PERGAMON

Phytochemistry 62 (2003) 39–46

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Expression of a *Streptomyces* 3-hydroxysteroid oxidase gene in oilseeds for converting phytosterols to phytostanols

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Received 22 May 2002; received in revised form 29 August 2002

Abstract

Plant sterols and their hydrogenated forms, stanols, have attracted much attention because of their benefits to human health in reducing serum and LDL cholesterol levels, with vegetable oil processing being their major source in several food products currently sold. The predominant forms of plant sterol end products are sitosterol, stigmasterol, campesterol and brassicasterol (in brassica). In this study, 3-hydroxysteroid oxidase from *Streptomyces hygroscopicus* was utilized to engineer oilseeds from rapeseed (*Brassica napus*) and soybean (*Glycine max*), respectively, to modify the relative amounts of specific sterols to stanols. Each of the major phytosterols had its C-5 double bond selectively reduced to the corresponding phytostanol without affecting other functionalities, such as the C-22 double bond of stigmasterol in soybean seed and of brassicasterol in rapeseed. Additionaly, several novel phytostanols were obtained that are not produced by chemical hydrogenation of phytosterols normally present in plants.

Keywords: *Glycine max*; Leguminosae; *Brassica napus*; Cruciferae; Transgenic; Sitosterol; Campesterol; Stigmasterol; Sitostanol; Campestanol; Stigmastanol; 3-Hydroxysteroid oxidase

1. Introduction

Phytosterols and their hydrogenated forms, the phytostanols, are beneficial in lowering serum and LDL cholesterol (**1**, Fig. 1), thus reducing the risk of cardiovascular disease (Ling and Jones, 1995). These compounds, in turn, are poorly absorbed and most likely interfere with intestinal cholesterol (**1**) absorption by decreasing its micellar solubility. Phytosterols, such as campesterol (**2**) and sitosterol (**3**) differ from cholesterol (**1**) in possessing an alkylated side chain bearing either a methyl or an ethyl group at C-24 (Fig. 1). Moreover many studies have demonstrated that phytostanols, such as sitostanol (**4**), are more effective than phytosterols in lowering cholesterol (**1**) levels in mammals (Sugano et al., 1976; Heinemann et al., 1991). While the intestinal absorption of phytostanols is lower than that of phytosterols, campesterol (**2**) is more efficiently absorbed than sitosterol (**3**). Thus, from the perspective of maximizing cholesterol (**1**) reducing activity of phytosterols, sitostanol (**4**) offers the best prospect as a component of a cholesterol (**1**) lowering dietary supplement and/or functional food.

Interest in the cholesterol (**1**) lowering properties of plant sterols and stanols has recently been galvanized by the development of food products enriched in fat-soluble plant sterol and stanol esters. Thus, incorporating plant stanols into the diet may become a common and highly effective means of reducing serum LDL cholesterol (**1**) levels. The currently available food products enriched with plant sterols and stanols use sterols derived from the deodorized distillate by product of vegetable oil refining. Vegetable oils, however, contain very low amounts of phytosterols ranging from 0.3% in soybean to 1.4% in corn (Gunstone et al., 1995), whereas phytostanols are either absent or are found at trace levels. Phytostanols are currently produced by processing deodorized distillate and hydrogenating the phytosterols; however, such chemical modifications add

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PII: S0031-9422(02)00431-4

to the cost of manufacturing the final product. Therefore, modification of phytosterols to phytostanols in planta would be a more economical means of producing vegetable oil distillates with significant nutritional value.

Plant sterols are synthesized from the mevalonate pathway similar to cholesterol (**1**) biosynthesis (Nes and Venkatramesh, 1997). However, plants make more than one form of sterol. The major plant sterol end products are sitosterol **3** (stigmasta-5-en-3β-ol), stigmasterol **5** [(24*E*)-stigmasta-5,22-dien-3β-ol] and campesterol **2** (campest-5-en-3β-ol) (Benveniste, 1986). Additionally, plants such as rapeseed contain brassicasterol **6** [(22*E*)-ergosta-5,22-dien-3β-ol] (Fig. 1). All of these sterols contain a double bond at the C-5 position, with stigmasterol **5** and brassicasterol **6** containing an additional double bond in the side-chain at C-22. Chemical hydrogenation, however, reduces all double bonds, i.e. from sitosterol **3** and stigmasterol **5** to sitostanol **4**, and campesterol **2** and brassicasterol **6** to campestanol **7**.

