94-090-01p

CALGENE

June 22, 1994

Dr. Sivramiah Shantharam
Biotechnology, Biologics and Environmental Protection
APHIS, USDA
6505 Belcrest Road
Federal Building
Hyattsville, MD 20782

Re: Docket P94-90-01, Petition for Determination of Nonregulated Status for Laurate Canola (Brassica napus), submission of corrected page 118

Dear Dr. Shantharam,

By this letter I would like to submit corrected page 118 to Docket P94-90-01, Petition for Determination of Nonregulated Status for Laurate Canola (Brassica napus). In the table of Mean Estimates, as originally submitted, the column "lines" had two rows inverted.

Sincerely,

Julianne Lindemann

Julianne Lindemann, Ph.D.
Regulatory Manager, Oils Division

Enclosure

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94-090-01P

PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR LAURATE CANOLA (BRASSICA NAPUS)

Calgene, Inc.

March 30, 1994

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PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR LAURATE CANOLA (BRASSICA NAPUS)

Calgene, Inc.
March 30, 1994

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, BBEP, make a determination that the article should not be regulated under 7 CFR Part 340.

signature Julianne Lindemann

Certification

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

(Signature) Julianne Lindemann

(Name of Petitioner) Calgene, Inc.
Julianne Lindemann, Regulatory Manager

Calgene, Inc.
1920 Fifth Street
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I. Overview

A. Request for determination of nonregulated status

We request that USDA determine that Laurate canola (Brassica napus) is not a regulated article based on factual information demonstrating that Laurate canola does not possess plant pest characteristics. Complete descriptions of the genetic modification process, inserted DNA sequences and the vector system are contained within this petition. The petition also contains a literature survey and experimental data to support a favorable environmental evaluation for Laurate canola.

Key evaluation criteria used in our assessment are summarized in Table 1. Results of the evaluation are unequivocal: Laurate canola does not have plant pest characteristics. Specifically, we have determined that:

1. Laurate canola is not weedier than nontransgenic canola varieties.
2. Laurate canola is not invasive.
3. Laurate canola seed is not more likely to persist in the soil seed bank than is seed from its nontransformed parent variety or other canola varieties.
4. The Laurate phenotype does not confer a selective advantage in B. napus.
5. Fatty acid composition of seed is entirely unrelated to plant pest characteristics in the plant family Brassicaceae.
6. Spread of pollen from Laurate canola to other B. napus and to weedy relatives will occur at a very low frequency. Outcrossing will not be environmentally significant because the Laurate genetic construct does not confer a selective advantage.
7. The introduced genetic construct will not increase outcrossing frequency.
8. The vast majority of pollinations of B. napus relatives by B. napus pollen do not result in the formation of viable seed. Pollen from Laurate canola is not more able than that of nontransgenic canola cultivars to produce seed on B. rapa.
9. Crop/weed hybrids are not expected to become established in the field because of poor fertility (even when backcrossed to the weedy parent) and lack of any selective advantage of the introduced genetic construct.
10. Crop/weed hybrids formed with B. rapa or B. juncea will revert to the B. napus form, and thus be nonweedy. This phenomenon will significantly reduce the potential for introgression of the laurate phenotype into weedy relatives.
11. B. rapa X Laurate canola hybrids do not have the persistence characteristics of weeds and demonstrated no other selective or competitive advantage relative to B. rapa or control hybrids.
12. The development of Laurate canola is of no different and no greater environmental significance than the development of canola from High Erucic Acid rapeseed (HEAR), and carries no additional environmental or plant pest risk.

Table 1. Summary of parameters used in evaluation: location of information on cultivated canola and Laurate canola in this document. Refer to the Table of Contents for the page number location of the sections indicated below.

Parameter	Background information on cultivated canola	Information on Laurate canola
Seed characteristics		
Laurate (lack of toxicity)	not applicable	VI.A.2
Fatty acid composition	VI.C.3.Issue 4	
Erucic acid content	VI.A.4	VI.A.4
Lack of relationship between seed fatty acids and plant pest characteristics	VI.C.3.Issue 4	
Glucosinolate content	VI.A.4	VI.A.4
Germination %	VI.C.3.Issue 3.	VI.C.3.Issue 3.
Germination from various soil depths	VI.C.3.Issue 4	VI.C.3.Issue 4
Baseline dormancy	VI.C.3.Issue 3.	VI.C.3.Issue 3.
Heat-induced dormancy	VI.C.3.Issue 3.	VI.C.3.Issue 3.
Cold-induced dormancy	VI.C.3.Issue 3.	VI.C.3.Issue 3.
Agronomic characteristics		
Seedling vigor	IV.B	IV.B, VI.C.3.Issue 2
Stand establishment	IV.B	IV.B, VI.C.3.Issue 2
Days to flowering	IV.B	IV.B
Days to maturity	IV.B	IV.B
Yield	IV.B	IV.B
Pest susceptibility	IV.C	IV.C., VI.C.3.Issue 2
Winter survival	IV.B	IV.B
Production and management	II.H	II.H
Biology of unmodified recipient plant	II	not applicable

Table 1, continued

Parameter	Background information on cultivated canola	Information on Laurate canola
Other Genetic characterization	II.D	III.E. and Appendices 2 and 3
Methods for identification of recipient plant species	II.C	not applicable
Potential weediness	II.G	VI.C.3.Issue 1
Impact on the weediness of other plants with which it can interbreed	VI.C.3.Issue 4	VI.C.3.Issue 4
Hybridization with wild relatives	II.E.3 & 4	VI.C.3.Issue 6
Hybridization with *B. rapa*	II.E.3 & 4	VI.C.3.Issue 6.
Seed dormancy in *B. rapa* × *B. napus* hybrids	VI.C.3.Issue 7	VI.C.3.Issue 7
Changes in plant metabolism	not applicable	III. Appendix 4
Effects on nontarget organisms	not applicable	VI.A
Gene transfer to organisms with which it cannot interbreed	VI.B	V.B VI.B
Invasive potential	II.G	VI.C.3.Issue 2
Overall assessment of selective advantage	VI.C.3.Issue 4	VI.C.3.Issue 4

D. History of product regulation by USDA/APHIS

Laurate canola has been field tested under permits from USDA/APHIS since May 1992 (Table 2). Sixteen (16) trials have been completed and the results are analyzed in this petition. Trials were conducted in three states (GA, CA and MI) which are representative of U.S. spring-type canola growing regions[1]. Trials were conducted for plant breeding and selection activities, for seed increase and for evaluation of environmental safety.

Table 2. Summary of field trials with Laurate canola by Calgene in the U.S. Sixteen (16) trials have been completed and the results are analyzed in this petition[1].

Location	USDA permit	Planting and Harvest Dates	Genes	Trial type
Huron, Michigan	91-346-01	5/92 - 9/92	Laurate and other	Breeding
Missaukee, Michigan	91-346-01	5/92 - 10/92	Laurate and other	Breeding
Presque Isle, Michigan	91-346-01	5/92 - 10/92	Laurate and other	Breeding
Six Sites in Georgia	92-156-01	10/92 - 5/93	Laurate and other	Breeding, Ecology
Brawley, California	92-163-01	10/92 to 4/93	Laurate and other	Seed Increase
Michigan	92-363-01	5/93 - 9/93	Laurate	Pilot production
5 Counties in Michigan	92-244-01	5/93 - 9/93	Laurate and other	Breeding, seed increase

[1] Spring-type canola is planted in the fall for spring harvest when cultivated in Georgia and California, and is spring planted in Michigan. Field trial reports for these trials were previously submitted to USDA/APHIS, and are also contained in Appendix 1.

B. Definition of Laurate canola

For the purpose of this petition, "Laurate canola" is defined as any Brassica napus cultivar or progeny of a B. napus line containing the 12:0 ACP thioesterase gene from California bay (bay TE gene) (Voelker et al., 1992) with its associated napin promoter and napin terminator regions (Kridl et al., 1991). Laurate canola may also contain the *kan*^{r} gene (Beck et al., 1982) with its associated 35S promoter and *tml* 3' terminator, the ori pRi (Jouanin et al., 1985; McBride and Summerfelt, 1990), the left T-DNA border and right T-DNA border (Barker et al., 1983), a Tn5 transposon segment (Auerswald et al., 1981; Beck et al., 1982) and a Lac Z' polylinker sequence (Yanisch-Perron et al., 1985). Laurate canola was produced using one of the Agrobacterium-derived binary vectors described in McBride and Summerfelt (1990). The bay TE gene was isolated from California bay (Umbellularia californica) and encodes the 12:0 ACP thioesterase enzyme (Davies et al., 1991; Voelker et al., 1992). Activity of the bay TE enzyme results in the accumulation of the 12 carbon, saturated fatty acid, laurate, in the canola seed. The bay TE gene is controlled by a seed specific, napin promoter from Brassica rapa.

Laurate canola has been field tested under one or more of the following USDA APHIS permits: 91-346-01, 92-156-01, 92-163-01, 92-244-01 and 92-363-01. Field testing data are available for genetic lines derived from two transformation events: 18 and 23. Progeny from these lines may include progeny from crosses between the transgenic lines and other canola cultivars.

C. Rationale for development of product

Laurate (lauric acid) is a major component of consumer products such as laundry detergent, shampoo and other personal care products. Edible uses of high laurate oils include nondairy coffee whitener, nondairy whipped toppings and uses in confectionery. The only available sources of laurate worldwide are coconut and palm kernel oils from the tropics. There is no domestic (U.S.) source of laurate, and both supplies and prices are unstable. Calgene is developing Laurate canola lines to address this market condition.

At this point in product development, it is crucial that we gain USDA's concurrence that Laurate canola is not a plant pest. This will allow Calgene the necessary latitude to continue improvement of Laurate canola through plant breeding, to freely grow and ship the seed, and to commercialize the products.

II. The Parent Plant: Rapeseed (*Brassica napus*)

A. Rapeseed as a crop

History of Domestication. (see also Table 3). The term rapeseed may refer to several oilcrop species in the genus Brassica: B. rapa (syn. campestris[2]), B. juncea , or B. napus.

Of the three species, B. rapa has the longest history of cultivation with mention in the ancient Sanskrit writing *circa* 2000 B.C. in India. Both vegetable and oilseed forms are in cultivation, and each form evolved independently over a wide range of adaptation. As a crop, B. rapa exhibits a noncentric pattern of variation, that is to say, native populations were domesticated over a wide area and at different times.

The origin of B. juncea as a crop is believed to be the Middle East; however, additional centers of diversity in China and India are known. Like B. rapa, B. juncea is noncentric in its variation. The cultivation of B. juncea for oil is common on the Indian subcontinent. In North America, the species is cultivated for edible greens and for production of condiment mustard.

The domestication of B. napus was more recent with a single center of origin in the Eastern Mediterranean. Cultivated first in Southern Europe, B. napus was introduced into China, Japan and India. Today B. napus is the most common rapeseed species grown in China, Korea and Japan. Introduction of B. napus and B. rapa to North America occurred in the twentieth century. The B. napus cultivars have better yield potential; however, different environmental adaptations keep both species in cultivation (Downey, 1983; Raymer et al., 1990).

History of oil uses. Cultivated by the Romans and Greeks for its oil, rapeseed was an important vegetable based source of cooking oil, lamp oil and of soap in Europe by the end of the middle ages. The high erucic acid content of all non-canola rapeseed (the only type available before 1968)[3] makes it especially suited to nonfood uses. Rapeseed cultivation declined in the nineteenth century, when it was replaced by petroleum oil. During the twentieth century, rapeseed oil production has expanded in Europe and North America. China produced most of the world's supply prior to 1938. Large scale production began in Canada in 1942 in support of the war effort; the oil was used for marine engine lubricants. After the war, production of summer cultivars expanded to serve other industrial uses. The first B. napus introduced into Canadian production was a mixture of seed stocks of Argentine origin. By 1954, the first cultivar of summer rapeseed developed for Western Canada was released, cv. Golden (Stefansson, 1983). In the late 1960's, improvements in processing technology, new cultivars and higher price supports in the European Economic Community combined to produce large increases in crop areas and yields in Europe. Previously, oil had been imported into European markets.

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[2] Throughout this Petition, B. rapa syn. campestris will be referred to as B. rapa.

[3] Low erucic acid cultivars were first available in 1968, and the first cultivar low in both erucic acid and alkenyl glucosinolates was introduced in 1974.

For the 1990-91 production season, the total worldwide production of edible vegetable oils was 40.8 million metric tons. Of that total, rapeseed oil was 8.8 million metric tons, second only to soybean at 15.6 million metric tons (USDA, 1990b).

Introduction of Canola. For most of its history, rapeseed oil served as a fuel, a lubricant, and a food ingredient. The meal was used primarily as fertilizer since glucosinolates limited the value of the meal as animal feed. The most significant recent achievements in rapeseed breeding and selection are the introduction of varieties low in erucic acid and alkenyl glucosinolates. Canola, by legal definition, has low levels of both of these naturally occurring toxicants[4]. Genetic sources of these important traits were the strain Liho, from Germany, and the Polish cultivar Bronowski, respectively. Characteristics of the different types of rapeseed are summarized in Table 4.

The first canola cultivar was introduced in Canada in 1974, B. napus cv. Tower (Stefansson, 1983). Candle, a canola quality B. rapa cultivar, was introduced in 1976 (Daun, 1983; Fribourg et al, 1989). By 1981, all of Canada's crushing capacity and 87% of Canadian rapeseed production (2 million hectares) was devoted to canola (Pigden, 1983). For the 1991-92 season, 3.2 million hectares were in cultivation in Canada (Mielke, 1992).

In 1985, the U.S. Food and Drug Administration (FDA) recognized that rapeseed and canola were different products and granted GRAS (generally recognized as safe) status to canola (21 CFR 184.1555). This action opened the United States to canola imports. Only 12,000 metric tons of canola oil were imported into the U.S. in 1985 compared to an estimated 200,000 metric tons in 1990. In the fall of 1990, U.S. production of canola was quite modest: 24,000 hectares. Within ten years the U.S. production may be as high as 4.5 million hectares (Raymer et al., 1990).

The first canola quality cultivar developed specifically for the southeastern U.S. was released in 1990, A112 (B. napus.) Cultivar 212/86, developed by ProDana of Denmark, and licensed to Calgene for use in the U.S. is also suited to production in the U.S. and is the parent of Laurate canola. These are spring-type canola suitable for either fall planting in the southeastern U.S. or spring planting in northern tier states such as Michigan.

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[4] The term "Canola" was trademarked in 1978 by the Canola Council of Canada. By legal definition, canola seed contains less than 2% erucic acid as a percentage of the total fatty acids in the oil, and less than 30 μmoles/g of alkenyl glucosinolates in the oil-free meal. These are discussed in Section VI.A.4.

Table 3. Selected History of Cultivated Rapeseed.

2000 BC	Civilizations in India and China use rapeseed oil to fuel lamps and for cooking.
Middle Ages	Rapeseed is introduced and grown in Europe for oil and soap.
1769	Rapeseed oil is widely used in steam engines because it clings to water and steam-washed metal surfaces better than any other lubricant.
1800's	Rapeseed oil uses are replaced by petroleum oil.
1942	*Brassica napus* varieties of rapeseed are introduced to Canada.
World War II	European and Asian sources of rapeseed oil blocked. Canadian growers plant both *B. napus* and *B. rapa* to supply oil for naval and merchant ship steam engines.
1956	Nutritional aspects of oil examined by Health and Welfare, Canada.
1958	Objection to food use removed in Canada after data shows no harmful effects.
1963	Crop demand reflected by futures market on Winnipeg Commodity Exchange.
1965	400,000 hectares of rapeseed planted in Canada. Production areas in Europe began to increase.
1970	Health and Welfare Canada calls for lower erucic acid in oil for human consumption.
1974	Tower, first double low *B. napus* variety (reduced erucic acid and glucosinolate levels) released in Canada.
1978	The term Canola is trademarked in Canada.
1979	3.4 million hectares planted in Canada.
1980	World-wide edible vegetable oils production is 31.7 million tons, of which 4.1 million tons is rapeseed (13%).
1981	Canola oil accounts for more than half the vegetable oil processed in Canada.
1982	Canola defined by law in Canada as containing less than 2% erucic acid in oil and less than 30 µmole/g alkenyl glucosinolates in defatted meal.
1984	Double low cultivars Rubin, Darmor and Tandem registered in Germany and France.
1985	FDA in the United States grants GRAS status to canola and opens the U.S. market for the vegetable oil. Canadian growers produce over half a million tons.
1990	World wide edible vegetable oils production is 40.9 million tons, of which 8.8 million tons is rapeseed (22%). First canola developed for the Southeastern U.S., A112, a *B. napus* cultivar, is released.
1991	First field test of transgenic, oil-modified canola (High Stearate A112).
1992	Expansion of field testing with transgenic oil-modified canola producing either stearate or laurate.

Table 4. Oil and meal characteristics of classes of rapeseed cultivars[1] .

Class	Oil (weight % erucic acid)	Defatted Meal (µmol/g alkenyl glucosinolates)
00 (Canola quality)	less than 2	less than 30
0 (LEAR)	less than 2	greater than 30
Industrial (HEAR)	optimally > 50	variable

[1] LEAR = Low Erucic Acid Rapeseed; HEAR = High Erucic Acid Rapeseed.

B. Geographic distribution

In 1990, rapeseed oil crops were produced on 17.8 million hectares of the world's agricultural lands and provided over 22% of the world's edible vegetable oil (Mielke, 1991; USDA, 1990b). Areas of cultivation of mustards (e.g.. B. juncea) and winter and spring forms of rapeseed are shown in Figure 1. Of the three Brassica oil seed species, B. napus and B. rapa predominate in the temperate regions. B. rapa and B. juncea are major sources of vegetable oil in the semitropical areas of Asia. China (5.3 million hectares), Canada (2.6) Western Europe (2.2), Eastern Europe (0.9) and former USSR (0.4) account for most of the temperate production for the 1990-91 season, 11.4 million hectares. India (5.3), Pakistan (0.3) and Bangladesh (0.3) account for 5.9 million hectares in the semitropical production area (Mielke, 1991).

The rapeseed crops, B. napus and B. rapa are well adapted to the temperate range. They have the ability to survive and grow at relatively low temperatures and then in warmer temperatures, to quickly flower and produce seed. Two forms exist, the biennial or winter form and the annual or spring form (Table 5). The winter form remains in the vegetative stage until the vernalization requirement is met (~40 days at near freezing temperatures). This adaptation is useful for colder climates with short growing seasons. The biennial form is established in the fall, meets vernalization requirements over the winter and once the warmer temperatures of spring arrive, quickly begins the reproductive phase. Annual forms do not require vernalization prior to flower and are planted in the spring for late summer harvest in Canada and the northern tier of the U.S. In the southern regions of the U.S. where winter temperatures are moderate, annual forms may be planted in the fall for late spring harvest.

Rapeseed as a crop can be widely adapted. Plant breeders have used the wide range of variation to introduce the crop in many regions. Today, B. napus is the predominate rapeseed species in North America, having greater yield potential. However, B. rapa matures more rapidly and cultivars have been developed for the shorter growing seasons in northern latitudes.

Figure 1.

