered soybean and *Arabidopsis* seeds can be proposed. For example, it is possible that the introduced conjugases were mistargeted to domains of the ER that are not actively involved in TAG synthesis in soybean and *Arabidopsis* seeds. However, little evidence exists for discrete domains of PC and TAG synthesis in seeds.

To date, fatty acid conjugases have been identified in plants from six different families (Cahoon et al., 1999, 2000, 2001; Qiu et al., 2001; Hornung et al., 2002; Dyer et al., 2002; Iwabuchi et al., 2003; Cahoon and Kinney, 2004). These fatty acid conjugases are all divergent forms of FAD2, but their primary structures do not display close phylogenetic relation. For example, the *V. fordii* conjugase shares 74% amino acid sequence identity with a typical FAD2 from *Euphorbia lagascae* but <60% identity with Δ^{12} conjugases from *M. charantia*, *P. granatum*, and *I. balsamina* and 46% identity with the Δ^{9} conjugase from *C. officinalis*. This suggests that conjugated fatty acid biosynthetic ability has evolved independently multiple times in the plant kingdom. It is therefore likely that metabolic mechanisms that selectively exclude the accumulation of conjugated fatty acids in membrane phospholipids in seeds of these species (as shown in Fig. 4) have also arisen independently multiple times in plants. As such, it is difficult to envision that complicated pathways have evolved for the selective flux of conjugated fatty acids to TAG. This channeling may, in fact, involve only one or a very small number of specialized metabolic enzymes, and alternative mechanisms for channeling of conjugated fatty acids may have arisen in the different, unrelated species that accumulate high levels of conjugated fatty acids in their seeds.

Finally, the most notable secondary effect of conjugated fatty acid production on the fatty acid composition of transgenic seeds was the elevation of the oleic acid content. This was most evident in seeds that produce α-eleostearic acid. This phenotype has been previously observed in soybean somatic embryos that produce α-eleostearic acid (Cahoon et al., 1999) and in *Arabidopsis* seeds that produce punicic acid (Iwabuchi et al., 2003). Elevation of oleic acid content accompanying the transgenic expression of divergent FAD2 hydroxylases (Broun and Somerville, 1997; Broun et al., 1998; Smith et al., 2003), epoxygenases (Singh et al., 2001), and acetylenases (Thomæus et al., 2001) as well as a cytochrome P450 epoxygnesase (Cahoon et al., 2002) is also well-documented. The best evidence to date suggests that the fatty acid product that contains modifications (e.g. hydroxylation and epoxygenation) at or in the vicinity of the Δ^{12}position inhibit the activity of the native FAD2 (Cahoon et al., 2002). The less pronounced elevation in oleic acid content in *Arabidopsis* seeds that express the *C. officinalis* conjugase may be explained by the lack of variant chemical modification at the Δ^{12} position of calendic acid. However, it cannot be excluded that the more modest increase in oleic acid content in transgenic seeds that express the *C. officinalis* conjugase results from the build-up of calendic acid in pools of PC that are normally associated with polyunsaturated fatty acid biosynthesis. Inefficient removal of calendic acid may reduce the rates of turnover of these pools and result in less desaturation of oleic acid.

3.1. Concluding remarks

In conclusion, we have shown that seeds of transgenic plants can be engineered to produce moderate levels of conjugated fatty acids. However, one or more specialized enzymes that mediate the selective flux of these fatty acids to TAG following their synthesis on PC in plants such as *M. charantia* and *C. officinalis* is absent from or deficient in soybean and *Arabidopsis* seeds, as evidenced by the accumulation of conjugated fatty acids in PC. The identification of these enzymes will likely be essential for achieving high levels of conjugated fatty acids in metabolically engineered seeds and for maintaining the agronomic viability of these seeds. Understanding the mechanisms associated with the channeling of conjugated fatty acids may also provide useful information for the discovery of specialized metabolic enzymes for other unusual fatty acids, such as epoxy and acetylenic fatty acids, that are also synthesized by divergent FAD2s.

4. Experimental

4.1. Preparation of plant expression constructs

cDNAs for the *C. officinalis* (Cahoon et al., 2001) and *M. charantia* (Cahoon et al., 1999) conjugases were linked on their 5′ ends to the strong seed-specific promoter for the α′ subunit of the β-congylcinin gene from *Glycine max* and on their 3′ ends to the 3′ UTR for the phaseolin gene. Detailed descriptions of the preparation of these gene expression cassettes were previously reported (Cahoon et al., 1999, 2001). For the production of transgenic *Arabidopsis*, the expression cassettes for the *C. officinalis* and *M. charantia* fatty acid conjugase cDNAs were cloned as *Asc*I fragments into the corresponding restriction enzyme site of the binary vector pKR92. This binary vector contains a neomycin phosphotransferase II gene under control of the cauliflower mosaic virus 35S promoter for kanamycin selection of transgenic plants.