In cotton plants engineered for insect resistance by expressing a 3-hydroxysteroid oxidase from *Streptomyces hygroscopicus* A19249 in plastids, there was a significant increase in campestanol **7** and sitostanol **4** levels (Corbin et al., 1994). In leaves from transgenic cotton plants, up to half the phytosterols were converted to phytostanols, whereas untransformed cotton leaves did not contain phytostanols (Corbin, unpublished data). Moreover, when seeds from the same plants were analyzed for levels of 3-hydroxysteroid oxidase and sterol composition, a direct correlation between enzyme levels and phytostanol levels were observed, suggesting that the enzyme is responsible for formation of the latter (data not shown). However cotton plants were engineered with the same gene without the chloroplast target peptide (CTP), growth was severely impaired (data not shown). By including the CTP there was no such impairment on vegetative and seed development, and seed yield and seed size were not affected. In this report, *Streptomyces* A19249 3-hydroxysteroid oxidase was expressed in the plastids (leucoplasts) of *Brassica napus* and *Glycine max* seeds. This resulted in each of the major phytosterols with a C-5 double bond being reduced to its corresponding phytostanol, with hydrogenation being restricted to the C-5 double bond and not affecting the C-22 double bond of

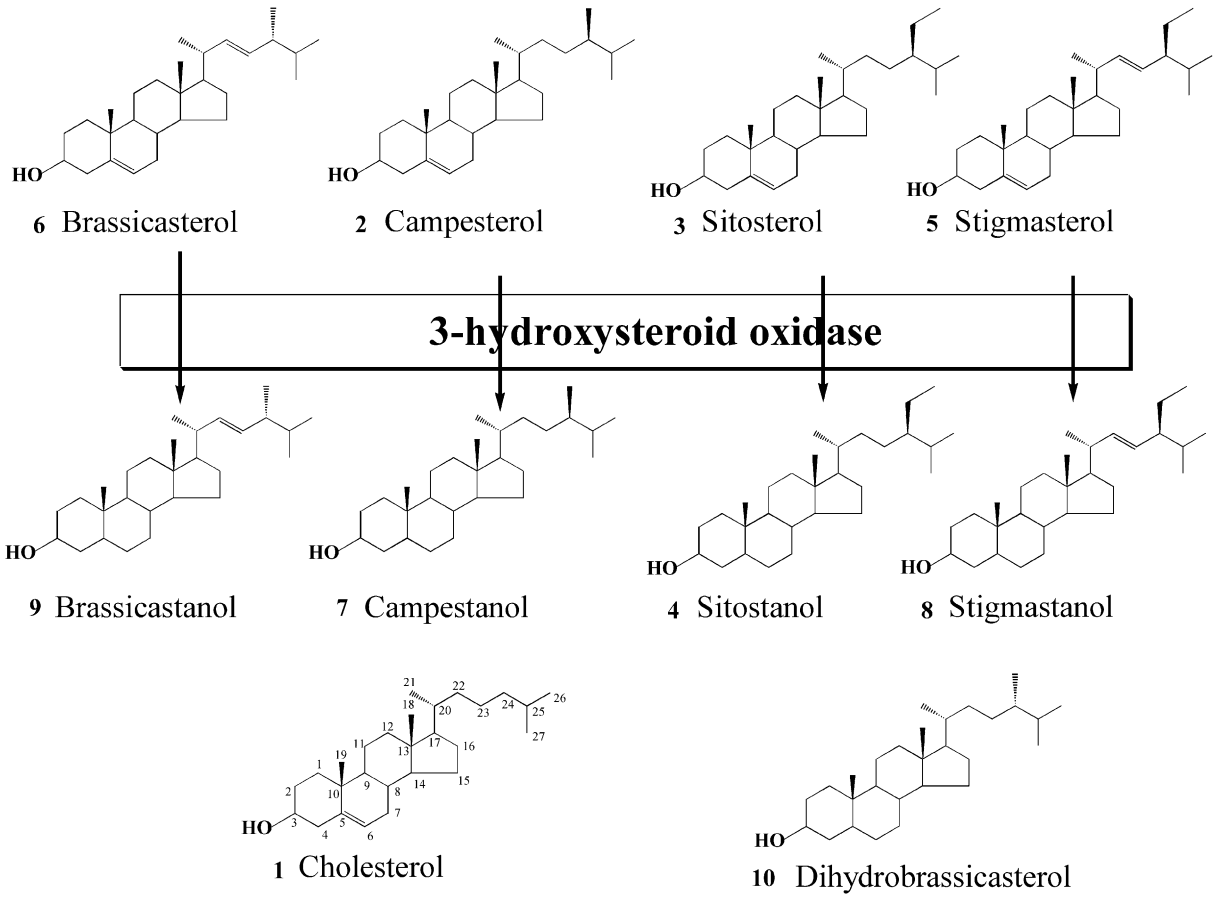

Fig. 1. Conversion of phytosterols to phytostanols by 3-hydroxysteroid oxidase and structural comparison of the phytosterols and phytostanols with structures of cholesterol and dihydrobrassicasterol. Compounds illustrated are: **1** cholesterol, **2** campesterol, **3** sitosterol, **4** sitostanol, **5** stigmasterol, **6** brassicasterol, **7** campestanol, **8** stigmastanol, **9** brassicastanol, **10** dihydrobrassicasterol.

stigmasterol **5** in soybean and brassicasterol **6** in rapeseed. This has, consequently, led to the formation of the phytostanols, stigmastanol **8** and brassicastanol **9**, normally not observed in nature.

2. Results and discussion

2.1. Conversion of sterols to stanols in Brassica napus seeds expressing 3-hydroxysteroid oxidase gene

Seeds of *B. napus* (rapeseed; Canola) usually contain three major sterols, viz. as percent of total sterols, brassicasterol **6** (~12%), campesterol **2** (~30%), and sitosterol **3** (~57%) (Kochhar, 1983). Rapeseed oil is the major source of brassicasterol **6** which is not present in other vegetable oils such as soybean, cotton or sunflower (Kochhar, 1983; Gunstone et al., 1995). The *Streptomyces* A19249 3-hydroxysteroid oxidase was expressed in *B. napus* using the embryo-specific napin promoter and targeted to the leucoplasts using the chloroplast targeting signal of the soy RUBISCO small subunit (Berry-Lowe et al., 1982) to determine the effect on seed oil sterols.