World's Oilseed Brassica Production

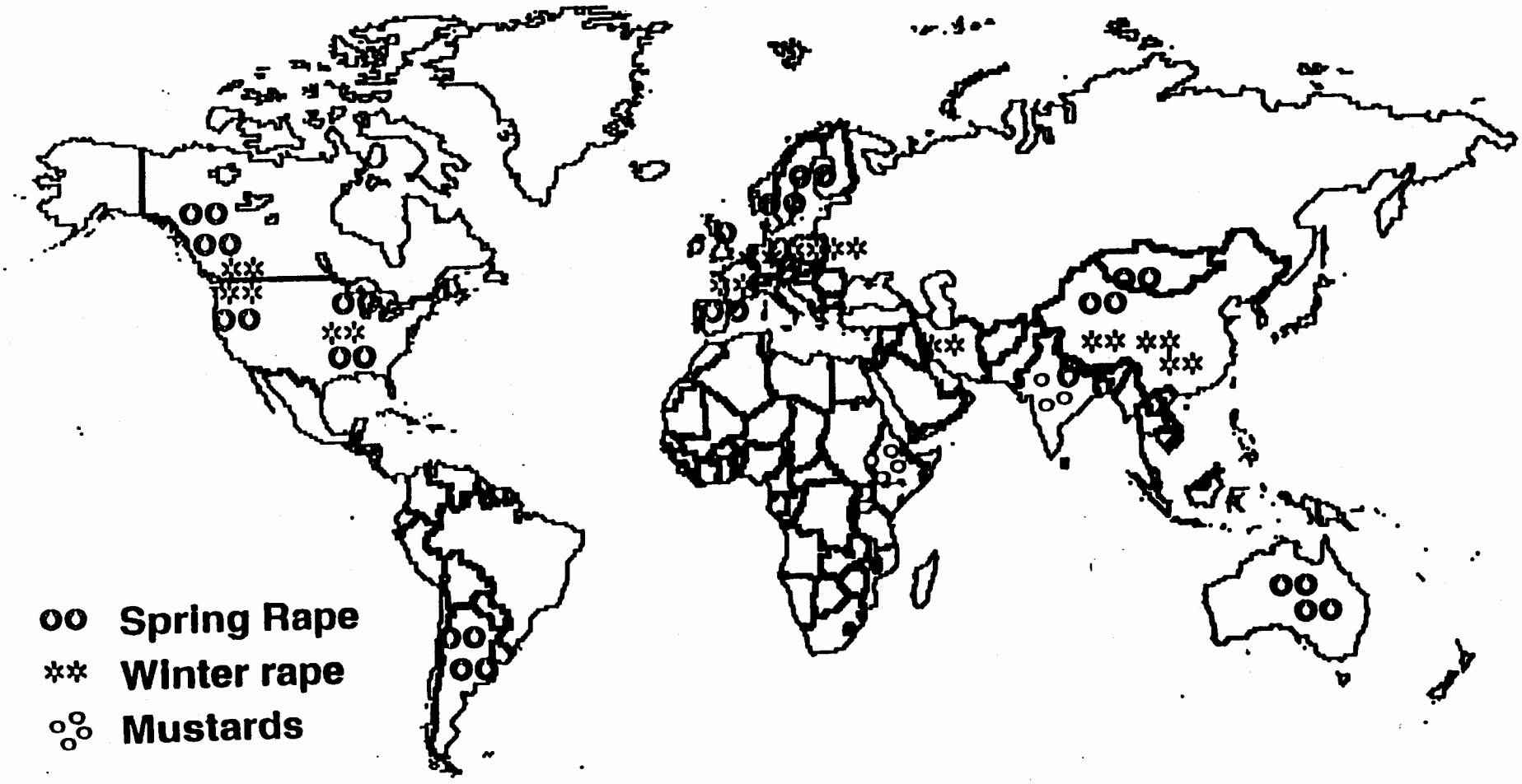

Table 5. Geographic distribution of winter and spring forms of rapeseed (Downey and Rakow, 1987).

Oilseed Species	Form	Region
Brassica napus	winter (biennial)	Northern Europe Northwest U.S. China, Chile
	spring (annual)	Canada, Southeast and Midwest U.S., Northwest China Denmark
Brassica rapa	winter	Sweden Finland
	spring	Western Canada Northwest China

C. Taxonomy and nomenclature of rapeseed

Taxonomy of Brassica napus. The genus Brassica belongs to the plant family *Brassicaceae (Cruciferae)*, the mustard family (*Hortus Third*, 1976.) The family contains approximately 375 genera and 3200 species. At least 123 species in 56 genera are found in the U.S. and Canada (Rollins, 1981; Appendix 9).

The family *Cruciferae* is characterized by flowers borne on a terminal branching inflorescence. The flowers have four petals arranged in the shape of a cross. The family is of world-wide distribution.

The genus Brassica contains 100 species. Members of the genus can be distinguished by their yellow flowers and seed pods (siliques) terminating in a beak. This genus contains many domesticated crops grown for oil, seed, food or forage, as well as some weeds. All of the 9 Brassica species in North America have been introduced : none are natives (Table 6).

Brassica napus is closely related to 5 other species, which share genomes in an interrelated manner (Robbelen et al., 1989; USDA, 1992 Appendix 6; see also Figure 3):

B. carinata A. Braun, n=17 amphidiploid[5], derived from a cross between B. nigra and B. oleracea. Center of origin believed to be northeast Africa. Cultivated forms include Abyssinian mustard.

B. juncea (L.) Czerniakowska et Cosson, n=18 amphidiploid, derived from a cross between B. nigra and B. rapa. Centers of origin throughout Old World. Cultivated forms include brown mustard.

B. napus L., n=19 amphidiploid, derived from a cross between B. oleracea and B. rapa. Center of origin in Mediterranean area. Cultivated forms include oilseed rape and rutabaga.

B. nigra (L.) Koch, n=8 diploid. Centers of origin in Europe and Asia. Cultivated forms include black mustard, however most important worldwide as a weed.

B. oleracea L., n=9 diploid. Center of origin in Mediterranean area. Cultivated forms include broccoli, cauliflower and cabbage. No naturalized forms known.

B. rapa L. (syn. B. campestris L.), n=10 diploid. Centers of origin Europe, Asia, northern India and northern Africa. Cultivated forms include rapeseed and turnip.

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5 Amphidiploid Brassicas are diploid species containing two separate genomes, and are formed by a cross between 2 diploid species, followed by chromosome doubling.

Table 6. Examples of members of the genus Brassica[1] found in North America, including the common names of cultivated and naturalized[2] or wild forms. (Fribourg et al., 1989; *Hortus Third*, 1976; Rollins, 1981).

Brassica species	cultivated form	naturalized or wild form
B. carinata	abyssinian mustard (rarely cultivated in U.S.)	no naturalized forms known
B. elongata	not cultivated	not applicable
B. hirta syn. *B. alba*	white mustard	white mustard
B. juncea	brown mustard leaf mustard mustard greens	Indian mustard
B. napus	canola, oilseed rape	wild rape
B. nigra	pungent table mustard	black mustard
B. oleracea		none known
var. *alboglabra*	Chinese kale	
var. *botrytis*	broccoli, cauliflower	
var. *capitata*	cabbage, head & savory	
var. *gemmifera*	brussels sprouts	
var. *caulorapa*	kohlrabi	
var. *italica*	Italian broccoli	
var. *tronchuda*	kale	
B. rapa	fodder turnip, turnip rape, canola, rapeseed	field mustard, wild turnip
B. tournefortii	not cultivated	wild turnip

1 Hirschfeldia incana, referred to as Brassica adpressa in Europe, is not included in this table, but is discussed later, for example, refer to Table 10.

2 Naturalized forms are those that have developed self-sustaining populations after escape from cultivation. Wild forms are forms of the species that were not subject to domestication. Species that have weedy members are listed in Table 10.

Rapeseed Species Identification.

Rapeseed grown for seed and for oil in North America is either from the genus, B. napus or B. rapa. *Hortus Third* (1976) describes B. napus as

> "**Napus L. RAPE, COLZA.** Ann., but late-sown plants overwintering and flowering the following spring, making thin taproot; lvs. glaucous, lower lvs. lyrate-pinnatifid, sparsely bristly, petioled, middle and upper lvs. oblong-lanceolate, thick, clasping and sessile; fls. pale yellow; siliques to 4 $^1/_4$ in. long, ascending, on rather slender pedicels, beak to 1 in. long."

Plants remain in a rosette form until the onset of flowering. Depending on form, species, and climate, the plant may remain in the rosette stage for as short a period as 30 days (annual or spring form) or as long as 210 days (biennial or winter form). However, once the day length and temperature trigger floral initiation, the plant bolts rapidly. Yellow flowers are borne on a terminal branching inflorescence. The two species, B. napus and B. rapa can be distinguished at flowering by the position of the opened flowers to the unopened floral buds. In B. napus the buds are above the open flowers, while in B. rapa the buds are below. Leaf attachment of the flowering stock to the stem is also a distinguishing trait. In B. rapa the leaf blade clasps the stem completely, while in B. napus the leaf partially clasps the stem (Figure 2). The siliques of B. napus are 4.5 in. long with 1 in. long beaks, while the siliques of B. rapa are 2.5 in. long or less and the awl-beak is 1/4 to 1/2 in. long. (Downey, 1983; *Hortus Third*, 1976).

A.

B.

Figure 2. Distinguishing characteristics of rapeseed leaves. (A) The blade of the upper leaves of B. rapa fully clasp the stem, (B) B. napus leaves partially clasp the stem (from Downey, 1983).

D. Genetic nature of the amphidiploid B. napus.

Amphiploids, a special case of polyploidy, are formed by mating two species with different genomes and doubling the chromosome number of the hybrid. The origin of the species Brassica napus can be traced to natural crossing between two diploid species, B. oleracea and B. rapa growing in close proximity, followed by spontaneous chromosome doubling of the hybrid. Such a doubled chromosome configuration would be stable at meiosis and thus allow the new polyploid species to reproduce. Crossing without chromosome doubling results in sterile progeny. Cytological studies of B. napus have shown that it contains both the aa and the cc genome, and is an amphidiploid derived from the monogenomic species, B. oleracea (cc genome) and B. rapa (aa genome), (Mizushima, 1980; U, 1935). Such a crossing/doubling event probably occurred only once for B. napus, since it has a discrete center of origin in the Mediterranean area. Both B. napus and B. juncea have the aa B. rapa genome in common (Fig. 3).

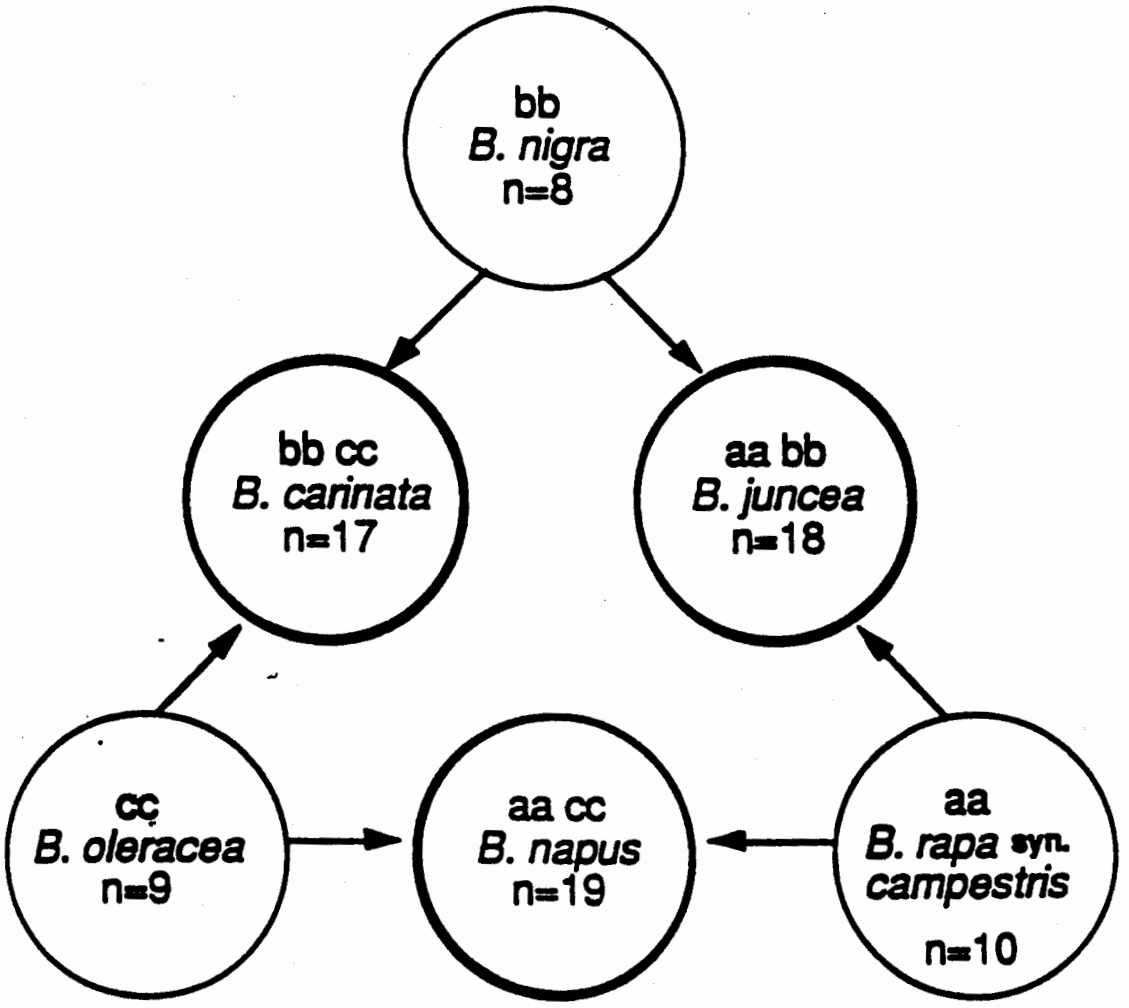

Figure 3. Genome relationships of some economically important Brassica species. After U (1935).

E. Gene transfer in Brassica napus

Overview.

We conclude from our literature survey that B. napus, B. rapa, and B. juncea are the only species with which Laurate canola is liable to cross-pollinate and form fertile hybrids under field conditions in the U.S. Hybrids formed with B. nigra are male sterile. Hybrids are not formed with Sinapis arvensis. Production of field hybrids with Diplotaxis muralis, Raphanus raphanistrum or Hirschfeldia incana (syn. B. adpressa) is extremely unlikely. Using controlled pollination under greenhouse conditions hybrids can be formed with B. carinata. Since B. carinata is neither naturalized nor widely cultivated in North America, production of field hybrids is extremely unlikely. Hybrids can be formed with difficulty with B. oleracea using manual pollination and embryo rescue, but there is little chance of field crosses since B. oleracea is not naturalized. Hybrids have been formed with other species, but in all cases intervention by man with techniques such as embryo rescue or bud emasculation were required. Since outcrossing is dependent upon the proximity of a compatible plant that is flowering simultaneously with the canola, the use of standard practices for identity preserved production of specialty oils and maintenance of germplasm integrity can significantly reduce outcrossing.

The formation of interspecific hybrids *per se* does not represent a plant pest or an environmental risk. Since Laurate canola does not possess plant pest characteristics, and displays no competitive or selective advantage relative to B. napus varieties, interspecific hybrids formed with Laurate canola pose no different and no additional risks than interspecific hybrids formed between weedy species and traditionally bred B. napus varieties. The lack of relationship between fatty acid composition in the seed and plant pest characteristics is easily demonstrated by a survey of pestiferous and nonpestiferous species within the family Brassicaceae (Section VI.C.3. Issue 4).

1. Pollination biology of B. napus

B. napus is self-compatible and thus, primarily self pollinating, although some crossing between individual plants of B. napus (outcrossing) does occur under field conditions. As the flower bud matures and the petals open, the stigma grows past the stamens loaded with pollen, and shedding pollen easily lands on the stigmatic surface. By virtue of the lack of pollen inhibition and the large pollen load on stigma surface, foreign pollen is often swamped by self-pollen. Fertilization is complete within 24 hours of pollination.

2. Outcrossing (Intraspecific crossing) in B. napus

Brassica napus canola readily crosses with other types of cultivated B. napus, including other rapeseed, fodder rape and rutabaga. The probability of gene introgression from Laurate B. napus into fodder rape or rutabaga is very low. Both are very minor crops in North America and are harvested before seed is set. Gene introgression into rapeseed can be deterred using

standard agronomic practices for production of that crop. For example, it has always been necessary to isolate B. napus canola fields from fields of high erucic acid rapeseed (HEAR) to prevent the canola from producing higher than acceptable levels of erucic acid after receiving pollen from a HEAR cultivar[6]. Since breeders and growers have been successful in maintaining canola quality oil (i.e. low erucic acid), they must have also been successful in preventing the bulk of gene transfer between these types of B. napus.

Outcrossing frequencies depend on proximity of sexually compatible plants, the availability of insect pollinators (i.e., honey, leaf-cutter, and bumble bees), weather, and the genotype of the crop. High plant populations and the physical contact of racemes in full bloom account for much of the natural cross-pollination observed within adjacent field plantings. Wind is not an effective rapeseed pollinator and has impact in pollination mainly for enhancing self-pollination. Thus, the role of wind in cross pollination is in causing direct physical contact between adjacent plants, not in the movement of free pollen. Cross-pollination at greater distances is primarily dependent on insects, as the pollen is fairly heavy and sticky and cannot travel more than a few yards without insect pollinators (Downey and Röbbelen, 1989). Successful pollination is also dependent upon pollen viability, which decreases rapidly with time.

Outcrossing frequencies measured within field plots of cultivated, nontransgenic B. napus vary, but average 20% - 30% (Downey, 1992; Huhn and Rakow, 1979; Rakow and Woods, 1987; see also review in Bing, 1991). Outcrossing to neighboring areas has been more difficult to measure, but one study reported a frequency of 5 - 15% outcrossing to an adjacent plot (Huhn and Rakow, 1979). Outcrossing to greater distances using a strain of B. napus with a recessive mutation as the pollen recipient and commercial variety of B. napus as the pollen donor has been reported in Downey and Bing (1990) and in Downey (1992). Table 7a provides a data summary of this study using nontransgenic lines. Recipient blocks were 46m square in size and placed 46, 137 and 366m away from a field planting of B. napus. The scale of this study provides outcrossing rates that can be expected from commercial plantings of B. napus.

Outcrossing rates have been measured using B. napus genetically engineered for herbicide tolerance as the pollen parent, and nontransformed plants as pollen recipients (Table 7b). Since the herbicide tolerance trait is expressed throughout the plant life cycle and is dominant, large numbers of progeny can be reliably screened at the seedling stage. Data, such as those in Table 7b confirm that outcrossing rates fall off with distance and are generally quite low, although still measurable and quite variable at distances > 50 m. Alleged outcrossing events reported by Darmency and Renard (1992) at 450 m

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[6] Oil quality is determined by the genotype of the seed embryo, not the genotype of the maternal parent. Also, genes that control erucic acid content are additive (Downey and Craig, 1964). This means that if pollen from a high erucic acid plant were to fertilize a canola flower, the F_1 seed would have elevated levels of erucic acid.

and 800 m have not, to our knowledge, been confirmed to be transgenic, but are acknowledged here for the sake of completeness.

Outcrossing rates obtained from Calgene's transgenic High Stearate canola field plots in two locations in the U.S. show a similar trend, as distance from the transgenic plots increases, pollen transfer and successful pollination drops (Table 7c). Outcrossing rate was measured using a seed germination assay to detect resistance to the antibiotic, kanamycin (Morris, Kareiva and Raymer, 1993). A description of these studies is included in section VI.C.3. Issue 5, below.

Outcrossing rates for transgenic B. napus measured in the U.S. are comparable to those measured in the UK., Canada and France (compare Tables 7b and 8). Of even greater relevance to this document, Calgene's and other transgenic B. napus do not exhibit greater rates of outcrossing than nontransgenic lines (compare values in Tables 7a, 7b, and 8).