The binary vector containing myc-epitope-tagged *V. fordii* fatty acid conjugase cDNA was constructed by PCR amplification of the myc-tagged conjugase cDNA from pRTL2-mycTFADX (Dyer et al., 2002) with primers that added *Bgl*II and *Sac*I sites before and after the start and stop codons, respectively. The PCR products were subsequently digested with *Bgl*II and *Sac*I, gel-purified, and subcloned into the corresponding sites of vector pS-5(BglII). pS-5(BglII) contains a seed-specific promoter derived from the phaseolin gene (Kawagoe et al., 1994). The phaseolin promoter-myc-tagged *V. fordii* conjugase cDNA cassette was excised with *Hin*dIII and *Sac*I and cloned into the corresponding sites of pBI121 (Clontech) to generate the plasmid pBI-Phas-mycFADX.

4.2. Production of transgenic Arabidopsis and soybean plants

Binary vectors containing expression cassettes for the *C. officinalis*, *M. charantia*, and *V. fordii* conjugase cDNAs were introduced into *Agrobacterium tumefaciens* strain C58 (pMP90) by electroporation. *Agrobacterium* cells harboring these plasmids were used for transformation of *Arabidopsis* by use of the previously described floral dip method (Clough and Bent, 1998). A *fad3/fae1* mutant of *Arabidopsis thaliana* (Columbia) (Smith et al., 2003) was used as the background for these experiments. Transgenic plants were identified by kanamycin selection. The seeds used for these experiments were collected from homozygous lines from $\geqslant T_4$ generation.

Soybean (*G. max* cv Jack) plants transformed with the gene expression cassette for the *C. officinalis* conjugase were regenerated from the previously reported "CoFADX-2" somatic embryos (Cahoon et al., 2001). Regeneration of somatic embryos was conducted as described (Kinney et al., 2001). Soybean seeds used for these experiments were from a transgenic line of $>T_5$ generation.

4.3. Analysis of the fatty acid composition of lipid classes

Lipid compositional analyses were conducted on mature seeds collected from transformed *Arabidopsis* plants maintained at 22 °C and 50% humidity under a 16 h light (100 μmol irradiance $m^{-2}\,s^{-1}$)/8 h dark cycle. Biological replicates for experiments with *Arabidopsis* consisted of seeds collected from individual pots that contained 3–4 plants. Independent analyses of soybean seed lipids were conducted on extracts from single seeds collected from plants maintained under greenhouse conditions at 24 °C with 14 h day length.

Lipids were extracted from approximately 100 mg of *Arabidopsis* seeds by homogenization with a glass rod in MeOH:$CHCl_3$ (3 ml, 2:1 v/v) or from a single soybean seed ground with a mortar and pestle and transferred to the same volume of the organic solvent mixture in a 13 × 100 mm test tube. After 30 min incubation, $CHCl_3$ (1 ml) and H_2O (1.8 ml) was added. The sample was then mixed thoroughly and spun at 1000*g* for 5 min in a clinical centrifuge. The organic layer was recovered and used for lipid compositional analyses. The lipid extraction protocol was based on that previously described by Bligh and Dyer (1959). The same procedure was used for the isolation of lipids from immature soybeans at early to mid-development (approximately 100–150 mg FW). Two seeds were used for each analysis.

Triacylglycerol (TAG) and diacylglycerol (DAG) were resolved by TLC of the total lipid extract using Silica 60 plates (0.25 mm layer thickness; Merck) with a solvent system consisting of heptane:Et_2O (60:40 v/v). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were resolved by TLC of the total lipid extract on the same phase with a solvent system consisting of $CHCl_3$:MeOH:H_2O:30% ammonium hydroxide (65:35:3:2.5 v/v/v/v). Lipid standards were chromatographed on the opposite edge of the TLC plates. Because of the sensitivity of the double bonds of conjugated fatty acids to *cis–trans* isomerization, iodine vapor was applied only to the lipid standards for detection. Bands from the TLC plates corresponding to TAG, DAG, PC, and PE were scraped onto wax paper and quickly transferred to 13 × 100 mm glass tubes that contained two ml of 1% (w/v) sodium methoxide in MeOH. The tubes were evacuated with N_2 prior to capping and heated at 85 °C for 45 min. Fatty acid methyl esters (FAMEs) generated from each lipid class were subsequently recovered as previously described (Cahoon et al., 2002). FAMEs were analyzed by GC using an Agilent 6890 gas chromatograph with flame ionization detection. Resolution of FAMEs was achieved with an HP-INNOWax column (30 m length × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent), and H_2 was used as the carrier gas with an inlet flow rate of 30 ml/min. The oven temperature was programmed from 185 °C (1 min hold) to 230 °C (3 min hold) at 7 °C/min. The inlet and detector temperatures were 250 and 260 °C, respectively.