R1 seeds from 27 transgenic plants and one untransformed control plant were harvested at maturity and the sterols were analyzed. Results from the nine best performing plants (Table 1) indicated that significant amounts of the phytostanols, sitostanol **4,** campestanol **7** and brassicastanol **9,** in addition to the normally present phytosterols, sitosterol **3**, campesterol **2** and brassicasterol **6,** accumulated in seeds of transgenic *B. napus* expressing the 3-hydroxysteroid oxidase gene. Phytosterols and phytostanols, including brassicastanol **9** were separated from the sterol mixture using reversed phase HPLC and structural confirmation was done by GC–MS as described by Goad and Akihisa (1997). Phytosterols and phytostanols were then separated from one another based on their structural properties, such as the number of double bonds in the rings and side chain, and also based on the number of methyl groups on the side chain, i.e. 24-methyl from 24-ethyl. Calculated as weight percent of sterols, in the highest stanol accumulating plants, about 18–22% of sitosterol **3** was converted to sitostanol **4**, about 17–24% of campesterol **2** was converted to campestanol **7**, and about 26–43% of the brassicasterol **6** was converted to brassicastanol **9**. Thus, significant amounts of phytostanols not normally present in seed of *B. napus* were produced and accumulated in seed of the transgenic plants. The appearance of the reduced stanols was due to the reduction of the C-5 double bond in sitosterol **3**, campesterol **2** and brassicasterol **6**, presumably due to the activity of the 3-hydroxysteroid oxidase enzyme introduced into the transgenic plants.

Table 1
Phytosterol and phytostanol composition of transgenic *Brassica napus* seeds expressing a 3-hydroxysteroid oxidase gene[a]

Event number[b]	Brassicasterol **6** % of total sterol	Brassicastanol **9** % of total sterol	Brassicastanol **9** % of Brassicasterol[c] **6**	Campesterol **2** % of total sterol	Campestanol **7** % of total sterol	Campestanol **7** % of Campesterol[d] **2**	Sitosterol **3** % of total sterol	Sitostanol **4** % of total sterol	Sitostanol **4** % of Sitosterol[e] **3**
Control	10.9	–	–	34.1	–	–	50.0	–	–
1	5.6	3.3	37	25.9	6.4	20	43.5	9.6	18
2	6.1	3.5	36	25.4	7.2	22	42.1	7.9	16
3	7.6	4.4	37	26.6	7.4	22	39.3	8.8	18
4	5.5	4.1	43	25.5	8.1	24	40.9	11.8	22
5	7.4	3.5	32	23.1	5.4	19	46.4	8.8	16
6	7.3	3.1	30	25.9	6.1	19	41.8	9.3	18
7	7.0	4.4	39	24.9	6.9	22	39.4	10.5	21
8	8.7	3.1	26	26.3	5.2	17	46.2	3.8	8
9	6.7	3.6	35	26.1	7.4	22	40.5	8.7	18