Table 7a. Outcrossing rates measured using a strain of B. napus with a recessive mutation as the pollen recipient and a commercial variety of B. napus as the pollen donor (Downey, 1992). Both lines are nontransgenic.

Outcrossing %	Distance (M)
2.1	46
1.1	137
0.6	366

Table 7b. Outcrossing rates measured using transgenic B. napus as the pollen parent and nontransgenic B. napus as the pollen recipient.

Tolerance to	Outcrossing %	Distance (M)	Reference
Glyphosate	0.17	50	Muench, 1990
	0.125	225	
Glufosinate	0.06	4.5	Renard et al., 1990
	0.001	48.5	
Glufosinate	5.0	within plot	Scheffler et al., 1992
	1.5	1	
	0.02	12	
	0.0003	47	
Phosphinotricin (maternal parent was male sterile)	11.3	0.1	Mesquida et al., 1992
	2.7	1	
	0.8	2	
	0.7	3	
	0.1	6	
	0.1	12	
	0	24	
	0	48	
Phosphinotricin (maternal parent was male fertile)	0.54	0.1	Mesquida et al., 1992
	0.18	1	
	0.095	2	
	0.064	3	
	0.015	6	
	0.007	12	
	0.001	24	
	0.00	48	

Table 7c. Outcrossing rates measured in transgenic High Stearate canola, using nontransgenic B. napus as the pollen recipient. Full materials and methods, and results for this experiment are given in Section VI.C.3. Issue 5.

Location	Outcrossing %	Distance (M)
California	1.9	0.0
	1.0	0.3
	0.8	0.6
	0.7	3
	0.5	4.5
Georgia	3.4	0.0
	1.5	0.3
	1.2	0.6
	0.5	3
	0.6	4.5

3. Spontaneous, interspecific crossing [7]

Overview.

The Laurate genetic construct could potentially move into wild or naturalized species of Brassica by production of hybrid progeny from crosses with the Laurate canola as either the pollen or maternal parent. The most likely path for movement of the construct into naturalized species would be through pollen from the Laurate canola, followed by backcrossing to the naturalized species in areas outside of cultivated fields. If the Laurate canola were the maternal parent, the vast majority of the seed would be rendered nonviable by crushing for oil production after harvest[8]. Hybrid seed that remained in the field after harvest could germinate soon after or the following year[9], but would likely be controlled by cultivation and/or herbicide application to the new (non-canola) crop (See section II.H).

After a thorough review of the literature, we have concluded that only three species are likely to produce fertile hybrids after receiving pollen from genetically modified B. napus plants under field conditions in the U.S. and Canada: B. napus, B. rapa and B. juncea (Bing, 1991; Bing et al., 1991; Downey and Bing, 1990; Downey, 1992; Kerlan et al., 1991; Salam and Downey, 1978; USDA, 1992; Table 8). Of these, successful crosses are most likely with cultivated B. napus, and the likelihood of these crosses can be significantly reduced with proper agronomic practices, as discussed above in section II.E.2.

Crosses between B. napus and either B. carinata or B. oleracea would be possible in the field, (although very unlikely due to incompatibility - Fernandez-Serrano et al., 1991; Kerlan et al., 1992; Downey et al., 1980) except that neither species occur in the wild (are naturalized) in the U.S. Standard isolation practices prevent hybrid production. There is no significant production of B. carinata anywhere in the U.S. The vegetable Brassicas (e.g.. broccoli) are not taken to seed intentionally, except in geographically isolated seed production areas. Finally, crosses between B. napus and B. oleracea are extremely difficult when B. napus is the pollen parent, even using manual pollination and embryo rescue techniques (0.002 - 0.0067 plants produced per fertilized ovary, Kerlan et al., 1992). Thus, we conclude that there is no significant probability of these species crossing with B. napus in the field.

Recent studies using controlled and natural pollination conditions in western Canada (Saskatchewan) for rapeseed and its weedy relatives (B. nigra and Sinapis arvensis syn. B. kaber) have concluded that the natural barriers for gene flow into the weedy relatives are formidable and that gene flow would not occur (Bing et al., 1991). The PROSAMO group in the UK. has also studied rapeseed and its wild relatives with similar conclusions: hybrids are not made under a variety of field conditions and laboratory produced hybrids

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[7] Throughout this petition we will use the convention that the first parent in a genetic cross refers to the maternal parent; eg., in the cross A X B, A is the maternal parent.

[8] A small percentage of seed would also be disseminated along roadsides during transport from the field to the crushing plant. The ability of canola to compete under these conditions is addressed in section VI.C.3.Issue2. In production areas most volunteers are controlled with mowing or herbicide application.

[9] Dormancy in such F_1 seed would not be comparable to the level of dormancy in the weedy parent (see section VI.C.3. Issue 7).

are sterile in the field (Cherfas, 1991). Recent studies in France with additional wild relatives (Hirschfeldia incana syn. B. adpressa and Raphanus raphanistrum) show that field crosses can occur under very unusual circumstances but are extremely unlikely (Baranger et al., 1992; Kerlan et al., 1992; Chevre et al., 1992).

Hybrids between B. napus and H. incana, B. nigra, Sinapis arvensis or Raphanus raphanistrum are either not produced, or are sterile, or insufficiently fertile to maintain themselves by self-pollination, and/or are unable to backcross to their weedy parent or lose the B. napus genome during backcrossing. Crosses with Diplotaxis muralis have only been reported from laboratory studies (Ringdahl et al., 1987; Salisbury, 1988). Field crosses with D. muralis are extremely unlikely since it is not a common agricultural weed (based on description of distribution in Rollins, 1980; also the species is not listed in the Weed Control Manual, 1992). Further, D. muralis is highly self-compatible and most fertilization is complete before the flower opens: in laboratory studies, 95% of fertilization occurred before bud emasculation (Ringdahl et al., 1987), which is normally done 24-48 hours before the flower would open.

Thus, we view the potential to hybridize with H. incana, B. nigra, S. arvensis, R. raphanistrum or D. muralis as not posing any concern.

Crosses with B. juncea

B. napus is capable of acting as the pollen donor in crosses with B. juncea, cultivated as Indian or brown mustard although fertility of the hybrids is $\leq$ 10% (Bing, 1991; Dhillon et al., 1985; Heyn, 1977; Roy, 1980; Table 8). Most pollinations yield few if any seed (0-7), although genotype combinations have been identified that give 100% seed when manually pollinated (Heyn, 1977; Roy, 1980). Viability of pollen from hybrid plants is less than 10% (Bing, 1991).

Under field conditions in western Canada with B. napus and B. juncea interplanted, an average of 4 hybrid seed per plant (4.7% of seeds tested) were produced on the maternal B. juncea plants. Many of these F_1 plants were completely infertile and produced no seed, 50% produced only 5 seed, 10% produced up to 25 seed and the remainder produced intermediate amounts of seed (6 to 15 seed per plant) under open pollinating conditions in a greenhouse (Bing, 1991). Using herbicide tolerant B. napus as the pollen parent, 0.3% and 0.1% of seed were hybrid in two years of field trials. Fertility of the hybrids was very low, but actual values were not given (Bing, 1991).

Hybrids between B. napus and B. juncea have been generated in field experiments (described in paragraph immediately above), but we did not find any published reports of natural field hybrids being formed. The distribution of naturalized B. juncea is sparse (although widespread) throughout temperate North America.

Table 8. Summary of results of experimental crosses under field conditions between various Brassica species and wild relatives[1] (female parent) and B. napus (pollen parent). Supporting data and references are in the text.

Female Parent	Field Hybrids Produced? (Yes or No)	Fertility of Hybrids
B. napus	Yes	normal
B. rapa	Yes	≤10%
B. juncea	Yes	≤10%
B. nigra	Yes	male sterile
B. oleracea (Species is not naturalized)	No	n/a
B. carinata (Species is not naturalized)	No	n/a
Hirschfeldia incana (syn. B. adpressa)	Not determined[2]	<<10%
Raphanus raphanistrum	Experiment in progress[3]	< 1 seed/plant
Sinapis arvensis	No	n/a

[1] Weed status of Brassica species and wild relatives is given in Table 10.

[2] Dale (1992) notwithstanding, Kerlan et al., (1991) did not demonstrate field hybrids formed with B. napus as the pollen parent, and further stated that no seed were produced without embryo rescue. Field hybrids of low fertility have been produced on male sterile B. napus (Lefol et al., 1991; Darmency and Renard, 1992; Baranger et al., 1992; Chevre et al., 1992). It has not been determined whether hybrids would ever be produced under conditions typical of rapeseed production, although it seems highly unlikely. Laboratory produced hybrids are unable to backcross into H. incana (Baranger et al., 1992). Hirschfeldia incana occurs in Oregon, Southern Nevada, central and southern California.

[3] R. raphanistrum is able to pollinate male sterile B. napus containing a Raphanus cytoplasm and experiments are under way to determine if Basta tolerance can be transfered from B. napus to R. raphanistrum via pollen (Baranger et al., 1992). Pass of backcross progeny is described in the text.

Crosses with B. rapa

B. napus and cultivated B. rapa can be inter-fertile and spontaneous crosses have been observed where the two species have been grown in adjacent cultivation (Downey, 1992). In experimental field trials, these crosses are most successful with B. napus as the pollen recipient, which gives B. napus canola growers a strong incentive to ensure isolation, especially for a niche market or specialty crop. Many B. rapa crops, such as turnips, fodder rape and chinese cabbage, are harvested before seed is set in production fields, so that the likelihood of gene introgression from Laurate B. napus into any of these crops is negligible, and crossing into B. rapa rapeseed is the only concern. With B. napus as the pollen parent in B. rapa crosses, an average of 9 viable seed were produced per flower pollinated under greenhouse conditions (Bing, 1991). None of the F_1 hybrids had > 10% fertility, and they produced an average of 2 seed per plant. Under field conditions with B. napus and B. rapa interplanted, 0.7% - 1.3% of seeds produced on the maternal B. rapa plants were hybrid. These F_1 plants produced only "a small amount of viable seed" when grown under open pollination conditions (Bing, 1991).

Rapeseed seed production is commonly carried out using isolation conditions (e.g.. AOSCA standards). Thus, with proper agronomic practices (especially crop rotation and field separation), introgression of genetic material into cultivated B. rapa from the Laurate B. napus should be rare.

Since wild or naturalized B. rapa is common and widespread throughout temperate North America, and occurs in cultivated and disturbed areas, it is much more likely than either B. juncea or cultivated B. rapa to be in close enough physical proximity to receive pollen from cultivated B. napus. Natural field hybrids between B. napus and B. rapa are often identified in western Canada (Downey, 1992).

Crosses with B. nigra (black mustard)

Under field conditions, hybrids were either not produced at all (Baranger et al., 1992) or were produced in very low numbers and were male sterile (Bing, 1991).

Crosses with Sinapis arvensis (wild mustard, charlock)

Under field conditions, hybrids were not produced (Baranger et al., 1992; Bing, 1991).

Crosses with Raphanus raphanistrum (wild radish)

We conclude from the studies described immediately below that under normal conditions of B. napus canola cultivation few or no hybrids would ever be produced between B. napus and R. raphanistrum, and that such hybrids would neither persist nor stably transfer the B. napus genetic material into wild Raphanus populations.

Field studies. No studies to date have demonstrated that hybrids are formed under field conditions when B. napus is the pollen donor. R. raphanistrum (R.r.) was able to produce hybrid seed on male sterile B. napus containing a Raphanus cytoplasm[10] under field conditions when the two types of plants were adjacent and planted in equal numbers (Baranger et al., 1992)[11]. These hybrids were barely able to self-pollinate, and produced fewer than 1 seed per plant[12]. When 15 of the B. napus X R.r. hybrids were backcrossed to R.r. in the field, a total of four seed were produced or an average of 0.26 seed per hybrid (Lefol and Darmency, 1993). BC_1 X R.r. had much increased fertility, 1162 seed per plant, (Lefol and Darmency, 1993) indicating that the R.r. genome was probably reconstituted with loss of the B. napus genome (support for this interpretation is from the laboratory studies described immediately below).

Laboratory studies. Laboratory hybrids (manual pollination and embryo culture) were produced in reciprocal crosses between Basta tolerant B. napus and wild R.r. When these F_1 hybrids were backcrossed to R.r., progeny were only produced on plants containing the B. napus cytoplasm, and at a very low frequency (0.004 hybrids per manual pollination). F_1 hybrids produced using B. napus pollen were unable to backcross to R.r. When the BC_1 progeny were pollinated by R.r., numerous progeny were produced, but all had lost both the B. napus phenotypic traits and Basta tolerance, indicating that most or all of the B. napus genome had been lost.

Crosses with Hirschfeldia incana (syn. B. adpressa)[13]

Manual crosses made with Hirschfeldia incana as either parent yield few progeny (0.01 - 0.03 hybrids per cultured, fertilized ovary). The F_1 hybrids are exclusively male sterile (Baranger et al., 1992; Kerlan et al., 1992). The F_1 hybrids are either female sterile (Kerlan et al., 1992) or nearly so (produced 0.003 seed per pollination, Baranger et al., 1992). Laboratory hybrids produced using B. napus as the pollen parent (i.e. containing H. incana cytoplasm) were unable to backcross to H. incana (Baranger et al., 1992).

Under field conditions, very low numbers of hybrids (ca. 5 seed per plant) have been produced on male sterile B. napus interplanted with equal numbers of H. incana (Lefol et al., 1991; Darmency and Renard, 1992; Baranger et al., 1992; Chevre et al., 1992). Field hybrids produced on male sterile B. napus are predominantly sterile when backcrossed to H. incana: they produced 0.003 viable seed per pollination (Baranger et al., 1992). Since the hybrids are male sterile, they would not be able to maintain themselves by selfing. The probability of such hybrids being produced is vanishingly small

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10 Laurate canola does not contain a Raphanus cytoplasm.

11 The use of male sterile B. napus in outcrossing experiments overestimates the frequency of hybrids by a factor of 10 - 20 (Mesquida et al., 1992).

12 If a given genotype produces less than 1 seed per plant, that genotype will become extinct.

13 In the european literature, Hirschfeldia incana is referred to as Brassica adpressa.

when B. napus pollen is present and weed infestation is low to moderate. H. incana does not occur east of Nevada (Rollins, 1981; Warwick, 1993).

Fate of hybrid progeny.

F_1 hybrids formed between either B. juncea or B. rapa and B. napus have an intermediate number of chromosomes. In subsequent generations, the most likely scenario is for the hybrids to revert to a chromosome number close to one of the parents. Fertility of individual hybrid plants is a function of chromosome number. Salam and Downey (1978) found that a disproportionate number of F_2 hybrids formed between B. rapa and B. napus had chromosome numbers close to B. napus and that these plants were more fertile than F_2 plants with lower chromosome numbers (more like B. rapa), Figure 4. Further, F_1 plants backcrossed to B. rapa were still highly infertile and exhibited high (25-33 %) seedling mortality. Under field conditions, in the absence of an introduced selective advantage and proper selection pressure, the hybrids most likely to persist are those with the greatest seed output, and these will be the ones with genotypes most like B. napus. The phenomenon of hybrids reverting to cultivated forms was also observed by Bing (1991).

With B. napus as the pollen parent, F_1 hybrid plants exhibit < 10% fertility in B. rapa crosses (Bing, 1991), and both F_2 plants and backcrossed F_1 plants exhibited both low fertility (Figure 4) and 10-33% seedling mortality (Salam and Downey, 1978). Thus, the persistence of progeny from such a cross may still be considered rather unlikely unless the progeny can backcross into B. napus. Backcross progeny would quickly reconstitute a B. napus form and thus not be weedy like B. rapa.

The compatibility of B. juncea / B. napus varies from 0 to 100%, depending upon the individual genotypes (Roy, 1980; Sacristan and Gerdemann, 1986; Bing, 1991). However, regardless of the ease with which hybrids are formed initially, most F_1 progeny from B. juncea /B. napus crosses are either sterile or only slightly fertile (producing 2 - 10 seed per plant) when self- or open-pollinated (Dhillon et al., 1985; Roy, 1980). F_1 plants also display abnormal root morphology (Dhillon et al., 1985; Sacristan and Gerdemann, 1986; Roy, 1978). F_2 plants retain a high degree of infertility (producing only a "few seeds" per plant) or are sterile. F_1s backcrossed to B. napus were still largely (89%) infertile or partially fertile, producing 0 - 4 seed per pollination, although a few were highly fertile (the reciprocal backcross was not performed), (Roy, 1980). Hybrids formed with B. juncea that possessed moderate fertility (producing 1 - 8 grams of seed per plant) were reported to preferentially revert to the B. napus form by the F_2 generation (Roy, 1980).

Under natural (non-experimental) field conditions, hybrids between B. napus and B. juncea have not been reported. These should be much rarer than B. napus/B. rapa hybrids, not only because B. juncea is so much less prevalent, but also because self-pollination in B. juncea occurs as the flower opens or very shortly thereafter, whereas B. rapa is self-incompatible.

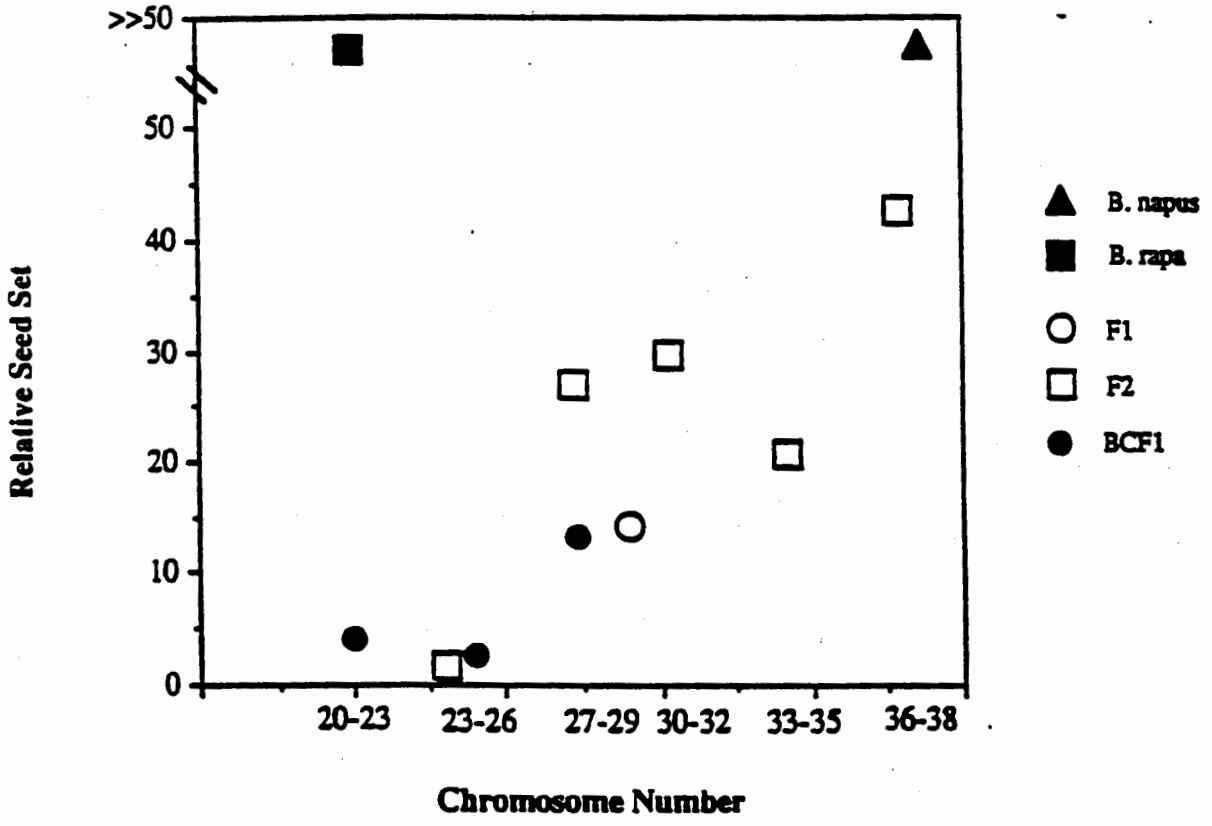

Figure 4. Relative number of seed produced in parental lines, F1, F2, and backcross hybrids as a function of chromosome number (after Salam and Downey, 1978).