Lipids were also extracted from mature seeds of *I. balsamina*, *C. speciosa*, *C. officinalis*, *M. charantia*, and *P. granatum* as described above. Seeds were either collected or purchased from commercial sources. The total lipid extracts from these seeds were partitioned into neutral lipid, glycoplipid, and phospholipid fractions by silica solid phase extraction columns as described previously (Lynch and Steponkus, 1987). TAG and PC were resolved by TLC from the neutral lipid and phospholipid fractions, respectively, and FAMEs were prepared and analyzed from these lipids as detailed above.

4.4. Stereospecific analysis of the fatty acid composition of phosphatidylcholine from transgenic Arabidopsis and soybeans

PC was purified by TLC from the total lipid extract of soybean seeds expressing the *C. officinalis* conjugase or *Arabidopsis* seeds expressing the *M. charantia* conjugase. The TLC conditions were the same as above. PC was eluted from silica scrapings from TLC plates as previously described (Lynch and Steponkus, 1987). The purified PC

was then subjected to phospholipase A_2 digestion by using the enzyme from *Naja naja* (Sigma). The reaction and subsequent TLC analyses of the products was conducted according to the procedure reported by Wu et al. (2005). The fatty acid composition of the *sn*-1 lyso-PC product was determined by GC analysis following transesterification in 1% (w/v) sodium methoxide/MeOH. The fatty acid composition of the *sn*-2 position of PC was determined by subtraction of the lyso-PC fatty acid composition from the total PC fatty acid composition [PC fatty acid composition minus (lysoPC fatty acid composition/2)]. Of note, the fatty acids released from the *sn*-2 position were not analyzed because of the sensitivity of conjugated double bonds to acidic reagents required to methylate free fatty acids. Reactions were observed to go to near completion, and little or no palmitic acid or stearic acid was determined to be at the *sn*-2 position using these procedures.

4.5. Analysis of the fatty acid composition of the sn-2 position of triacylglycerols from transgenic Arabidopsis and soybeans

TAG was purified from total lipid extracts from soybean seeds expressing the *C. officinalis* conjugase or *Arabidopsis* seeds expressing the *M. charantia* conjugase by TLC using the conditions described above for resolution of DAG and TAG. TAG was also purified from total lipid extracts from *C. officinalis* and *M. charantia* seeds. TAG was eluted from silica TLC scrapings with Et_2O. The fatty acid composition of the *sn*-2 position of TAG was determined from *sn*-2 monoacylglycerols (MAG) generated by lipase digestion of TAG. TAG was dissolved in Et_2O (1 ml) in a 13×100 mm glass screw cap test tube, and 0.8 ml of a buffer consisting of 50 mM borate, pH 7.8, and 5 mM calcium chloride was subsequently added. Reactions were started with the addition of 200 μl of a purchased solution of *Rhizomucor miehei* lipase (Sigma, Catalog no. L4277) to the aqueous phase. The samples were mixed thoroughly and incubated at 37 °C for 1 h with shaking at 350 rpm. The reactions were stopped with the addition of 2 ml of MeOH:$CHCl_3$ (1:1, v/v). Following mixing and centrifugation at 1000*g* for 5 min, the organic layer was recovered and concentrated under N_2. MAG was resolved from the reaction products on silica 60 TLC plates using a solvent system of Et_2O:heptane (80:20 v/v). Bands that co-migrated with a MAG standard were transesterified and the fatty acid composition determined by GC as described above. To assess the validity of the *R. miehei* lipase for the analysis of conjugated fatty acid-enriched TAG, the fatty acid composition of residual TAG from digestion of *C. officinalis* and *M. charantia* TAG was found to be nearly identical to that of the TAG prior to digestion. These results suggest that the lipase did not discriminate against conjugated fatty acids. In addition, palmitic and stearic acids were not detected in the fatty acids from the recovered MAG, indicating that the enzyme is specific for the cleavage of *sn*-1 and *sn*-3 fatty acids from TAG.

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