[a] Typical ranges for brassicasterol, campesterol, and sitosterol in Canola seed are 115–366, 289–930, and 645–1477 μg/g, respectively. The changes in the sterol and stanol composition in % reflects the relative changes occurred in their native levels due to the expression of 3-hydroxysteroid oxidase without affecting the total sterol level.

[b] 1–9 are independent transgenic events (plants) from which 10 R1 seeds per plant were analyzed for sterol composition.

[c] Expressed as brassicastanol/(brassicasterol + brassicastanol)×100.

[d] Expressed as campestanol/(campesterol + campestanol)×100.

[e] Expressed as sitostanol/(sitosterol + sitostanol)×100.

Table 2
Phytosterol and phytostanol composition of transgenic *Glycine max* seeds expressing a 3-hydroxysteroid oxidase gene

Event number[a]	Campesterol **2** (μg/g seed)	Campestanol **7** (μg/g seed)	Campestanol **7** % of Campesterol[b] **2**	Stigmasterol **5** (μg/g seed)	Stigmastanol **8** (μg/g seed)	Stigmastanol **8** % of Stigmasterol[c] **5**	Sitosterol **3** (μg/g seed)	Sitostanol **4** (μg/g seed)	Sitostanol **4** % of Sitosterol[d] **3**
Control	79.3	0	0.0	74.0	0	0.0	236.5	17.1	6.7
1	20.1	51.7	72	31.9	36.2	53	121.0	200.5	62
2	21.1	32.1	60	26.4	25.6	49	110.0	124.6	53
3	15.0	64.8	81	29.5	55.0	65	78.2	227.8	74
4	26.8	48.4	64	28.0	31.4	53	95.2	143.7	60
5	13.2	70.1	84	18.9	54.8	74	63.1	253.2	80
6	41.3	52.5	56	40.2	28.2	41	152.9	180.4	54
7	69.9	49.1	41	58.3	35.2	38	246.7	178.7	42
8	77.5	126.6	62	79.2	88.5	53	302.1	466.8	61

[a] 1–8 are the lead independent transgenic events (plants) out of a total of 30 from which 10 R1 seeds per plant were analyzed for sterols.
[b] Expressed as campestanol/(campesterol + campestanol)×100.
[c] Expressed as stigmastanol/(stigmasterol + stigmastanol)×100.
[d] Expressed as sitostanol/(sitosterol + sitostanol)×100.

Table 3
Summary of results from screening R1 *Glycine max* transgenic plants showing transformation of plant sterols to stanols in R2 seeds

Event number[a]	Number of				Average sterol composition (μg/g seed)[b]						Stanol % of respective sterol[c]		
	Transgene insert	Plants screened	NPTII positive plants	Plants produced stanols in seeds	Campesterol **2**	Campestanol **7**	Stigmasterol **5**	Stigmastanol **8**	Sitosterol **3**	Sitostanol **4**	Campestanol[d] **7**	Stigmastanol[e] **8**	Sitostanol[f] **4**
Control	–	1	0	0	85.0	0	83.7	0	252.3	0	0	0	0
1	1	21	14	14	22.5	52.2	30.9	34.3	150.5	196.9	70	53	57
2	1	11	9	9	48.8	64.8	56.6	48.0	243.0	242.2	60	46	51
3	>1	14	12	12	27.2	60.2	39.0	49.4	160.1	224.1	68	54	57
4	1	20	15	15	38.3	40.0	47.9	31.5	188.3	142.1	52	40	44
5	>1	20	19	19	17.4	42.1	28.2	36.8	126.1	175.1	72	58	59
6	1	23	18	18	26.5	35.3	33.5	24.7	144.2	133.8	65	43	50
8	1	24	19	19	24.9	34.0	22.5	22.9	116.4	116.4	62	51	51

[a] 1, 2, 3, 4, 5, 6, and 8 are the R1 transgenic events out of a total of 8, from which the NPTII positive R1 parent, R2 seeds were analyzed for sterols.
[b] Average sterol composition of R2 seeds from NPTII positive R1 parents per event.
[c] Average % of sterol to stanol conversion of R2 seeds from NPTII positive R1 parents per event.
[d] Expressed as campestanol/(campesterol + campestanol)×100.
[e] Expressed as stigmastanol/(stigmasterol + stigmastanol)×100.
[f] Expressed as sitostanol/(sitosterol + sitostanol)×100.