As discussed above, hybrids between B. napus and Hirschfeldia incana, B. nigra, Sinapis arvensis or Raphanus raphanistrum are either not produced, or are sterile, or insufficiently fertile to maintain themselves by self-pollination, and/or are unable to backcross to their weedy parent or lose the B. napus genome during backcrossing. Thus, we view the potential to hybridize with these weedy species as not posing any concern and we do not discuss them further.

Persistence of the transgene in naturalized hybrids or wild relatives.

Persistence of naturalized hybrids, especially in a perennial community, will be dependent upon sufficient seed output, ability to compete, and ability to form seed banks in the soil. We have already discussed above the relative infertility of F_1 and F_2 hybrids formed with B. rapa and their greater seedling mortality. Introgression of any gene that does not confer a selective advantage into persistent populations of B. rapa through backcrossing is also unlikely, given the infertility of such backcross progeny (Salam and Downey, 1978). Formation of seed banks from such hybrids will also be unlikely, due to low seed output of hybrids and backcross progeny. Hybrids exhibit significantly less seed dormancy than naturalized forms of B. rapa (a range of 0 - 13 % versus a range of 50 - 93 % for the hybrids and B. rapa parent, respectively, $P < 0.01$) (Linder and Schmitt, 1993a). The combination of lower fertility and lower seed dormancy reduce the potential to form persistent seed banks and thus, make it unlikely that hybrid forms would persist in the environment.

The situation with B. juncea hybrids is somewhat different. Hybrids are quite infertile as discussed above. In addition, since naturalized B. juncea does not exhibit levels of seed dormancy normally associated with weeds, hybrids would not be likely to form large seed banks even if they were highly fertile.

The tendency for hybrids to revert to a B. napus form upon selfing further lessens the probability that persistent populations of weedy genotypes containing the Laurate genetic construct will be produced.

4. Artificial crossing

B. napus can be artificially crossed with most other Brassica species (Prakash and Hinata, 1980), especially with the aid of embryo rescue techniques (Table 9a). Spontaneous crossing under field conditions with distantly related species is extremely unlikely due to differences in blooming time, inhibition of pollen tube growth (Röbbelen, 1960), and disturbed endosperm development (Eenink, 1975). Refer to Table 9b for information on the relative frequency of manual crosses between closely related Brassica species.

Table 9a. Summary of successful manual hybridizations performed under laboratory conditions between Brassica napus and related members of the Brassiceae tribe. Weed status for most species is given in Table 10.

Name & chromosome number	Method of hybridization	Parent	F_1 progeny produced?	F_1 fertility
Brassica carinata Abyssinian mustard 2n=34	manual cross	Male or female	0.0002 hybrids per pollination	reduced fertility, but able to be backcrossed to *B. carinata*
Brassica juncea Indian mustard 2n=36	manual cross	Male	0 - 7 hybrids per pollination	Pollen fertility <10%. One author reported 19.7 seed per 100 pollinations of the backcross F_1 x B. napus
	manual cross	Female	0.05 hybrids per pollination	reduced fertility, but able to obtain some F_2 progeny
Brassica nigra Black mustard 2n=16	manual cross and ovary culture	Male only	0.029 hybrids per pollination	Pollen fertility 0 - 2.4 %
Brassica oleracea cabbage 2n=18	manual cross and ovule culture	Male & Female	0.004 - 0.128 hybrids per pollination	7 - 59 % male fertility in ACC genome. 96.4 % fertility in AACCCC genome.
Brassica rapa[1] turnip 2n=20	manual cross	Male & Female	0.5 - 9 hybrids per pollination	Pollen fertility <10%, but relatively easy to obtain F_2 progeny with manual crosses

[1] Hybridization is more successful when *B. rapa* is the pollen parent. Many of the seeds produced are nonviable when *B. rapa* is the female parent.

Table 9a. continued.

Name & chromosome number	Method of hybridization	Parent	F_1 progeny produced?	F_1 fertility
Hirschfeldia incana (syn. B. adpressa) hoary mustard	ovary culture	Male & Female	0.01 - 0.025 hybrids per pollination	Male sterile. A few with partial female fertility
Raphanus sativus radish 2n=18	ovule culture	Male only	yes, but only 1 plant	unknown
Raphanus raphanistrum wild radish 2n=18	ovule culture	Male & Female	0.007 - 0.01 hybrids per pollination	0 - 7.1 % male fertile. Able to backcross to wild parent but loses B. napus genome
Sinapis arvensis wild mustard, charlock	ovule culture	Male only	0.022 hybrids per pollination	0 - 40 % male fertility, F_2 obtained by open pollination
Diplotaxis muralis sand rocket	bud emasculation manual cross	female	0.1 hybrids per pollination[2]	able to backcross to *B. napus*
Diplotaxis erucoides [3]	bud emasculation manual cross	female	0.005 hybrids per pollination[2]	able to backcross to *B. napus*

Manual hybridizations that were attempted with no success:
Brassica cossoneana
Sinapis alba
Sinapis pubescens
15 *Diplotaxis* species

2 Both D. muralis and D. erucoides are highly self-compatible and self-fertilization occurs before the flower opens (Ringdahl et al., 1987).

3 D. erucoides does not occur in the U.S. (Holm et al., 1991; Warwick, 1993).

Table 9b. Relative crossability among Brassica species using manual pollination techniques (after Downey et al., 1980).

Average number of hybrid seed produced per flower pollinated

Pollen Parent	Maternal Parent				
	B. napus	B. rapa	B. juncea	B. carinata	B. oleracea
B. napus	25	0.5	0.5	0	0.02
B. rapa	>2	25	1	0.02	0.002
B. juncea	0.02	0.002	25	0	0
B. carinata	0.0002	0.02	0.02	25	no data
B. oleracea	0.02	0.02	0.002	no data	25

F. Weed characteristics

The term "weed" has been variously defined, depending on the different perspectives of ecologists, agronomists and the public. Weeds may occur in disturbed areas (cultivated lands, roadsides) or natural areas. Their importance is often classed as 1) pestiferous to man or 2) displacing native species in habitats where alien and native species are in competition (Rollins, 1981), thus defining agricultural and ecological weeds, respectively. A domesticated species that establishes a naturalized form is not necessarily a weed. Weedy traits are most often manifested as displacement of or competition with a crop or a native species.

In his classic work, Baker (1965 *as cited in* Keeler 1985) identified characteristics typically found in "successful" weed species:

- Germination requirements fulfilled in many environments.
- Discontinuous germination (internally controlled)[14] and great longevity of seed.
- Rapid growth through vegetative phase to flowering.
- Continuous seed production for as long as growing conditions permit.
- Self-compatible, but not completely autogamous or apomictic.
- When cross-pollinated, unspecialized visitors or wind is utilized.
- Very high seed output in favorable environmental conditions.
- Produces some seed in a wide range of environmental conditions; tolerant and plastic.
- Has adaptations for short- and long-distance dispersal.
- If a perennial, has vigorous vegetative reproduction or regeneration from fragments.
- If a perennial, has brittleness, so not easily drawn from ground.
- Has ability to compete interspecifically by special means (rosette, choking growth, allelochemics).

Often such characteristics, if they were originally present, have been intentionally altered during the development of crop varieties from their wild ancestors. For example, most crops do not exhibit much seed dormancy. On the other hand, characteristics such as high seed output may be desirable in crops as well as weeds. We can find nothing in Baker's list to suggest that modifying seed oil composition would increase weediness, and expert opinions support this view (USDA, 1990; Boyce Thompson, 1987; Dale, 1993.)

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[14] Seed dormancy and the ability to respond to environmental cues to either induce dormancy or trigger seed germination.

G. Weediness Potential in Rapeseed

1. Brassica napus is not a weed in North America.

B. napus is not a weedy pest in North America (Table 10). Brassica napus is not listed as a weed by the Weed Science Society of America (1989) or in the 1992 Weed Control Manual. B. napus is the only naturalized Brassica that is not noted as a pestiferous weed by Rollins (1981). B. napus is not listed in *Weeds of the United States* (Lorenzi and Jeffrey, 1987). No Brassica is noxious (Federal Noxious Weed Regulation, 7 CFR 360). B. napus is not listed as a serious, principal, or common weed in the U.S., Canada, Mexico, or any European or Asian country with a comparable latitude, i.e. between 25 and 49° north latitude (Holm et al., 1991). Listed as a common weed only in Finland (60° north latitude) and Kenya (5° north to 5° south latitude), B. napus is not a serious or principal weed anywhere in the world (Holm et al., 1991).

All of the Brassica species currently present in North America have been introduced. Those species that are weedy either escaped from cultivation or were introduced into fields as seed contaminants (Table 10). B. napus has a slower relative growth rate than either B. juncea or B. rapa (Tsunoda, 1980), which may reduce its competitive ability and thus its weediness potential, in spite of high seed production. Half of the B. napus genome is derived from B. oleracea, an extremely slow growing species that has never naturalized. B. juncea, on the other hand, is derived from B. nigra and B. rapa, both of which are weedy.

Tsunoda (1980) reports that without favorable, intensive cultivation, domesticated forms of B. napus cannot compete with common crop plants and that its naturalized forms are quite distinct from domesticated forms. In the U.S. and Canada, naturalized forms of B. napus are sporadically distributed. In the UK., naturalized forms of B. napus are more widespread (Mitchell-Olds, 1992). Repeated introduction of B. napus may have been important in establishing records of its occurrence outside of cultivation, rather than one or a few escape events forming the basis of self-perpetuating naturalized populations (van der Meijden and de Vries, 1992).

Table 10. Members of the genus *Brassica* and some wild relatives found in the continental United States (B. oleracea and B. carinata are not naturalized). Status as a weed in the U.S. is denoted by S (serious), P (principal), C (common), or X (present, weed status unknown) after Holm et al., (1991). Species denoted by R are considered pestiferous weeds by Rollins (1981). Those denoted by LJ are considered weeds by Lorenzi and Jeffrey (1987). Those considered weeds by the Weed Science Society of America (1989) are indicated by WS.

species	weed status	common name of naturalized form	distribution of naturalized form
Brassica elongata	R	none	roadside weed of eastern Nevada
B. juncea	X, R, WS	Chinese Mustard Indian Mustard	sparse, but widespread throughout temperate North America, occurs in cultivated and disturbed areas
B. napus	none	rape	sporadic in temperate North America, waste places
B. nigra	C, R, LJ, WS	black mustard	widespread in temperate North America, especially common in the Central Valley of California, sporadic in the more northerly areas of the continent
B. rapa syn. campestris	X, R, LJ, WS	field mustard bird's rape	common and widespread throughout temperate North America, occurs in cultivated and disturbed areas
B. tournefortii	R	wild turnip	roadsides and old fields of the Southwest
Diplotaxis muralis	X, R, WS	sand rocket	widely scattered, waste places, roadsides, abandoned land, heavily grazed grassland, beaches
Hirschfeldia incana syn. B. adpressa	X, R, WS	shortpod mustard Mediterranean mustard	roadsides, ditch banks and waste areas of California and Nevada
Raphanus raphanistrum	C, R, LJ, WS	wild radish, jointed charlock	widely distributed, especially in the eastern North America and in the Central Valley of California
Sinapis alba syn. B. hirta	X, R, WS	white mustard	widespread but sporadic in North America, abundant in some localities
Sinapis arvensis syn. B. kaber	P, R, LJ, WS	wild mustard charlock	abundant throughout the temperate agricultural areas of North America, especially in newly disturbed areas

2. Persistence of cultivated B. napus

In agricultural systems, B. napus seed may remain in the soil following harvest due to seed that shatter prior to harvest and inefficiencies in the harvesting operations. In areas with a rapeseed growing history (such as Canada) volunteer rapeseed is an issue for farmers only when crop rotation practices are not followed, and a new rapeseed variety is introduced to a field that produced rapeseed the previous year.

B. napus is easily controlled in other crops since it is sensitive to substituted ureas, norflurazon, triazines, imidazolinones, most sulfonylureas, and other classes of herbicides. Volunteer rapeseed can be controlled using agronomic practices such as 1) rotation of fields out of rapeseed and concomitant use of selective herbicides and/or 2) field cultivation after germination of volunteer seed. A more detailed discussion is provided in section II.H. Production and Management of B. napus canola.

Species may be characterized as having either transient or persistent seed banks. Cultivated B. napus forms a transient seed bank after pod shattering and/or harvest loss of seed. Species that form persistent seed banks must possess seed dormancy and the ability to respond to environmental cues to trigger seed germination under appropriate conditions.

3. Seed Dormancy

One of the reasons that cultivated forms of B. napus are not weedy is because they lack the dormancy characteristics of true weeds and do not form persistent seed banks. For example, weedy forms of B. rapa commonly exhibit 60 - 90 % baseline dormancy at conducive temperatures whereas cultivated forms of B. rapa and B. napus exhibit <10% baseline dormancy (see Section VI.C. Issue 3, below). Seed of another weedy Brassica napus relative, Sinapis arvensis, retained 60% dormancy after 1 year burial in soil (Crawley et al., 1993). Seed dormancy in weedy forms also typically increases in response to environmental conditions such as shade, whereas cultivated types are relatively nonresponsive to such conditions and have $\leq$10% induced or nonenforced dormancy (Adler et al., 1993). Finally, successful weeds possess sophisticated means to detect when the environment is suitable for growth so that dormancy should be broken (otherwise seeds would germinate at inappropriate times and not survive long enough to set seed). For example, dormancy in weed seed is typically broken by exposure to freezing temperatures (stratification).

In naturalized settings, seed rain marks the end of a plant's life cycle and the renewal of the seed bank (Fig. 5). There are two types of seed dormancy expressed in the soil seed bank. The enforced dormancy type is imposed by unfavorable conditions (for example, freezing temperatures or drought) and requires a return to conducive conditions for germination to occur (release of dormancy). The second type, innate or induced dormancy, requires a cue to break and is often termed "true" dormancy. For the purpose of assessing whether Laurate canola poses plant pest characteristics, both baseline dormancy and environmentally-induced dormancy were evaluated. As illustrated in Figure 5, the two dormancy types are subsets describing the

state of viable seed in the seed bank. Dormant seed may exit the seed bank by either germination, death or predation. Seed expressing induced-dormancy may either enter the enforced-dormant seed bank after responding to a cue or may germinate directly once dormancy is broken. If induced-dormant seed never receive a cue to germinate, or cannot properly interpret the cue, the seed are likely to exit the seed bank by death.

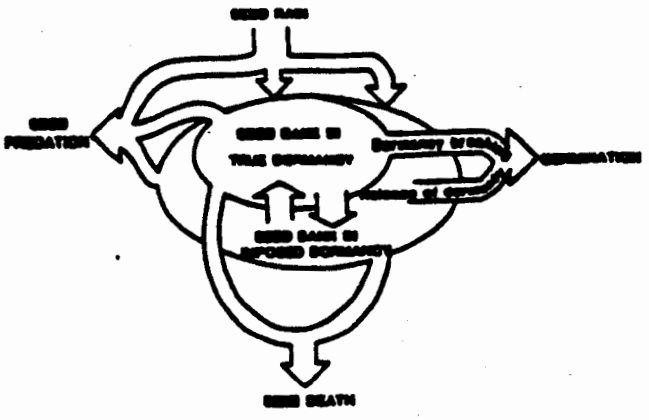

Figure 5. Seed Bank Dynamics (after Bradbeer, 1988).

H. Production and management of B. napus canola

In this section we discuss the production and management of both nontransgenic, and transgenic, oil-modified B. napus canola. Production and management practices for both types of crop are identical, and no additional practices are required to control volunteers of the transgenic, oil-modified B. napus canola. The numbers of volunteers of the transgenic, oil-modified B. napus canola, including laurate canola, seen during field trials were within the expected range. Experiments designed specifically to evaluate the potential for laurate canola to be either persistent or invasive are reported in Section VI.C.3 Issues 2 and 3.

1. Production

References for this section are: Raymer and Thomas, 1990; Raymer et al., 1990; Fribourg et al., 1989; Ameri-Can,1990.

B. napus canola requires fertile, well-drained soils. It should not be grown in fields that a) have heavy infestations of Brassica weed species; b) have produced a Brassica crop within the past four years; or c) have residual levels of substituted ureas, norflurazon, triazines, imidazolinones, most sulfonylureas, and other classes of herbicides to which canola is susceptible. Rotation away from Brassica crops is important to avoid volunteers and reduce the incidence of disease.

Crop establishment. High plant populations are necessary to compete with weeds in the stand establishment phase. Well-prepared seed beds and shallow sowing depth are necessary for this small seeded crop. The establishment of canola using reduced tillage practices has been difficult.

Fertility and soil requirements. Supplemental nitrogen is required. Deficient soils may require sulfur, boron and lime to bring the soil pH range into 5.8-6.2 for optimum production.

Pests. Common insect pests include flea beetles, aphids, cabbage seed-pod weevils, and a broad range of foliar feeders. Potential diseases include sclerotinia white mold, powdery mildew, and blackleg. See also section IV.C.

Harvest. Timing of harvest is critical since harvesting too early can result in heavy dockage due to green seed, high moisture and high foreign matter. Delays in harvest often result in excessive shatter and harvest losses. Harvested seed must be stored at no more than 8% moisture to maintain crop quality and seed viability, and to minimize spoilage.

2. Isolation in seed production and identity preserved production

Seed purity is important both for the production of certified seed and specialty oil production. The greatest risks to the quality of the crop are contamination from volunteer rapeseed or weeds and admixture or mislabeling of the seed.

Common agronomic practices used to insure quality and purity of the seed are:

- Isolation from other types of rapeseed
- Isolation from Brassica weed species
- Crop rotation

It is imperative to know the crop history of any potential B. napus production field site and its surrounding land. Seed certification requirements have been established for seed purity by the Association of Official Seed Certifying Agencies (AOSCA, 1971). For seed production of self-compatible rapeseed such as B. napus to be grown, the required isolation distance from any contaminating pollen source is 660 feet (200 m) for foundation seed (the most stringent category), and 330 feet (100 m) for certified seed.

3. Crop rotation

Rotation requirements for foundation grade seed are four years without a rapeseed and/or mustard seed crop. For certified seed, the requirements are reduced to the previous two years. Crop rotation allows volunteer B. napus to be controlled after deposition of seed back to the field during harvest.

The potential for increases in disease and pest incidence is another reason to rotate fields out of B. napus. A current recommendation for control of blackleg and white mold diseases is a four year rotation with non-susceptible crops such as soybean or cereals (Ameri-Can, 1990)

Depending upon the growing region and crops in the rotation program, the field either remains fallow for the season or is planted to another crop. In a fallow management scheme, the field would be tilled and/or mowed on a periodic basis to prevent weed seed development. When the field is planted to another crop, herbicide and/or other weed control measures are used.

Crop rotation options for spring planted B. napus are usually fallow for the winter season followed by corn or wheat. Fall planted B. napus canola in the southeastern U.S. may be followed by peanuts, wheat, small grains, soybeans, sorghum or held as fallow for the summer season. In California, the fall planted B. napus canola rotation program may include wheat, sugar beets, winter vegetables and fallow.