Brassicastanol **9** is a novel phytostanol which has not been reported to date to occur in nature (Akihisa et al., 1992). The other phytostanols observed in these transgenic seeds, i.e. sitostanol **4** and campestanol **7**, occur to some extent in many seed oils, and such phytostanols can be made commercially through chemical hydrogenation of vegetable oil distillates. However, in the process, brassicasterol **6** is hydrogenated to 22-dihydrobrassicastanol **10**, in which both C-5 and C-22 double bonds are reduced. It is, therefore, not commercially feasible to produce brassicastanol **9** by hydrogenation of brassicasterol **6** without the use of sophisticated regiospecific hydrogenation catalysts that can be very expensive and/or not commercially feasible.

2.2. Conversion of sterols to stanols in Glycine max seeds expressing 3-hydroxysteroid oxidase gene

Seeds of *G. max* (soybeans) usually contain three major sterols, viz. as percent of total sterols, stigmasterol **5** (~17%), campesterol **2** (~18%), and sitosterol **3** (~64%) (Kochhar, 1983; Fig. 1). The *Streptomyces* A19249 3-hydroxysteroid oxidase was expressed in *G. max* using the embryo-specific 7S promoter and targeted to the leucoplasts using the chloroplast targeting signal of the soy RUBISCO small subunit (Berry-Lowe et al., 1982) to determine the effect on seed oil sterols.

R1 seeds from 30 transgenic plants and one untransformed control plant were harvested at maturity. Sterols were extracted and analyzed and results from the eight best events are shown in Table 2. Significant amounts of phytostanols, sitostanol **4**, campestanol **7** and stigmastanol **8**, in addition to the phytosterols, sitosterol **3**, campesterol **2** and stigmasterol **5** normally present, accumulated in seeds of transgenic *G. max* (Fig. 2). The phytostanols and phytosterols peaks in the GC-chromatograph of the transgenic soy seed were identified by GC–MS data collected from the samples as described by Goad and Akihisa (1997). Calculated as weight percent of sterol, in the highest phytostanol accumulating plants, about 42–80% of sitosterol **3** was converted to sitostanol **4**, about 38– 74% of stigmasterol **5** was converted to stigmastanol **8**, and about 41–84% of campesterol **2** was converted to campestanol **7**. Thus, significant amounts of phytostanols not normally present in seeds of *G. max* were produced and accumulated in seeds of transgenic plants. Based on these results it also appears that greater amounts of phytosterols were converted to phytostanols in seeds of *G. max* than seen in *B. napus*. It is unclear if this is because of apparent differences in the two promoters or some other factor.

Because of the relatively high level of phytostanols produced in *G. max*, R1 seeds from 7 events were carried forward to the R2 generation. Thirty R1 seeds from each event were planted in the greenhouse and R2 seeds were collected at maturity. Leaf samples from each plant were also collected during the early stages of growth and were used to screen for marker gene