4. Management of volunteer B. napus in the next crop

Volunteers are best managed by keeping the seed on or near the soil surface to promote germination. After germination, the seedlings are killed by tillage and/or herbicide application. Although "B. napus varieties have no dormancy in the seed and, therefore, present little problem in volunteering" in subsequent seasons (Canola Council of Canada, 1984, p 715) it is always best to avoid deep burial, which could promote longevity of seed. In B. napus production, it is standard practice to soil-incorporate, by a shallow tillage operation, the plant biomass remaining in the field after harvest. This may

be done either before or after the first flush of volunteers has germinated. Before planting the next crop, herbicide is applied, which also reduces volunteers.

Seed losses during harvest are commonly in the range of 25 - 50 lb/acre (Ameri-Can, 1990), but can be as high as 300 lb/acre (Freibourg et al., 1989). We estimated the number of volunteer canola plants one should expect to see based on expected harvest losses, known seed weights of laurate canola (whose seeds weigh on average 3 g/1000 seed), and the assumptions that the seed were not deeply buried after harvest, that seed showed 100% survival, and that no pre-emergence herbicide was used on the field prior to the first rains. The normal loss levels correspond to 80 -160 seed per square foot (sq. ft) of Laurate canola, whose seeds weigh on average 3 g/1000 seed. During canola production, herbicides are normally applied before planting the next crop, which accounts for levels of volunteers below 80 per sq. ft (i.e. lower levels are not due to seed dormancy, which is virtually absent in B. napus).

Experience with nontransgenic canola. Based on Calgene's experience with the commercial production of nontransgenic B. napus canola in the U.S., a flush of volunteer canola germination follows the first good rain after harvest. The seedling density of this first flush is normally 20-100 plants/ sq. ft, which is within the range expected. Usually the field is tilled which destroys the volunteer canola and prepares the land for the next phase of the rotational program. Following the next rain or irrigation, the density of volunteer canola has declined to 1-8 seedlings per sq. ft, if any. The crop rotational practice controls B. napus canola volunteers within the first year after harvest.

Experience with transgenic oil-modified canola. It has been our experience with the transgenic canola field sites to observe the expected pattern; a dense flush of germination in response to rainfall or irrigation and subsequent germination(s) of greatly reduced seedling density. In order to allow some worst-case evaluation of volunteer management at the transgenic field trials, we did not sterilize the soil after plot destruction except at three sites (see Table 11 below and monitoring reports in Appendix 1). Instead, we allowed germination of the seed remaining in the field after harvest, removed the seedlings by tillage and/or herbicide treatment, depending upon the crop rotation, and continued to monitor the sites.

As expected from harvest loss estimates and experience with nontransgenic canola, typically ~20 seedlings per square foot of transgenic, oil-modified canola were observed in the first flush of germination, with fewer thereafter. In trials where nontransgenic border plantings of several varieties were used to provide a broad range of flowering, the mixed maturity but single harvest resulted in heavy seed losses (the early flowering border controls were overly mature, and shattered before and during harvest). In those sites, the area formerly occupied by the nontransgenic border planting was evident by the greater density of volunteer seedling observed in the first germination flush. Typical tillage and herbicide practices have been sufficient to eliminate the seedlings of both transgenic (including laurate canola) and nontransgenic canola at the field sites. In addition, controlled studies

specifically designed to evaluate persistence and invasiveness potential of the laurate canola were conducted (methods and results are reported in Section VI.C.3 Issues 2 and 3).

Conclusions. Production and management practices for B. napus canola are well established. Transgenic, oil-modified canola does not require additional or altered practices during cultivation or after harvest. Post-harvest numbers of volunteers of Laurate canola have been within the expected range.

Table 11. Summary of Calgene's experience with various agricultural management practices to monitor and control volunteer, transgenic oil-modified canola in three regions; a fall planted production area (GA), a spring planted production area (MI) and a fall planted, breeder seed increase area (CA). More detailed accounts may be found in Appendix 1.

Production system	Post-harvest practice	Observations
Fall plant /Spring harvest	Soil sterilization	No volunteers
	Summer fallow, no tillage	Volunteers germinate but cannot establish due to weed competition.
	Shallow incorporation of biomass into soil, plant to peanut using standard agricultural practice	Volunteers germinate with rainfall, 29 volunteers per sq. ft. Tillage + herbicide removes seedlings.
Spring plant / Fall harvest	Shallow tillage and plant to winter grains using standard agricultural practice	Volunteers germinate with rainfall, 12-20 volunteers per sq. ft. Tillage + herbicide removes seedlings.
	No fall tillage. Winter fallow with corn planted in spring	Volunteers germinate with spring rainfall noted as ~100 in the 0.46 acre site. Tillage and /or herbicide removes seedlings.
Winter nursery	Summer fallow (no rains), irrigation and tillage. Plant to winter vegetables in fall.	Volunteers germinate with irrigation. 2-3 cycles of irrigation and tillage is sufficient to control volunteers.

III. Description of the Genetic Modification to produce Laurate Canola

A. Introduction

The T-DNA region of the genetic construct contains two coding regions: one containing the 12:0 acyl carrier protein (ACP) thioesterase gene from California bay (Umbellularia californica) and one containing the selectable marker gene for resistance to kanamycin (Voelker et al., 1992). All other DNA is non-coding and has genetic regulatory, rather than coding, function. The gene constructs are chimeric, in that the coding regions are joined to regulatory regions from different genes. The construct borders are composed of DNA from Agrobacterium tumefaciens that functions to move the DNA from the bacterial cell into the plant cell chromosome, where the DNA becomes stably integrated.

The gene of interest, bay 12:0 ACP thioesterase (bay TE) , codes for an enzyme in the fatty acid biosynthetic pathway found in developing seeds. Use of the napin promoter restricts synthesis of the enzyme to the developing seed of transgenic canola.

The result of this genetic modification is the accumulation of the medium-chain fatty acids, laurate and myristate. Bay TE cleaves lauroyl-ACP to release free laurate. Laurate is a medium chain fatty acid (12:0) that accumulates naturally in seeds of only a few plant species, the most important ones (for commercial use) being coconut (Cocos nucifera L.), oil palm (Elaeis guineensis), and certain Cuphea species (see also Table 14b in section VI.A for a discussion of the occurrence of laurate in seeds of plants growing in the United States). In rapeseed, lauroyl-ACP is an intermediate in the synthesis of other fatty acids, but laurate is not normally present at more than 0.1 % (Sebedio and Ackman, 1979). Laurate biosynthesis is described more fully in Appendix 4.

The amino acid sequence of the bay TE enzyme is substantially similar to a long chain thioesterase enzyme native to canola. 25-30% of the sequences are identical and the proteins are the same length. ACP thioesterase enzymes are present throughout the plant kingdom where they are essential for fatty acid biosynthesis. Canola contains ACP thioesterase (TE) enzymes that cleave ACP from longer chains (e.g.. C16 and C18). Thus, this type of enzyme function and this class of enzyme is known to be native to rapeseed. At least one variety of canola, Tower, has been reported to contain very low levels of laurate (less than 0.03 weight %) in the seed (Sebedio and Ackman, 1979). It is theoretically feasible to alter the specificity of rapeseed's native thioesterase using mutagenesis so that it cleaves ACP from 12:0 more efficiently and laurate accumulates in the seed. It is also probable that rapeseed mutants that accumulate laurate due to altered specificity of this enzyme have arisen in nature through natural means over an evolutionary time scale but have either not persisted or not been discovered.

B. Plant transformation with binary vectors

Hypocotyl explants from 7-day-old seedlings of Brassica napus cv. 212/86 were incubated with Agrobacterium tumefaciens strain EHA101 (Hood et al., 1986) containing a binary vector to accomplish plant transformation (Radke et al, 1988). The Agrobacterium strain contains the vector construct that is to be transferred (T-DNA) on a separate plasmid from *vir* DNA sequences necessary for DNA transfer. This assures that the transformed plants do not contain *vir* DNA sequences required for further movement of the DNA (Hoekema et al., 1983). Thus, the T-DNA becomes stably integrated into the plant chromosome. The vector plasmid in EHA101 is disarmed, i.e. it does not contain oncogenic DNA sequences responsible for plant pathogenesis (Hood et al., 1986).

The binary vector, pCGN1578, is described in McBride and Summerfelt (1990). The vector contains right border (RB) sequences 13992 to 14276 and left border (LB) sequences 626 to 1205 of T-DNA from Agrobacterium plasmid pTiA6 (Houck et al., 1990), and the selectable marker gene, *kan*^{r} (described below). The binary vector also contains the lacZ' gene segment from pUC18 (Vieira and Messing, 1982) as a source of unique restriction sites (and multiple cloning sites) as well as an insertional inactivation marker for cloned DNA. In the final construct pCGN3828 (see below), i.e. after insertion of the cassette containing the gene of interest, fragments of the inactivated lacZ' gene are located between tml 3' and napin 3' DNA, and between napin 5' and RB DNA. No protein is produced from the inactivated lacZ' DNA fragments. Small portions of linker DNA are contained in the binary vector as described by McBride and Summerfelt (1990). The nucleotide sequence of the T-DNA portion[16] of the binary vector, pCGN1578, with identification of functional regions, is in Appendix 2.

C. The construct map of pCGN3828.

The construct map of pCGN3828 is given in Figure 6. It contains one copy of the bay 12:0 ACP thioesterase gene coding sequence under the control of the napin promoter, as well as one copy of the region that confers resistance to kanamycin. Each of the functional segments of DNA are described below.

[16] Nucleotide sequence of T-DNA between the nick sites.

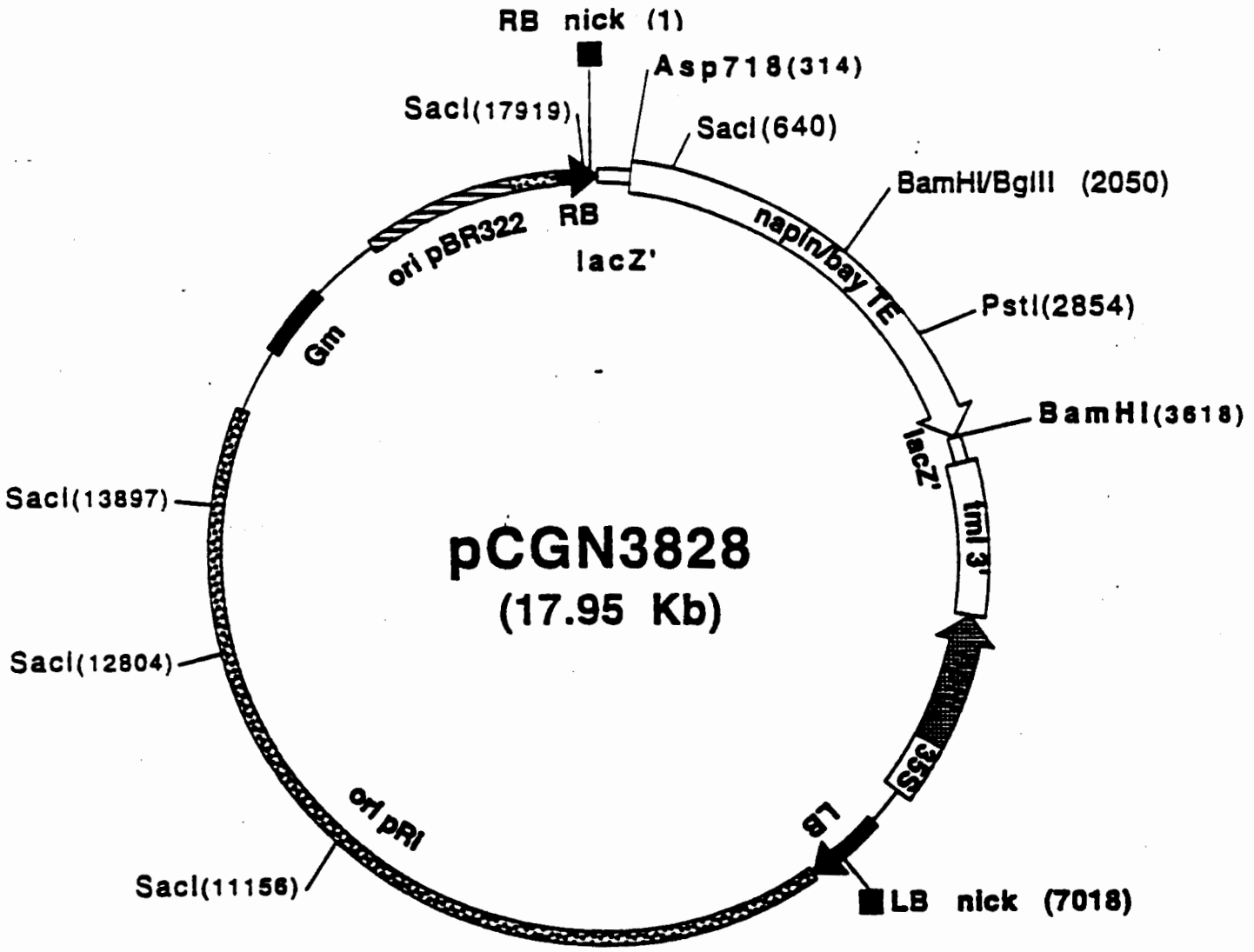

Figure 6. Plasmid Map of pCGN3828

Only the restriction sites important for the discussion are shown. Orientation of the respective genes are indicated by arrows. RB and LB, are right and left border of the T-DNA, respectively. Gm, extent of the gentamicin resistance gene. The pRi replicon from Agrobacterium rhizogenes (immediately adjacent to both RB and LB) is designated ori pRi. The the napin promoter extends from bp314 to bp2050, the bay TE reading frame directly follows from bp2050 to bp3272. A napin 3' fragment covers from bp3272 to bp3618.

D. Genes, regulatory regions and their sources.

Gene of interest.

The reading frame encoding bay TE was isolated from a cDNA clone from California bay (Umbellularia californica). Isolation of this gene from a cDNA library using a polymerase chain reaction-generated probe is completely described in Voelker et al., (1992).

Identity of the cDNA clone as ACP thioesterase was confirmed by (Davies et al., 1991; Voelker et al., 1992) :

1. comparison of known long-chain TE cDNA sequences[17] and amino acid sequences derived from them
2. expression of the cDNA in Escherichia coli and demonstration of 12:0 ACP thioesterase enzyme activity and production of laurate
3. expression of 12:0 ACP thioesterase activity in Arabidopsis thaliana and Brassica napus seeds
4. accumulation of laurate in triacylglycerol molecules in Arabidopsis thaliana and Brassica napus seeds.

The sequence of the bay TE gene was determined and data are available in the GenBank data base (M94159) and in Appendix 2. The clone contains an open reading frame of 1.2 kb (coding for 382 amino acids). The complete reading frame (1221 bases) of the bay TE precursor protein was used in the production of seed-specific chimeric constructs. The clone also contains 5 bases of 5' untranslated DNA and 71 bases of 3' untranslated DNA.

Promoter and terminator regions.

A clone encoding a napin storage protein (BcNa1) was isolated from a cDNA library from a cultivar of Brassica rapa, as described in Kridl et al., (1991). This clone was used as a probe to obtain the corresponding gene and flanking sequences from B. rapa genomic DNA. After complete DNA sequencing, 1.75 kb of promoter (napin 5') and 0.32 kb of terminator (napin 3') regions of the napin gene were isolated. These were used to construct expression cassettes for the bay TE gene. The napin promoter functions in developing seed embryos but not in leaf tissue (Radke et al., 1988) and not in pollen (see Table 14c in Section VI.A).

The kanamycin resistance marker gene.

A gene conferring resistance to the antibiotic, kanamycin, is a component of the binary vector used to transform the Brassica napus plants. The gene coding for APH(3')II was originally isolated from the transposon Tn5, which is found in enteric bacteria such as Escherichia coli and Klebsiella pneumoniae (Berg et al., 1975; Courvalin et al., 1978). Kanamycin resistance in Tn5 was discovered and characterized at the University of WI, Madison.

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[17] DNA sequence similarity is approximately 50%.

The complete DNA sequence for the gene has been determined (Beck et al. 1982; Jorgensen et al., 1979). In the binary vector, pCGN1578, the kanamycin resistance gene is controlled by the 35S promoter from the plant pathogen Cauliflower Mosaic Virus (Odell et al., 1985). The terminator region, tml 3', is from an Agrobacterium tumefaciens plasmid, pTiA6 (Houck et al., 1990.) Safety in use of the kanamycin resistance gene has been extensively studied and data are contained in the references "Request for Advisory Opinion. *kan*^{r} gene: Safety and use in the production of genetically engineered plants" submitted to the U.S. Food and Drug Administration Nov. 26, 1990, submitted to USDA APHIS as Appendix 7 of FLAVR SAVR Tomato: Petition for Determination of Regulatory Status, May 31, 1992 (copies not enclosed).

E. Genetic characterization.

Genetic characteristics of the Laurate plants were evaluated in several ways:

1) Multi-generational greenhouse and field performance studies, and breeding characteristics were used to evaluate the stability of gene expression and heritability of the trait,
2) Multi-generational Southern blot analysis was used to evaluate genetic stability, number of insertion loci, and to confirm the presence or absence of various portions of the construct and binary vector in the transformed plants, and
3) Segregation analysis was used to evaluate linkage of insertions.

1. Gene expression. The genetic modifications were designed to modify oil composition in the seed, and to date, these modifications have been exhibited in every generation. There is no indication from plant performance in the field that they are genetically unstable (Appendix 1, Field Trial Results).

2. Southern blot analysis.

For the detection of bay TE, we used a 804 bp probe spanning essentially the entire coding region of the bay TE cDNA (from BamHI/BglII, pos. 2050 to PstI, pos. 2854 of pCGN3828, see map, Fig.6). As the map of pCGN3828 shows, (SacI, pos. 640) and BamHI (pos. 3618) flank the transgene and are unique for the T-DNA. Therefore, if the T-DNA inserts into plant genome, a plant genomic restriction site is necessary for the production of a BamHI or SacI restriction fragment carrying the bay TE gene. Therefore it is expected that essentially each transgene copy will lead to a fragment with a different electrophoretic mobility, allowing a precise determination of genomic copy numbers in most cases. Only in the cases of three or more T-DNA copies inserted at the same genomic site, the DNA fragments created by two T-DNA located enzymes will all be the same length, resulting in a Southern band stronger than the one derived from the bordering T-DNA copies.

As Fig. 7 shows, the digestion of genomic Brassica napus control DNA with BamHI does not lead to any detectable background using our Southern conditions. The plant from transformation event 18 shows two bands of unequal intensity, indicating that the upper band might be a doublet. Most likely, this plant has three copies of bay TE in its genome. The plant from transformation event 23 shows 14 bands, one of which is most-likely a doublet. Therefore we calculate the total copy number in this event as approximately 15. As is shown in the T2 generation (23-91, 23-132, 23-198), selfing leads to the expected reduction in genomic complexity, obviously not all copies are essential for maximal phenotype. The genetic construct has been stably maintained in the plant chromosome in progeny of transformation events 23 and 18 which are the subject of this petition.

Southern blot analysis[17] performed on T_1 plants from events 18 and 23 showed that there were multiple copies of the construct inserted. Analysis of segregating T_2 populations of event 23 indicated that insertions were segregating in a Mendelian fashion as do all plant genes. No new bands appeared after selfing, indicating that the locations of the inserts were not changing. The band pattern in the progeny of event 23-198 in T_4 and T_5 plants that were examined are identical to those in the T_2 generation, indicating that the insertions have been stable during the last 3 generations. Insertions in event 18 have also been stable since the T_2 generation in the line examined at T_5.

Southern blot analysis was also used to screen for the presence of non-T-DNA vector sequences in the transformed plants. Information on the presence of vector sequences in the laurate events is included in Appendix 3, and discussion of the lack of plant pest risk associated with the vector sequences is included in Section V.

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[17] Southern blot methods are described in Maniatis et al., 1982 and specifics for Calgene's protocol are given in Appendix 2. DNA isolation procedure is included in Appendix 2.

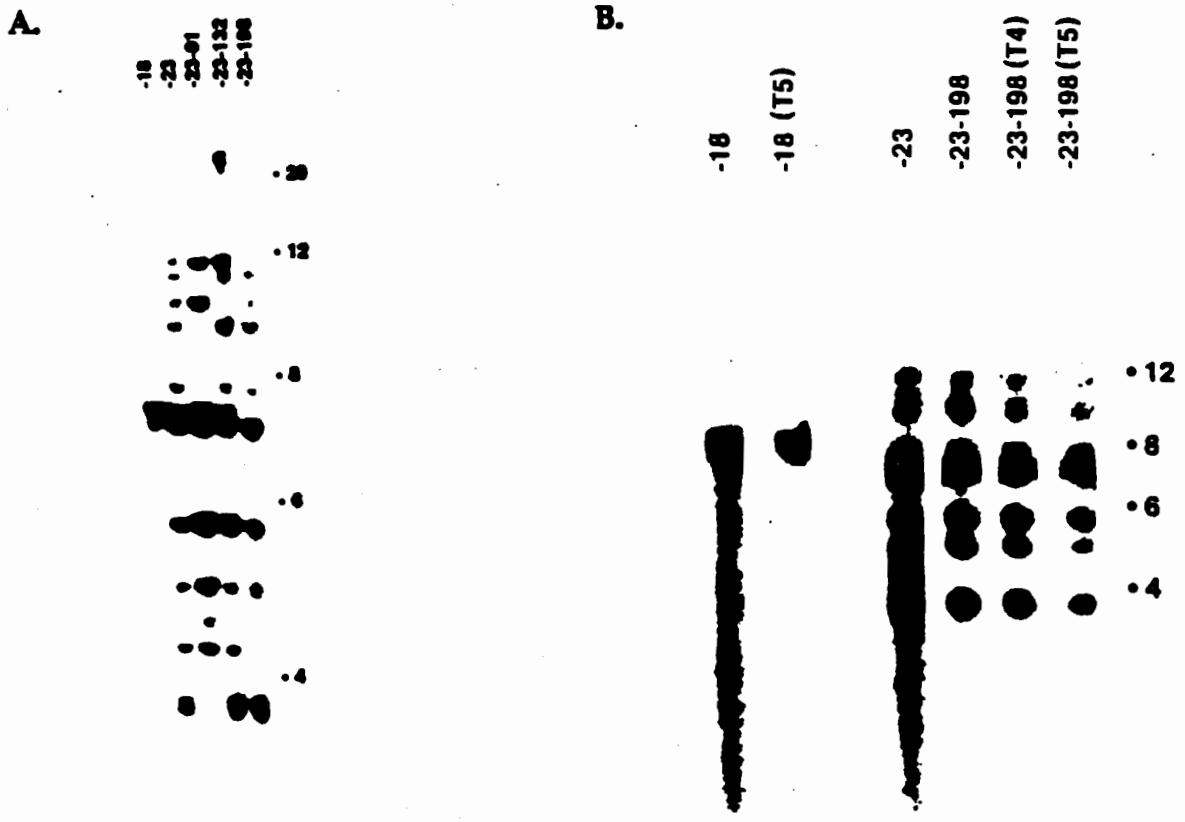

Figure 7. Southern blots of DNA from Laurate canola. For each lane, 10µg of genomic DNA was digested with BamH1 (cuts only once in the transferred DNA), separated on an 0.5% agarose gel and subsequently transferred to a nylon membrane. The membrane was probed with a 0.9kb bay TE cDNA fragment covering most of the bay TE reading frame (random priming). Brassica control DNA does not give any signal under the conditions used (not shown).

All plants were transformed with the construct pCGN3828. In Figure A, the plants -18 and -23 were two different independent primary transformants and plants 23-91, -23-132, -23-198 were second-generation plants (seeds obtained by self-pollination of event 23). In Figure B, lines -18 and -23 are the same T_1 generation plants shown in Figure A, in a replicate Southern analysis. Line 23-198 is a T_2 plant from event 23. The plants from generations T_4 and T_5 of events 18 and 23 are indicated in parentheses. Numbers on the right indicate molecular weights (in kb).

These results indicate that the inserts are stable in Laurate canola events 18 and 23 by the second generation in precommercial lines.

3. **Segregation analysis.** Segregation analysis, performed by testing antibiotic (G418, a kanamycin analog, Geneticin, Sigma) resistance of germinating T_2 seeds from pCGN3828 events are shown below. These results are consistent with the results of Southern blot analysis.

Methods. Segregation assay:

kan^{r}:*kan*^{s} — *Brassica* microspore-derived embryos selected on kanamycin-containing medium (25 mg/l) were derived from T_1 Laurate plants containing T-DNA from pCGN3828. Segregation ratios for haploid plants are one insert, 1:1; two, 3:1; three, 7:1; four, 15:1 and five, 31:1.

Laurate ±: — Laurate content from half-seed analysis of T_2 seed derived from T_1 plants. Segregation ratios for diploid plants are one insert, 3:1; two, 15:1; three, 63:1; and four, 255:1

Since the kanamycin assay was performed on haploid embryos whereas the Laurate analysis was performed on diploid seed, the segregation ratios are not expected to match. Rather, Chi square analysis was used to determine the most probable number of genetic loci in each transformation event.

Results. Results for two pCGN3828 events are given in Table 12. The number of gene inserts was estimated by laurate content in half-seed analysis, and by survival of microspore-derived embryos cultured in 25 mg/l kanamycin. The material undergoing field evaluation contains multiple inserts. Improvement of the Laurate material with multiple genetic loci requires the use of quantitative inheritance techniques.

Table 12. Number of independent genetic insertions in Laurate canola estimated using segregation ratios derived from one haploid and one diploid assay. The Chi square critical value with one degree of freedom for $\alpha = 0.05$ is 3.84.

T_1 event	Segregation ratio	Probable number of genetic loci	Chi square value for each potential number of inserts: one	two	three	four	five
pCGN3828-212/86-18							
kan^r:*kan*^{s}	76:24	two	27.04	0.05	12.09		
Laurate $^+$: Laurate $^-$	57:3	two	12.8	0.16	4.6	32.7	
pCGN3828-212/86-23							
kan^r:*kan*^{s}	401:19	four or five			24.43	2.14	2.71
Laurate $^+$:Laurate $^-$	214:0	$\geq$four	71.3	14.6	3.4	0.8	

IV. Characteristics of Laurate Canola Plants

A. Overview

The best Laurate lines closely resemble the parent cultivar, 212/86, except that the fatty acid profile of the seed has been modified in the way we intended. Significant levels of laurate are produced in the transformed lines[18].

This section describes behavior of the laurate canola plants in replicated field trials. Such trials are designed to evaluate the commercial potential of the plants and to make selections for further breeding and development. Variety trials also provide some information suitable for risk assessment.

B. Agronomic traits and quality characteristics

1. Materials and Methods

The phenotype of various lines of Laurate canola was determined during field trials and growth in the greenhouse compared to the untransformed parent variety, 212/86, and other commercial, nontransgenic varieties such as A112, Bingo and Cyclone. Quantitative traits were measured, and an analysis of variance was performed using the General Linear Models Procedure of SAS (SAS Institute Inc. *SAS/STAT® User's Guide*, Release 6.04 Edition. Cary, NC:SAS Institute Inc., 1988. GLM pp. 549-640.).

2. Results

Table 13a presents a summary of results of statistical analyses. Table 13b presents representative pooled data from the 1992-1993 field trials in Georgia. Commercially suitable lines are vigorous, possess good winter survival characteristics, have yield potential within the range of commercial varieties, and do not exceed legal levels of erucic acid and alkenyl glucosinolates in the seed.

Traits of particular interest for the assessment of plant pest risk are addressed at length in later sections of the document (see section IV.D below).

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[18] The biosynthetic pathway for laurate is illustrated in Appendix 4. See also Somerville and Browse (1991).

Table 13a. Summary of characteristics of commercially suitable Laurate canola plants as compared to the parent variety 212/86, determined under field conditions.

Characteristic	Significantly different[1]	Not significantly different[1]
Agronomic Traits		
Seed germination %		+
Seedling vigor		+
Stand establishment		+
Days to flowering		+
Days to maturity		+
Yield		variable[2] +
Yield/Stand relationship		+
Seed Characteristics		
Oil content		+
Laurate content	+[3]	

[1] Based on analysis of variance, $p = 0.05$.

[2] Yields of Laurate canola lines have never been significantly greater than the parent: in some trials they have been equivalent and in some trials one or more Laurate canola lines yield less than the parent.

[3] Laurate content in Laurate canola lines is > 10% by weight of oil. Laurate is present in trace amounts (ca. 0.1 %) in control (nontransgenic) lines (limit of detection is 0.1%).

Table 13b. Summary of replicated trial results for Laurate canola in Georgia 1992-93. Data are combined from three field locations in Cook, Washington and Wayne counties. Trials conducted under USDA permit # 92-156-01.

Variety	Yield lbs / acre	Stand establishment, plants per sq. ft	Winter Survival Ratio	Seedling Vigor, scale 1 to 5	Days to Maturity	Days to Flower	% oil	% erucic acid (22:1)	Alkenyl Glucosinolate µmoles/g
A 112	1976	29	136.15	4	192	147	44.44	0.001	13.5
SP004	2203	24	102.09	4	208	152	45.00	0.003	8.9
SP007	1771	21	88.14	4	201	147	44.41	0.011	7
212/86	2159	22	96.44	4	210	160	42.85	0.003	12.9
Laurate event 23	1908	26	96.95	4	211	157	43.34	0.002	16.4
Laurate event 18	1380	23	101.98	3	212	159	43.43	0.001	13.8
Mean	1900	24	103.62	4	205	154	43.91	0.003	12.1±4
Least significant difference, p=0.05	494	7	47.41	0	4	6	2.04	0.013	
C.V.	18	21	31.26	8	1	3	3.17	256.413	
Degrees of freedom	71	71	47	71	23	47	71	71	

Varieties A112, SP004, SP007 and 212/86 are nontransgenic.
Yield is expressed in pounds per acre of clean seed at 8% moisture.
Stand establishment was determined as plants per square foot, 45 days post-planting.
Winter survival is a ratio of fall and spring stand counts.
Seedling vigor is rated on a scale of 1 to 5, with 5 being the most vigorous.
Maturity is reached when the seed has no longer increased in dry weight.
Beginning of bloom is recorded when at least 90% of the plants in a plot have begun flowering.
Measurement of alkenyl glucosinolates was done on 1 pooled sample per line. Maximum permissible level is 30 µmoles per gram of the defatted meal.

C. Susceptibility to pests and diseases

Commercial canola may be subject to pressure from pests such as flea beetles (*Phyllotreta* spp.) and aphids (*Myzus persicae* (Sulzer), *Brevicoryne brassicae* (L.), *Lipaphis erysimi* (Kaltenbach)), and from diseases such as blackleg (*Leptosphaeria maculans* (Desm.) Ces. et de Not.), Sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary) and black spot (*Alternaria* spp.). Although B. napus is susceptible to Cauliflower Mosaic Virus (CaMV), this pathogen does not produce economic losses in the U.S. or Canada to our knowledge.

During field trials and greenhouse propagation of Laurate canola, the following diseases were monitored:

Common name	Scientific name
Stem rot	*Sclerotinia sclerotiorum*
Blackleg	*Leptosphaeria maculans*
Black spot	*Alternaria* spp.
Winter decline syndrome (WDS)	abiotic/biotic complex
Black rot	*Xanthomonas campestris pv. campestris*
Powdery mildew	*Erisyphe brassicae*
Gray stem	*Pseudocercosporella brassicae*
Damping off	*Rhizoctonia solani, Pythium* spp.
Turnip mosaic	TuMV
Cauliflower mosaic	CaMV
Crown gall	*Agrobacterium tumefaciens*

Disease Monitoring Protocol

Presence of disease within trials is checked on a regular basis when other agronomic observations are collected. Confirmation of the disease is done using standard disease diagnostic techniques, including microscopic examination of disease signs, isolation and culturing of disease-causing organisms, taxonomic identification of the isolated organisms, and re-inoculation of the cultured organisms onto rapeseed plants to reproduce disease symptoms. Methodical disease estimates are recorded for each individual plot when significant disease damage occurs and when differences among individual plots within a trial are visible.

The following table lists the data collected for estimating diseases in transgenic rapeseed trials:

Disease	Data Collected
Stem rot	percent infected plants/plot at harvest
Blackleg	mean rating of 50 plants/plot at harvest using 0-5 rating scheme (attached)
Black spot	0-4 rating of pod damage/plot at harvest (attached)
WDS	percent dead plants/plot at harvest
Black rot	percent foliage destroyed/plot prior to ripening
Powdery mildew	percent foliage/plot covered by mildew
Gray stem	percent infected plants/plot at harvest
Damping off	percent stand reduction during plant establishment
TuMV	percent infected plants/plot prior to ripening
CaMV	percent infected plants/plot prior to ripening
Crown gall	percent infected plants/plot prior to ripening

To date, significant disease has not been observed in Calgene's transgenic rapeseed trials. This is reflective of the general situation of current US rapeseed production and is primarily due to the limited acreage grown. Therefore, there has not been sufficient opportunity to determine the differential susceptibilities between transgenic and their non-transgenic rapeseed counterparts in the natural situation. Some additional information is contained in the field trial reports (Appendix 1).

In greenhouse studies to evaluate invasiveness potential of Laurate canola, infestations and damage from aphids and lepidopteran larvae were quantified and found to be the same for Laurate canola and two commercial varieties, including the parent 212/86 (see Kareiva and Parker, 1993 in Appendix 5).

Susceptibility to Cauliflower Mosaic virus.

Due to the presence of the CaMV 35s promoter in Laurate canola, Calgene arranged for the Laurate canola, B. napus variety 212/86 and other transgenic and nontransgenic lines of B. napus canola to undergo evaluation for susceptibility to CaMV[19]. Theoretical aspects of the potential interaction between the 35s promoter and the virus itself are discussed in section V.B.

The following summary is abstracted from Daubert, Stephen D. 1994. "Susceptibility of transgenic Brassica napus to CaMV" a report prepared for Calgene (Appendix 5).

Introduction. The transgenic selections of B. napus characterized in this study carry a section of the genome of CaMV. Under field conditions wild strains of

[19] Experiments were conducted by Dr. Steven Daubert at the University of California, Davis. His report is contained in Appendix 5.

CaMV may infect the transgenic plants; the endogenous and the infectious viral DNA homologues may interact to alter the nature of the ensuing viral infection. In this study, CaMV infection is followed under two very different environments, one favorable and the other unfavorable to CaMV infection. The infection is characterized. In neither environment were differences in symptoms recorded between Laurate canola and non-transformed canola cultivars.

Methods and materials. Seed from blind coded samples were germinated and seedling plants were grown in the greenhouse, under a 16 hour photoperiod established by additional fluorescent night lighting and high humidity (conditions favorable to infection). Plants were thinned and transplanted for even spacing at the cotyledon stage, and grown to the 4 leaf state (2 true leaves of equal or greater size than the cotyledon) 12 days post planting. Then leaves were dusted with celite, and the plants from each selection were divided into two identical paired sets: those on one side of their flat were inoculated with 50µl of a homogenate (1g/ml, in 50 mM potassium phosphate, pH 7.0) of CaMV-infected B. rapa leaves. Using conditions unfavorable to infection, (18°C, 12 hour light/day, approx. 10% daylight, low humidity) a similar set of inoculations was carried forward in a growth chamber study.

Results. All selections were susceptible to CaMV infection, developing very faint symptoms on inoculated leaves in about 9 days, followed by faint clearing of the secondary veins on the first set of systemically-infected leaves in about 17 days. In the greenhouse, under daylight, indoor-humidity conditions, the systemic infection progressed to a final stage involving clearing of the primary veins and stunting of the plants. Growth continued until 50 days post planting, then the above-ground parts (green mass) were collected and weighed. All plants were taken from the mock-inoculated sets; only symptomatic plants were taken from the inoculated sets. These included plants on which systemic symptom coverage was incomplete. Asymptomatic plants in inoculated sets were assumed to have escaped infection. When present, uninfected plants in the inoculated grove were generally larger than the surrounding infected plants.

Based upon the mean green mass accumulation, a reduction of approximately 30% or less was recorded for the greenhouse study. This matches results reported in the literature (see review in Daubert, 1994, Appendix 5). No significant differences are found between Laurate and control (nontransgenic) selections (Table 13c).

In the growth chamber study, plants were able to recover from infection and to grow out of the initial systemic symptoms with the emergence of asymptomatic upper leaves. Green mass was harvested 65 days post-planting and the average infection induced reduction in green mass ranged from 12 to 19%.

Table 13c. Test of similarity in response of selections to CaMV infection. Probability of equivalence of response to infection assessed by Student's T analysis. Comparison are made of the deviations observed for each selection in the weights of the infected plants from the average weight of the mock-inoculated control. A probability of >0.05 is considered significant.

Pair-wise comparison	probability of similarity
cv. 212/86 vs laurate event 18	0.32
laurate event 18 vs laurate event 23	0.49
laurate event 23 vs cv. 212/86	0.59
cv. 212/86 vs cv. A112	0.21

Conclusion: All of the transgenic and nontransgenic lines became systemically infected after inoculation with CaMV, but had very mild symptoms. There were no significant differences in symptoms or degree of infection-induced stunting between the laurate lines and nontransgenic lines (Daubert, 1994. Report in Appendix 5). Thus, there is no evidence that Laurate canola has altered susceptibility to CaMV.

D. Assessment of plant pest risk

Calgene conducted field, growth chamber and greenhouse tests specifically designed to evaluate environmental and plant pest risk factors. These studies are described in the following sections of the petition document:

Section	Topics
VI.C.3.2	Invasiveness, stand establishment, seedling vigor, competitive fitness,
VI.C.3.3	Persistence, seed germination and dormancy
VI.C.3.4	Selective advantage
VI.C.3.5	Pollen movement
VI.C.3.6	Crossing to wild relatives
VI.C.3.7	Germination and dormancy of crop/wild hybrids

V. Use Of Genetic Material From Regulated Articles.

The Laurate canola plants have been considered to be regulated articles because they, or the vectors used to construct them, contain DNA sequences from the list of organisms in 7 CFR 340.2: right and left border sequences and the tml 3' terminator from Agrobacterium tumefaciens, the origin of replication from Agrobacterium rhizogenes, and the 35S promoter from Cauliflower Mosaic Virus. These organisms, in their native state, can be plant pests.

A. Agrobacterium tumefaciens

The molecular basis of pathogenesis in Agrobacterium tumefaciens has been exhaustively studied, and precise regions of DNA have been identified that confer virulence, while other regions have been identified that are not involved in plant pathogenesis (see for example Nester et al., 1984 and Zambryski et al., 1989.) This detailed knowledge has made it possible to select or construct strains that are unable to produce disease in plants, but still retain the capability to transfer DNA into a plant chromosome. These strains and their vectors are termed "disarmed". Transformation with nonpathogenic or disarmed strains of Agrobacterium is the cornerstone of plant molecular biology.