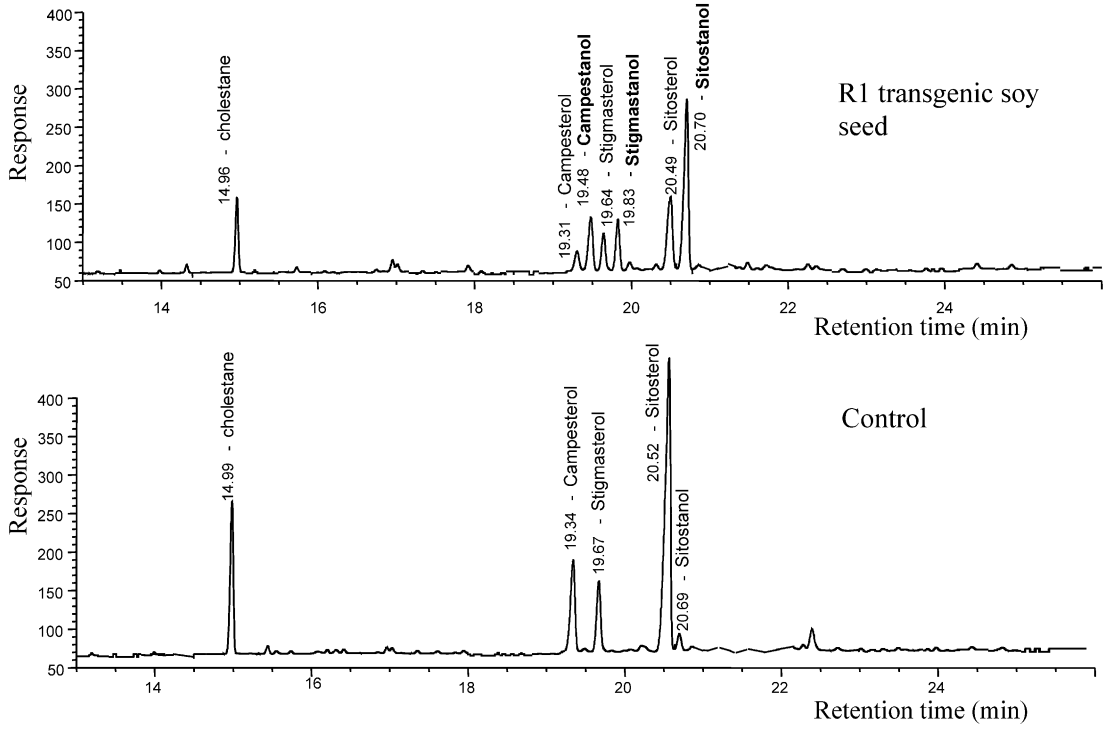

Fig. 2. GC–FID results of R1 transgenic soy seed harboring the pMON43008 and nontransgenic control. The peaks corresponding to campesterol **2**, campestanol **7**, stigmasterol **5**, stigmastanol **8**, sitosterol **3** and sitostanol **4** were confirmed by GC–MS.

expression by performing NPTII ELISA assays using commercial kits. Sterols were analyzed in the R2 seeds. A summary of the results is presented in Table 3. Based on NPTII ELISA results for each event, there were positive transgenic plants as well as negative. The ratio between the positives and negatives indicates the number of transgene inserts per event. When only one copy of the transgene is inserted there should be a 3:1 segregation ratio between positive and negative plants, respectively. Thus, of the seven events, events three and five have more than one transgene insert. The rest have single functional transgene insert. Further, in all the events there is a complete correlation between plants being NPTII positive and showing phytosterol to phytostanol conversion suggesting that it is unlikely that there are transgene insertions where the NPTII gene is functionally dissociated from the 3-hydroxysteroid oxidase gene. Calculated as weight percent of respective sterols, the transgenic events that produced large amounts of phytostanols in the R1 seeds also accumulated comparable amount of phyostanols in R2 seeds of the NPTII positive parent, indicating the stability of the transgenic trait over two generations.

The occurrence of stigmastanol **8** and brassicastanol **9** in transgenic *G. max* and *B. napus*, respectively, expressing 3-hydroxysteroid oxidase enzyme strongly indicates that this enzyme specifically reduces the C-5 double bond of phytosterols. The differences in the side-chain features of stigmasterol **5**, which is a 24-ethyl sterol, and brassicasterol **6**, which is a 25-methyl sterol, appear to have little effect on the catalytic ability of the enzyme.

This study demonstrates that it is possible to engineer plants converting high levels of phytosterols to phytostanols. It may be possible to produce these molecules at the desired levels in refined oil and deodorized distillate, and thus avoid downstream hydrogenation costs while increasing the cholesterol-lowering functional value of vegetable oils. One surprising observation in this study is that the chloroplast targeted enzyme is able to bring about changes in metabolites synthesized on the plasma membrane and endoplasmic reticulum. Whether this is due to improper targeting of the protein or degeneration of leucoplast integrity during seed development needs to be established.

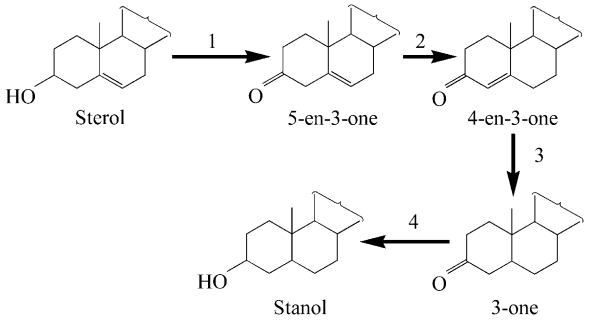

Fig. 3. Proposed scheme for sterol to stanol conversion in plants. 1 and 2 reactions are catalyzed by 3-hydroxysteroid oxidase, whereas reactions 3 and 4 are catalyzed by DET2 and 3-keto reductase, respectively.