1. Use of disarmed vectors.

The binary vector system used for genetic transfer of the Laurate construct into canola plant tissue is derived from a Ti plasmid of Agrobacterium tumefaciens. Genes essential for plant tissue disease expression (oncogenes) are not present in the vector system, i.e. it is disarmed (Hood et al., 1986). T-DNA border sequences that mediate DNA transfer from the vector into the plant chromosome are present in the binary vector (RB and LB in Appendix 2, map of pCGN3828). The Agrobacterium strain that is used for plant transformation (EHA101: Hood et al., 1986) contains two separate plasmids: one that contains the Laurate construct, and another, the helper plasmid, that contains the *vir* functions essential for physical transfer. This assures that the transformed plants do not contain *vir* regions required for further movement of the DNA (Hoekema et al., 1983). The helper plasmid is not transferred to the plant (Hood et al., 1986). Thus, the DNA carrying the Laurate construct will not be transferred again by the T-DNA mechanism after it has been inserted into the chromosome(s) of the canola cell.

2. Use of noncoding DNA sequences

The tml 3' terminator region consists of noncoding DNA from Agrobacterium tumefaciens plasmid pTiA6 (Nester et al., 1984; Houck et al., 1990). The noncoding region produces no protein and is itself incapable of causing disease in plants.

Within the DNA segment comprising the *tml* 3' gene, the 3' portion of the ORF of the *tml* gene is present as is the 3' portion of the A. tumefaciens gene 9 (Barker, 1983). Only 34 bp of the 1504 bp *tml* gene ORF is present and therefore this gene region is not expected to be expressed (refer to DNA sequence in Appendix 2). Some 502 bp of the 576 bp that comprise gene 9 are present in pCGN1578; however given that the gene 9 promoter is not present on the cloned segment of the *tml* 3' gene region, the truncated gene 9 ORF should not be expressed in the Laurate canola plants (refer to DNA sequence in Appendix 2).

3. Use in transformation

Transformation of hypocotyl explants from 7-day-old seedlings of Brassica napus cv. 212/86 was accomplished by incubation with Agrobacterium tumefaciens strain EHA101 containing the binary vector plasmid as described in Radke et al. (1988). EHA101 is a nonpathogenic strain (Hood et al., 1986). After initial selection of transformants, the plant tissues were counterselected with 500 mg/L carbenicillin to render nonviable any remaining cells of Agrobacterium.

B. Cauliflower Mosaic Virus (CaMV)

The CaMV 35S promoter is used extensively in recombinant DNA research because it is constitutively expressed in plants. We have no reason to believe that use of this promoter represents a plant pest risk.

1. Use of noncoding DNA sequence (35S promoter)

In the Laurate canola plants, the 35S promoter region, derived from CaMV (Gardner et al. 1981) controls expression of the selectable marker gene, *kan*^{r}. This promoter has been fully characterized and sequenced, produces no protein, and is not, by itself, capable of conferring plant pest characteristics (Odell et al., 1985; Benfey and Chua, 1990; Palukaitis, 1991).

The CaMV35S promoter sequence has not been shown to be a plant pest risk. Palukaitis (1991) concludes that, "while some of these plants [containing CaMV35S promoter] may have shown either unusual or abnormal responses, it has in every case been possible to delimit these host abnormalities to the expression of the gene and not to the presence of a promoter of viral origin. There is no evidence that the sequences of the CaMV promoters are in themselves inducers of pathogenicity. Thus, the major gene product rather than the well-characterized regulatory signals on the CaMV DNA are involved in the induction of pathogenicity in plants." This sequence, as used in producing Laurate canola, does not cause these canola lines to become plant pest risks.

Cauliflower mosaic virus is a double-stranded DNA caulimovirus with a host range restricted primarily to cruciferous plants. Genome size is about 8 kb. Since B. napus is a host for CaMV there are several types of potential interactions between the 35S promoter in the plant chromosomes and

infecting CaMV DNA (Daubert, 1994, Appendix 5). Each of 4 types of interactions are discussed below.

1) direct interaction and genetic recombination.

Cauliflower mosaic virus is a double-stranded DNA virus (Shepherd, 1981) and replicates in the nucleus of the plant cell. Virus replication involves production of many copies of its DNA genome, each of which would have some finite probability of recombining with plant DNA. Theoretically, it is possible that during infection of Laurate canola with CaMV, viral DNA could interact and recombine with DNA sequences in the plant chromosome via homologous recombination between the 35S promoter in the plant genome and the 35S promoter in the virus genome. Intermolecular recombination into a defective CaMV from plant chromosomal DNA containing the missing viral DNA has been reported (Gal et al., 1992). Such recombination events are likely to be extremely rare, "and likely undetectable under conditions where recombination is not selected for" (Daubert, 1994. Appendix 5).

Should the 35S sequences from the plant be introduced into viral DNA, such an event would have no consequence, since it would merely reconstitute the wild-type virus and could not make the virus more pathogenic.

Recombination from CaMV into plant DNA has not been reported. However, if recombination were to result in the introduction of additional viral DNA sequences into the plant (via imprecise recombination), this would also be of no consequence. CaMV is not seed borne. This means that any and all recombination events would take place in somatic plant cells only, and recombined DNA would never be passed to the next generation of plants or otherwise be preserved. Thus, for all intents and purposes, a Laurate canola variety could not come to possess additional plant pest DNA via a CaMV recombination event.

2) interaction of promoters at the function level.

The number of "plant" copies of the 35S promoter will be insignificant relative to the number of "virus" copies in an infected plant cell. Thus, any activity of a "plant" copy during viral transcription would not be significant. See Daubert, 1994 in Appendix 5.

3) indirect effect of altering plant susceptibility to CaMV.

A synopsis of results of inoculations of Laurate canola plants with a pathogenic strain of CaMV is reported in section IV.C. above. The Laurate canola plants exhibited no altered susceptibility to CaMV. There is no evidence to suggest that the 35S promoter has any influence on the susceptibility of B. napus to CaMV (Daubert, 1994. Appendix 5).

4) indirect effect of altering virus production.

There is no evidence that virus titre is altered in transgenic plants. See Daubert, 1994 in Appendix 5.

2. Gene VI fragment from CaMV.

Within the DNA segment comprising the 35S promoter, the 3' portion of the cauliflower mosaic virus gene VI is present (sequences 927 - 1114, Appendix 2). Gene VI is responsible for pathogenicity of the virus. Only 187 bp of the 1562 bp comprising the open reading frame of Gene VI (ORF VI) are present in the binary vector pCGN1578 (refer to DNA sequence in Appendix 2). As such, the sequence represents a large deletion mutation of ORF VI, and is not expected to be expressed. Zijlstra and Hohn (1992) note that transformed Arabidopsis plants expressing ORF VI were phenotypically abnormal. The complete lack of such abnormalities in the Laurate canola plants further supports the conclusion that no expression occurs from this small ORF VI gene fragment.

If there were to be recombination between an infecting strain of CaMV and the Laurate canola genome, this gene VI fragment could be substituted for the homologous fragment in the intact virus. Once again, this would simply reconstitute the wild-type virus, would not affect pathogenicity and should be of no consequence.

Potential imprecise recombination leading to inclusion of additional viral sequences into the plant genome would be very rare, would occur only in somatic cells (as discussed above for the 35S promoter) and therefore would be of no consequence.

C. Agrobacterium rhizogenes

1. Use of the pRiHRI replicon.

The origin of replication (ori pRi or *ori*) from plasmid pRiHRI of Agrobacterium rhizogenes strain HRI allows the binary vector to be stably maintained in the disarmed A. tumefaciens strain without antibiotic selection (Jouanin et al., 1985). Calgene's binary vector, pCGN1578, was constructed using the entire pRiHRI Bam HI-11 segment which confers additional stability over the *ori* sequence alone (Nishiguchi et al., 1987). This 8 kb fragment includes the *stb* locus (stability and copy number) as well as *ori* functions (Jouanin et al. 1985). Published data described below show that the *ori* sequences, and indeed the entire Bam HI-11 fragment, are both physically separated and functionally distinct from DNA sequences responsible for inducing plant disease.

HRI is an agropine strain. Agropine-type pRi plasmids harbor two T-regions (TL and TR) which can be transferred independently to the host plant genome, and can each independently induce proliferation of transformed roots (Vilaine and Casse-Delbart, 1987).

pRiHRI was mapped by Jouanin (1984). The *ori* and *vir* regions are nearly adjacent, but both are far removed from the T-DNA regions. The *vir*

region ends within BamHI fragment 3 based on homology with *vir* regions from pTiAch5 and pRi8196 (Birot and Casse-Delbart, 1988). Four additional fragments, 19a, 9, 23 and 33, separate fragments 11 and 3 by at least 12 kb. Thus, it is virtually certain that BamHI fragment 11 does not contain any of the *vir* region. BamHI fragment 11 is even more distantly located from the two T-DNA regions.

Huffman et al., (1984) completely mapped the restriction enzyme sites in pRiA4b, another Ri plasmid. All agropine-type Ri plasmids examined are very similar and quite separate as a group from the mannopine-type plasmids (Costantino et al., 1981; White and Nester, 1980). The origin of replication region in this Ri plasmid is also physically separated from the virulence region and both T-DNA regions, for example.

White et al. (1985) published a functional and molecular analysis of both T-DNA regions of pRiA4b. Neither of these regions contained the origin of replication sequences. Disarmed plasmids in nonpathogenic Agrobacterium strains contain *ori* sequences. Cosmids containing full length T-DNA's restored full virulence of such Agrobacterium strains and caused normal hairy root tumor morphology on Kalanchoe stems (White et al., 1985). Furthermore, A. rhizogenes was not used as a vector for transformation of B. napus. These results demonstrate that *ori* sequences do not confer pathogenicity or virulence in Agrobacterium.

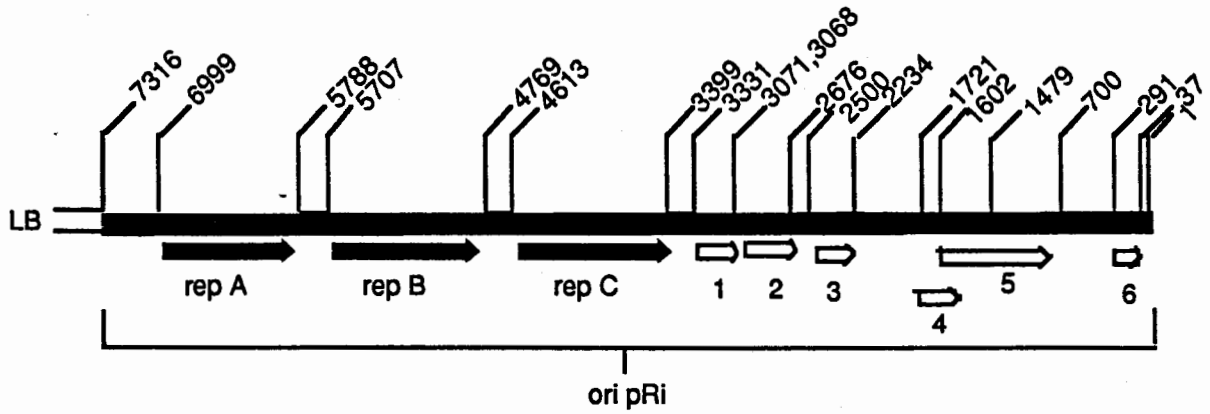
2. Open reading frames in the Bam HI-11 segment

The pRiHRI Bam HI-11 fragment contained in the binary backbone plasmid was analyzed for the presence of open reading frames. Putative open reading frames are shown in the following diagram Putative reading frames described below are contained within segments of bacterial DNA in the bacterial host, and would not be expressed in plant cells if transferred because there are no eukaryotic regulatory elements (promoters) available for the initiation of eukaryotic transcription.

The reading frames designated *rep*A, *rep*B and *rep*C encode proteins involved in the control of DNA replication of the plasmid pRiA4b from Agrobacterium rhizogenes (Nishiguchi et al., 1987). These three reading frames are also highly homologous to the same reading frames encoded by the Agrobacterium tumefaciens plasmid pTiB6S3 (Tabata et al., 1989). The *rep*A and *rep*B proteins also share considerable homology to the E. coli F-plasmid *sop*A protein (Mori et al., 1986) and the *kor*B protein of the broad host range plasmid RK2 (Theophilus and Thomas, 1987) respectively. All the above described proteins are involved in the replication and stability of these large plasmid elements in their respective prokaryotic hosts.

Analysis of the DNA sequence from 3331 to 37 also revealed the presence of six other putative open reading frames. In bacteria, these reading frames may encode small molecular weight proteins approximately 10,000 daltons in size. The reading frames designated 2 and 5 code for potential polypeptides 16,000 and 30,000 molecular weight respectively. There is no evidence to indicate that these proteins are actually expressed from the native pRi plasmid DNA when this plasmid element and its derivatives are maintained in Agrobacterium. Analysis

pRiHRI Bam HI-11 fragment: reading frames (described in text)



of possible polypeptides that could be produced do not share significant homology (greater than 10%) to any proteins that are available from DNA sequences stored in Sequence Data banks (Genbank, EMBL Data Library). If any of these putative polypeptides are expressed in the bacterial host, they are likely to be involved in the replication, maintenance, and stability of the pRi plasmid, because this region causes the plasmid to be more stably maintained than the region containing only *rep*A, *rep*B and *rep*C (Nishiguchi et al., 1987).

Analysis of the sequences 14613 to 14947 revealed no relevant open reading frames contiguous with this segment of pRi DNA.

In conclusion, these gene sequences will not be translated into proteins in the plant cell, due to lack of eukaryotic regulatory elements (promoters) that are required for the initiation of transcription. Information provided above demonstrates that the ori sequences do not confer pathogenicity or virulence in Agrobacterium, and are both physically separated and functionally distinct from DNA sequences responsible for inducing plant disease.

D. Conclusions for Section V.

Use of a nonpathogenic strain of Agrobacterium tumefaciens for plant transformation, the use of disarmed vectors, and the presence in the transformed plants of noncoding DNA sequences from Agrobacterium tumefaciens, A. rhizogenes and Cauliflower Mosaic Virus cannot, by any known mechanism, confer plant pest characteristics upon the Laurate canola plants.

VI. Potential for Environmental Impact by Introduction of Laurate Varieties

A. Safety of the gene products for nontarget organisms

This subsection covers concerns about "nontarget organisms" effects, which in this case is a somewhat awkward term, there being no "target organisms" associated with the Laurate canola. We have considered potential impacts of the gene products on humans, on vertebrate and invertebrate animals that could feed on the canola plants and seed, and on microorganisms. Potential impacts on plants are considered in later subsections of section VI.

Calgene continues to consult with appropriate staff at the U.S. Food and Drug Administration to assure that both oil and meal from Laurate canola are safe for consumption by humans and animals, respectively.

We conclude from the information provided below that products of the introduced genes in Laurate canola will have no significant impact on nontarget organisms.

1. Kanamycin resistance marker

a. APH(3')II safety and degradation

APH(3')II protein (aminoglycoside 3'-phosphotransferase II) is not toxic to humans. The protein occurs naturally, being produced by bacteria that colonize the human gut. Experiments have shown that the enzyme is inactivated (degraded) by pepsin in simulated gastric fluids and by simulated intestinal fluids, as is the case for any other typical protein (Appendix 7 of FLAVR SAVR Tomato Petition for Determination of Regulatory Status, May 31, 1992, not appended; Fuchs et al., 1993). Even if not degraded, APH(3')II will be inactive in the absence of the energy producing cofactor ATP and under the low pH conditions of the gut. Glycosylation and subsequent increase in the antigenic capacity of APH(3')II will not occur because APH(3')II does not contain the necessary sequence information for transport to the subcellular locations at which glycosylation reactions take place. Therefore, use of the *kan*^{r} gene in Laurate canola will not compromise efficacy of medical or veterinary use of kanamycin.

APH(3')II was shown not to have significant homology with known toxins and allergens (Appendix 7 of FLAVR SAVR Tomato Petition for Determination of Regulatory Status, May 31, 1992, not appended) and will not affect allergenicity of food products derived from Laurate canola.

There is no other known or hypothesized mechanism for the gene product, APH(3')II, to pose an environmental hazard.

b. Stability of *kan*^{r} Gene Product in the Environment

Calculations were made for potential release of APH(3')II into the soil from plant debris (FLAVR SAVR Tomato Petition for Determination of Regulatory Status, May 31, 1992. Appendix 2, Vol II, pages 312-314, not appended). It is not expected that such release of APH(3')II will pose an

environmental risk, since soil bacteria naturally produce this protein and much of it will likely be sequestered or degraded in the soil (FLAVR SAVR Tomato Petition for Determination of Regulatory Status, May 31, 1992. Appendix 2, Vol II, Sections E & G, not appended).

2. Laurate content of the plant

Laurate is a medium chain saturated fatty acid (12:0) that is accumulated in seeds of only a few cultivated plant species, the most important ones being coconut (Cocos nucifera L.) and oil palm (Elaeis guineensis). Laurate occurs in various foods, and there are a few concentrated sources of laurate in the human diet (Table 14a). Laurate is a safe food substance with no known toxicity to humans or animals.

Exposure by ingestion of seed. Fauna ingesting Laurate canola seed should experience no harmful effects from exposure to laurate. Laurate is a normal part of the diet of many animal species in the United States. Young mammals are exposed to laurate since mammalian milk (most species) contains laurate (Kuksis, 1978.) Laurate is also synthesized in avian uropygial glands (Kolattukudy et al., 1981), rat red blood cells (Jenik and Porter, 1981) and in insects (Dillwith et al., 1993). Seeds of several important native plant species in the continental U.S. accumulate laurate, principally American elm, Sassafras, and California Bay[20] (Table 14b). Such seeds are likely to be eaten by a variety of fauna, including deer, rodents and birds. Introduced ornamental plants, such as the common hedge plant, Bay Laurel, are also significant sources of laurate.

Stored fats are used as sources of metabolic energy in insects. The relative amounts of individual fatty acids in stored fats in insects are primarily a function of the insect species, rather than a function of their diet (McFarlane, 1985). Fatty acids that are essential for development in at least some insect species are linoleic (18:2) and linolenic (18:3) (McFarlane, 1985). Other fatty acids may promote growth if ingested (e.g.. oleic acid, 18:1) but need not be supplied by the diet, since they can be synthesized by the insect. Fats ingested are broken down in the midgut by lipases into diglycerides, monoglycerides and free fatty acids, which are then absorbed into the plasma. Free fatty acids are oxidized within the mitochondrion via the β oxidation pathway, which removes two carbons per cycle. Thus, (simplistically), C:16 is oxidized to C:14 + acetyl-CoA, C:14 is oxidized to C:12 + acetyl-CoA, and C:12 is oxidized to C:10 + acetyl-CoA, etc. The liberated acetyl-CoA are used as building blocks for *de novo* synthesis of fatty acids (Friedman, 1985).

The primary insects that feed on canola are pests: cabbage seedpod weevil (Ceutorhynchis assimilis) and various Lygus species (Fribourg et al., 1989; Raymer et al., 1990) feed on the developing seed and flea beetles (Phyllotreta spp.) feed on cotyledons. These insects are neither threatened nor endangered (U.S. Department of the Interior, 1993). The response of most animals is to accumulate lipid reserves when on an adequate diet, rather than to increase reproduction (a major exception to this is aphids who increase

[20] California bay is the source of the TE gene in Laurate canola.

reproduction on a rich diet) (Dillwith et al., 1993). Laurate canola does not contain elevated levels of oil, so differences in nutritional content of the seed should be slight. Thus, we do not expect that plant pest insects eating Laurate canola seed or cotyledons would have a diet that is altered in any important way (i.e. the diet would neither be toxic, nor accelerate the life cycle nor be more conducive to reproduction).