3-Hydroxysteroid oxidase is an FAD-dependent bifunctional enzyme that catalyzes both the oxidation of cholesterol **1** to the transient intermediate 5-cholesten-3-one with the reduction of molecular oxygen to hydrogen peroxide, and the isomerization of the steroid with a trans A:B ring junction to reduce 4-cholestan-3-one (Stadtman et al., 1954) (Fig. 3). Although it is a well characterized enzyme (Horii et al., 1990; Masami et al., 1990; Gadda et al., 1997; Yamashita et al., 1998), it is as yet unclear how this is able to catalyze sterol C-5 reduction in plants. It is quite likely that the sitosterol **3** analog of this intermediate would have been the end product of metabolism, but the presence of other 4-ene-3-one reducing enzymes metabolize this and other similar intermediates to their stanol forms. Recently, an *Arabidopsis thaliana* mutant, *sax1*, blocked in brassinosteroid biosynthesis was described (Ephritikhine et al., 1999a,b). Experiments with various intermediates of the pathway from campesterol **2** to campestanol **7** showed that the *sax1* mutation alters the step catalyzing the oxidation and isomerization of 3β-hydroxyl-$\Delta^{5,6}$ precursors (viz. sterols) to 3-oxo-$\Delta^{4,5}$-steroids (Ephritikhine et al., 1999a,b). This is the reaction catalyzed by 3-hydroxysteroid oxidases as well. Therefore, it can be predicted that a different enzyme or set of enzymes is required to complete the transformation from the 4-en-3-one product of hydroxysteroid oxidase reaction to the reduced stanol. Evidence for the existence of such an enzyme or enzymes was demonstrated from the identification of the brassinosteroid mutant *det2* in *A. thaliana* (Klahre et al., 1998). Metabolic experiments with ^{2}H-labeled intermediates involved in the formation of campestanol **7** from campesterol **2** in wild type and mutant plants demonstrated that DET2 catalyzes the 5α-reduction of the 4-en-3-one intermediate (Noguchi et al., 1999). Based on this, a scheme for the transformation of phytosterols to phytostanols can be postulated (Fig. 3). The final step in this scheme, the conversion of the 3-one to the phytostanol, requires a 3-keto reductase. This enzyme has not been described to date (Noguchi et al., 1999).

3. Experimental

3.1. Brassica napus transformation

The *Streptomyces* 3-hydroxysteroid oxidase gene was excised from plasmid pMON30423 by digesting with the restriction enzymes *AatII* and *NcoI*. The released fragment ~4 Kb contained the complete 3-hydroxysteroid oxidase gene, the NOS 3′ end, the bacterial ampicillin selection marker, and pUC origin of replication.

Plasmid pMON29141 was also the source for the napin promoter and chloroplast targeting signal sequence. The plasmid pMON29141 was digested with *AatII* and *SpeI* to release a 2.2 Kb fragment containing the M13-ori site, the napin promoter, and the soy RUBISCO small subunit chloroplast transit peptide.

The 4 Kb fragment containing the 3-hydroxysteroid oxidase gene obtained from pMON30423, the 2.2 Kb napin fragment from pMON29141, and a *SpeI-NcoI* linker (Life Technologies, Inc., Gathersburg, MD, USA) were ligated at 16 °C for overnight in a triple ligation mixture containing 1X T4 DNA ligase buffer [50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin] and 1 μl of T4 DNA ligase (400 units/μl) that resulted in the generation of the plasmid pMON43007. This plasmid was partially digested with *NotI* to release the cassette containing the napin promoter, the chloroplast transit peptide, the 3-hydroxysteroid oxidase gene, and the NOS 3′ termination signal sequence. This cassette was cloned into *NotI* site of the binary vector pCGN5139 to create pMON43011, which was used to transform *Agrobacterium tumefaciens*. *B. napus* hypocotyls were cocultivated with *A. tumefaciens* cells carrying pMON43011 on MS-1, B5-1 and B5-BZ media containing 0.7% Phytagar (Radke et al., 1992). Transgenic plants were selected by resistance to kanamycin, and were grown in the greenhouse after appropriate selection and rooting was achieved.

3.2. Glycine max transformation

The plasmid pMON43007 was digested with *Bgl*II and *Bam*HI to release a 1.8 Kb fragment containing the chloroplast transit peptide and the 3-hydroxysteroid oxidase gene. This cassette was cloned into the *Bgl*II site of the binary vector pMON29920 to create pMON43008, which was used to transform *A. tumefaciens*. Explants for transformation were prepared as follows: sterilized seeds were germinated on germination medium containing 0.8% agar under light at 28 °C for 5–6 days (Hinchee et al., 1999). One day prior to explant inoculation, the seedlings were placed in the dark at 4 °C for 24 h prior to excision. Seed coats were removed and hypocotyls of each seedling trimmed to a length of 0.5–1.0 cm. The cotyledons were then split open such that the hypocotyl was split down the middle. The primary leaves and apical region of each cotyledon were removed to expose the wounding region. Wounding was performed with 3 to 7 shallow, scalpel scores in line with the embryo axis, ensuring that the apical bud was damaged. Wounded explants were incubated for 1 h at room temp in the culture of recombinant *A. tumefaciens* carrying pMON43008. Inoculated explants were then transferred to a co-culture medium containing 1/10 MS salts and 1/10 B5 vitamins, 15 g/l glucose, 3.9 g/l MES, 4.68 mg/l naphthaleneacetic acid, 2.5 mg/l kinetin, 200 μM acetosryingone, and 1 mM galacturonic acid, at pH 5.4 and placed under light at 25 °C for 2 days (Hinchee et al., 1999).

After 2 days on co-culture, explants were transferred to a kanamycin selection medium containing 1×MS salts and 1×B5 vitamins (Sigma, M0404), 3% sucrose, 4.68 mg/l naphthalene-acetic acid, 2.15 mg/l kinetin, 500 mg/l ticarcillin, 100 mg/l kanamycin, and 0.7% purified agar (Sigma, B11853), at pH 5.7 and placed in a 25%deg;C light growth room for 2 weeks. At the end of 2 weeks explants were transferred to WP (Woody Plant) medium (Hinchee et al., 1999) and placed again in a 25 °C light growth room for another 2 weeks. Explants were transferred every 2 weeks to fresh medium for approximately 18–21 weeks. At the 6 weeks transfer, the cotyledons and any dead material were removed from the explants, and the petiole was cut. After every 2 weeks of transfer the petiole was cut to expose fresh cells to the medium.

Transgenic shoots (~1.25 cm in length) with two nodes, one open trifoliate and an actively growing point were selected, cut and transferred to rooting medium containing half the major and minor salts of B_5O (Hinchee and Conner-Ward, 1998). Once a root system was established the plants were sent to the greenhouse for transplant to soil.

3.3. Sterol analysis

Ten seeds from each plant were ground individually into a fine powder. A known amount of cholestane (usually 100 μg in 100 μl hexane) was added to each sample (~50 mg powder) as an internal standard. Sterols were hydrolyzed directly from the ground tissue by saponification with 10% KOH in MeOH (2 ml) by refluxing the material at 60 °C for 30 min. The resulting samples were then cooled to room temp and filtered through glass wool. An equal volume of water was added to each filtrate, and the nonsaponifiables were extracted by partitioning three times with equal volumes of hexane. The hexane phases were pooled and evaporated, with the residues obtained being resuspended in hexane (1 ml) and back-extracted with water (1 ml). The phases were separated by centrifugation and the hexane phases were transferred to GC vials and evaporated. The residue was resuspended in hexane (100 μl) and the vials were capped.

Sterols were analyzed by GC–FID using the following conditions: Inlet temp of 220 °C, detector temp of 320 °C, and column oven temp programmed from 220 to 320 °C with initial temp for 1 min and final temp for 16 min and ramp rate of 8 °C/min. The column used was a glass capillary DB-5 column (50 m) with a film thickness of 0.25 μm. The carrier gas was helium at a flow rate of 1.0 ml/min. A representative sample was

also analyzed by GC–MS to confirm identity of sterols where possible. The GC–MS had the same parameters as the GC–FID.

Acknowledgements

We thank Dr. David Corbin for providing the *Streptomyces hygroscopicus* A19249 3-hydroxysteroid oxidase gene and providing the cotton leaf data; Joann Turner and Kendra Williams for *Brassica napus* transformation and trait development; Melanie Fossum and Stephen Schrader for *Glycine max* transformation and trait development; and Dr. Dusty Post-Beittenmiller for valuable suggestions.

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