Exposure by ingestion of pollen. Honey bees (Apis mellifera) depend upon various pollens for most of their nutrients, and pollen is particularly important for rearing broods (Stanley and Linskens, 1974). One of the most important sources of pollen is the dandelion, Taraxacum officinale, (Standifer, 1966; Stanley and Linskens, 1974). Dandelion species are widely distributed in North America, South America, Europe and Asia. Dandelion pollen is regarded as an excellent nutrient source for honey bee brood rearing, and is relatively high in lipids (15%)[21], and in laurate (13% by weight of pollen lipid) (Standifer, 1966). In contrast, pollen from B. napus cultivar 212/86 has less than 0.5% laurate[22] by weight of pollen lipid (Table 14c). Dandelion pollens are available and collected for up to 7 months of the year (Stanley and Linskens, 1974), whereas canola blooms for less than 1 month per growing season. Finally, pollen from Laurate canola does not have significantly greater levels of laurate than its nontransgenic parent (Table 14c) since the napin promoter is seed specific. Thus, laurate is already a major component of the honey bee diet, exposure to laurate will not be increased by commercial cultivation of Laurate canola and honey bees will not be adversely affected by ingestion of pollen of Laurate canola.

Exposure by ingestion of green matter. Elevated levels of Laurate are not produced in green tissue, since the gene coding for the 12:0 ACP thioesterase enzyme is driven by the seed-specific napin promoter (Crouch et al., 1983; Scofield and Crouch, 1987; Radke et al., 1988). Thus, insect pests that feed on green tissues will not be exposed to elevated concentrations of laurate. The primary consumers of canola green matter are the cabbage aphid (Brevicoryne brassicae), turnip aphid (Lipaphis erycimi), and two species of armyworm (Spodoptera exigua and Pseudaletia unipuncta) (Fribourg et al., 1989; Raymer et al., 1990). These insects are pests and neither threatened nor endangered (U.S. Department of the Interior, 1993). Aphids synthesize medium chain, saturated fatty acids (up to 7% C:12 and 7 -70% C:14) using a fatty acid synthase and a thioesterase enzyme (Dillwith et al., 1993). Beneficial insects that feed on aphids, e.g.. Lady bird beetles (Coccinellidae), are already ingesting laurate since it is synthesized in the aphid body. These beneficial species will not be exposed to elevated levels of laurate in their diets and should not be adversely affected by feeding on insect pests from Laurate canola.

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[21] Lipid content in pollen varies from 1 to 20% of the dry wieght (Stanley and Linskens, 1974).

[22] Major fatty acids in B. napus pollen are 18:3 (60%), 16:0 (22%), 18:2 (6 %), 14:0 (4%), 18:0 (3%) and 18:1 (2 %).

Table 14a. Laurate composition of dietary fats, percentage by weight [23].

Butter	2.8 %
Canola oil	≤0.1 %
Cheese, cheddar	1.7 %
Coconut oil	45 %
Coffee whitener, powdered	38 %
Cottonseed oil	0.4 %
Milk: cow human	2.1 % - 4.1 % 3.1 %
Nondairy whipped toppings	≤16 %
Palm Kernel Oil	45 %

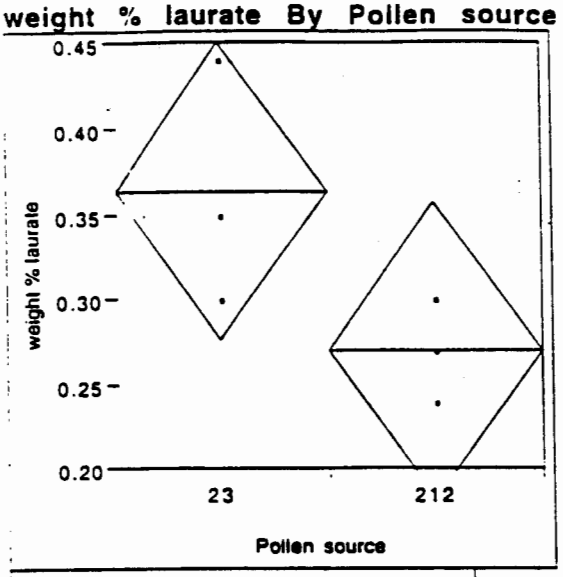
[23] References for Table 14a: CAST, 1991; Kuksis, 1978; Posati et al., 1975; Souci et al., 1990; Sebedio and Ackman, 1979.

Table 14b. Laurate composition of seeds of native* and introduced plant species in the U.S.[24].

American elm (Ulmus americana)*	5.9 %
Bay Laurel (Laurus nobilis)	35 - 45 %
California Bay, Oregon myrtle (Umbellularia californica)*	58 %
Sassafras (Sassafras albidum)*	17 %
Zelkova serrata	3 %

[24] Reference for Table 14b: Hilditch and Williams, 1964.

Table 14c. Laurate content (weight % Laurate methyl ester in lipid extracted from 20 mg pollen dry weight) in pollen collected from Laurate canola (event 23) and the nontransgenic parent variety, 212/86. Three replicates of 20 mg pollen samples per pollen source were analyzed by gas liquid chromatography. Data on laurate content were analyzed by a one-way analysis of variance using JMP, Version 2, SAS Institute Inc. Means are not statistically different at the 5% level.



Means

Summary of Fit

Rsquare	0.524064
Root Mean Square Error	0.054467
Mean of Response	0.316667
Observations (or Sum Wgts)	6

t-Test

t-Test	DF	Prob>|t|
2.09658873	4	0.1038

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	0.01306667	0.013067	4.4045
Error	4	0.01186667	0.002967	Prob>F
C Total	5	0.02493333		0.1038

Mean Estimates

Level	number	Mean	Std Error
23	3	0.363333	0.03145
212	3	0.270000	0.03145

3. The thioesterase enzyme

As described previously, the bay TE enzyme is functionally equivalent[25] to enzymes that are native to rapeseed and has 25 - 30% amino acid sequence identity to a long chain thioesterase native to canola. The function of this enzyme raises no safety concerns. Thioesterase enzymes that act to liberate medium chain fatty acids (12:0 and 14:0) are present in insects, birds and mammals (Knudsen et al., 1981; Dillwith et al., 1993; Kolattukudy et al., 1981). With very few exceptions, enzymes themselves are not toxicants or otherwise harmful (FDA, 1992). Enzymes are broken down during digestion in animal systems, as are most other proteins.

4. Naturally occurring toxicants

There are several types of naturally occurring toxicants in Brassica napus rapeseed and closely related species (Bell, 1984; Cheeke, 1989). Canola has been specifically bred to contain very low levels of these toxicants. By legal definition, canola seed contains less than 2% erucic acid as a percentage of the total fatty acids in the oil, and less than 30 μmoles/g of alkenyl glucosinolates in the defatted meal. These toxicants in canola have never been reported to cause illness when ingested in a normal human diet. These toxicants are discussed briefly below.

Glucosinolates (formerly termed thioglucosides) are found in all cruciferous crops including cabbage, broccoli and cauliflower (B. oleracea), turnip and Chinese cabbage (B. rapa), rapeseed (B. napus and B. rapa) and mustard seed (B. juncea.) More than 70 different glucosinolates have been identified. Levels of total glucosinolates in cabbage range from 264 - 1239 ppm fresh weight. Canola varieties have low levels (the upper limit in the specification is 30 μmol/g of alkenyl glucosinolates in the defatted meal).

Erucic acid is a monounsaturated fatty acid (C22:1) normally produced at concentrations of 20 - 60% in rapeseed oil. Canola oil, by definition, has 2% or less (by weight) of its fatty acid components as erucic acid (21 CFR 184.1555). This level is considered safe. Levels of erucic acid produced in seed of species in the family Brassicaceae are highly variable (Hilditch and Williams, 1964; Kumar and Tsunoda, 1980. See also Tables 22b and 22c in section VI.C.3. Issue 4). Field production of crops that produce high levels of erucic acid for industrial uses (e.g., high erucic acid rapeseed and crambe) is not restricted or otherwise regulated in the U.S.

The toxicants erucic acid and glucosinolates are the only known naturally occurring toxicants in rapeseed that have warranted regulatory action. Laurate canola is derived from a low erucic acid and low glucosinolate

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[25] Thioesterase enzymes cleave ACP from fatty acids. Different thioesterase enzymes demonstrate specificity for fatty acids of different chain lengths. We say that the bay TE enzyme is functionally equivalent to native enzymes because it cleaves ACP, even thought the native enzymes are active on longer chains and do not produce laurate.

canola variety. All canola varieties must meet specifications for levels of these toxicants. During field trials of the Laurate canola, different commercial varieties and transgenic lines exhibited location effects with regard to absolute levels of these components. All of the transformed lines with commercial potential (i.e., adequate yield and other agronomic traits) were within required specifications for erucic acid and glucosinolate levels at all locations (see for example Table 13b in section IV.B). Thus, there is no basis for concern over levels of these toxicants in the Laurate canola.

B. Gene transfer to organisms with which B. napus cannot interbreed

1. Potential transfer to microorganisms.

Concerns about potential transfer of DNA from transgenic plants to microorganisms primarily focus on the risk of pathogenic bacteria acquiring genes for antibiotic resistance, thereby compromising antibiotic therapy in human and veterinary medicine. To date, examples of such horizontal gene transfer are very few and difficult, if not impossible, to prove.

The probability of horizontal gene transfer is vanishingly small for the following reasons (IFBC, 1990):

1. The introduced genes are permanently incorporated into the plant chromosome,
2. Transgenic plants do not contain genes required for transport of DNA into bacteria, and
3. If bacteria acquired the DNA through passive uptake from decomposed plant tissue, the bacterial cell could not "read" the genes unless they had bacterial promoters.

Recently (Smith et al., 1992) arguments were presented for the occurrence of horizontal gene transfer. Two cases of transfer from eukaryotes to prokaryotes, and two cases of transfer from prokaryotes to eukaryotes are considered by the authors to be likely to have occurred throughout evolutionary time. If their assertions are true, these occurrences can still be considered to be extremely rare. For example, a worst case scenario for transfer of the *kan*^{r} gene from plant debris to bacteria predicts that the consequences of such transfer would be insignificant: the numbers of naturally occurring kanamycin resistant bacteria in the environment would continue to exceed any newly resistant forms by 7 orders of magnitude (Calgene, 1993).

Additional discussion of this issue was included in documentation provided to USDA/APHIS in a previous Petition (FLAVR SAVR Tomato Petition for Determination of Regulatory Status, May 31, 1992, not appended).

2. Potential transfer to plant species with which B. napus cannot interbreed.

The only potential mechanism for transfer of genetic material to plant species with which B. napus cannot interbreed would be through "bridging[26]." For example, within the genus Brassica, one could ask if a hybrid were formed between the Laurate B. napus and B. juncea, whether the hybrid could then cross into the weedy species B. nigra, since B. juncea and B. nigra share the bb genome. We discuss this potential immediately below, and conclude that the probability of this happening is essentially zero..

As we discussed in section II.E.3 above, formation of such hybrids (e.g.. B. napus / B. juncea hybrids) will be a rare event and persistence of hybrid individuals is extremely unlikely due to poor fertility, lack of any selective advantage of the introduced gene, high seedling mortality and poor germination. Further, the cross B. juncea X B. nigra is not fully compatible, and the cross between a B. napus / B. juncea hybrid and B. nigra should be even less compatible. Finally, since B. napus and B. nigra do not share a genome (refer to Figure 3), the introduced gene would have to be transferred via chromosomal crossing over to the bb genome in the B. napus / B. juncea hybrid in order to be stably introduced into B. nigra. Thus, we deem the probability for transfer and stable incorporation of the introduced genetic construct from B. napus to the closely related but not sexually compatible weedy species B. nigra via a bridging mechanism to be essentially zero. The probability of transfer of the introduced genetic construct to even less closely related species that do not share genomes, such as Sinapis arvensis, is also essentially zero[27].

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[26] Bridging is defined as "a mating made to transfer one or more genes between two reproductively isolated species by first transferring them to an intermediate species that is sexually compatible with the other two species," (King and Stansfield, 1985).

[27] Bing (1991) also concluded that "under the field environment of western Canada natural gene transfer from *B. napus* to *S. arvensis* and *B. nigra*, either directly or indirectly through bridging via *B. campestris* or *B. juncea*, would not occur."

C. Potential for plant pest introduction

1. Introduction

We have already discussed the safeguards employed in the utilization of DNA from plant pest organisms during the genetic modification process and the use of viable cells of a nonpathogenic strain of the plant pest, Agrobacterium tumefaciens, for plant transformation. These safeguards are sufficient to ensure that no plant pest characteristics are imparted to the transformants that could be attributed to the genetic modification process *per se*.

The other potential for plant pest introduction lies in the phenotype of the transformed plants. We have evaluated the potential for the transformed plants to be weedy pests, to transfer DNA to weedy and naturalized relatives, as well as the potential consequences of gene transfer to these relatives. In controlled experiments, the obvious experimental control is the parent canola line, 212/86. However, it is also instructive to look at characteristics of other canola varieties and to compare them to the general characteristics of Brassica species that are known to be weedy pests.

We used the issues identified by Tiedje et al. (1989) as a basis for our evaluation. For each of the issues listed below, we have consulted the published literature and in some cases conducted specific experiments designed to evaluate characteristics of the Laurate canola plants. The issues addressed are:

1. Weediness of Laurate canola
2. Invasiveness of Laurate canola
3. Persistence of Laurate canola
4. Selective advantage of the introduced genetic construct/phenotype
5. Pollen movement to nontransgenic B. napus
6. Outcrossing to wild relatives
7. Fate of B. rapa X Laurate canola hybrids

Based on our evaluation, we have concluded the following:

1. Laurate canola is not weedier than nontransgenic canola varieties.

2. Laurate canola is not invasive.

3. Laurate canola seed is not more likely to persist in the soil seed bank than is seed from its nontransformed parent variety or other canola varieties.

4. The Laurate phenotype does not confer a selective advantage in B. napus.

5. Fatty acid composition of seed is entirely unrelated to plant pest characteristics in the plant family Brassicaceae.

6. Spread of pollen from Laurate canola to other B. napus and to weedy relatives will occur at a very low frequency. Outcrossing will not be environmentally significant because the Laurate genetic construct does not confer a selective advantage.

7. The introduced genetic construct will not increase outcrossing frequency.

8. The vast majority of pollinations of B. napus relatives by B. napus pollen do not result in the formation of viable seed. Pollen from Laurate canola is not more able than that of nontransgenic canola cultivars to produce seed on B. rapa.

9. Crop/weed hybrids are not expected to become established in the field because of poor fertility (even when backcrossed to the weedy parent) and lack of any selective advantage of the introduced genetic construct.

10. Crop/weed hybrids formed with B. rapa or B. juncea will revert to the B. napus form, and thus be nonweedy. This phenomenon will significantly reduce the potential for introgression of the laurate phenotype into weedy relatives.

11. B. rapa X Laurate canola hybrids do not have the persistence characteristics of weeds and demonstrated no other selective or competitive advantage relative to B. rapa or control hybrids.

12. The development of Laurate canola is of no different and no greater environmental significance than the development of canola from High Erucic Acid rapeseed (HEAR), and carries no additional environmental or plant pest risk.

2. Literature Survey Summary

In order to evaluate the potential environmental effects from commercial scale cultivation of this Laurate canola, we have relied upon the following critical sources of information:

- Results from field trials in the U.S. with Laurate canola
- Results from field trials in the U.S. with transgenic High Stearate canola
- Environmental Assessment and Finding of No Significant Impact prepared by the United States Department of Agriculture for two such field trials conducted during the past two years in the U.S.
- Results from previous field trials in Europe with transgenic rapeseed
- Recommendations of participants at the USDA Workshop on Safeguards for Planned Introductions of Transgenic Oilseed Crucifers (USDA, 1990a)
- Evaluation of Agriculture Canada, Consultative Committee on Plant Biotechnology, 1990 and UK Dept. of Environment, 1993 (Appendix 6)
- Our literature survey.

These sources of information bolster our view that although there are environmental issues worthy of serious consideration relative to the widespread cultivation of transgenic canola, such concerns are thoroughly addressed for Laurate canola in this petition. We believe that the prospect of widespread cultivation of Laurate canola poses essentially no plant pest risk

As far as we have been able to determine, expert opinion is virtually uniform in assessment of the consequences of modifying seed oils in B. napus: genes regulating oil component profiles are not expected to confer a competitive advantage to the transgenic plants and are thought to pose little or no potential risk (Agriculture Canada, 1990; USDA, 1990a; UK DOE/ACRE, 1993). Thus, even when (or if) the laurate trait is passed to B. rapa or B. juncea through pollen, the modified oil trait is not expected to make these species weedier. Fatty acid composition in seed of Brassicas is quite variable, and is unrelated to whether the species is pestiferous (discussed in detail in section VI.C.3. Issue 4). As discussed above, B. napus exhibits only limited outcrossing potential under field conditions to naturalized and cultivated forms of B. rapa and B. juncea.

3. Discussion of issues raised by Tiedge et al., (1989)

Issue 1: Potential weediness of Laurate canola.

Literature: B. napus is the only Brassica species naturalized in the U.S. that is not considered to be a weed in the U.S. (Table 10; Holm et al., 1991; Weed Science Society of America, 1989).

Most crop plants are bred to express a set of traits that result in plants that are agriculturally useful. However, in general, these same plants are not very competitive in the natural environment without significant human intervention (e.g., cultivation and the application of fertilizers and pesticides). Canola is well adapted for cultivation, and poorly adapted for survival outside of cultivated areas. High plant populations are necessary for competition with weeds in the stand establishment phase. Well prepared seed beds and shallow sowing depth are necessary for this small seeded crop. The establishment of canola using reduced tillage practices has been difficult.

Tsunoda (1980) reports that without favorable, intensive cultivation, domesticated forms of B. napus cannot compete with common crop plants and that its naturalized forms are quite distinct from domesticated forms. In the U.S. and Canada, naturalized forms of B. napus are sporadically distributed. In the UK., naturalized forms of B. napus are more widespread (Mitchell-Olds, 1992) but the species is not classified as a weed in the UK (Holm et al., 1991). It remains unclear whether these UK populations are self-sustaining, or merely the result of repeated introductions (van der Meijden and de Vries, 1992). Research is underway to elucidate this (P. Dale, personal communication).

Experimental: In order to evaluate the weediness potential of Laurate canola, we have conducted numerous controlled field tests in locations representative of three U.S. growing regions (see Table 2) and directly compared characteristics of the transgenic and parental lines. Since the parent variety is not considered weedy, our experiments were designed to determine weediness of the transformed canola relative to the parent variety.

As indicated in Section IV above, the Laurate canola lines did not differ from the parent variety in their agronomic characteristics or morphology (Table 13a and 13b). Traits that might indicate greater potential for weediness such as earlier bloom or consistently higher seed yield were not present in the Laurate lines (Table 13a and 13b, and Field trial reports, Appendix 1). None of the transgenic Laurate lines had increased seedling vigor or winter survival compared to the parent (Table 13b). Persistence is discussed under Issue 3, below.

Conclusion: The Laurate lines are not more likely to be weedy than is the parent variety, 212/86.

Issue 2: Potential invasiveness of Laurate canola

Literature: The invasive potential of transgenic B. napus has been evaluated under field conditions in the UK. (Cherfas,1991) and it was found to be non-invasive. Preliminary data indicated "that these plants do not outgrow their competitors in the wild, nor is there any evidence that they pass on their foreign genes to other species." In these studies Westar, a widely grown canola variety, is compared to Westar engineered for resistance to the herbicide, Basta (syn. Ignite), or the antibiotic, kanamycin. Further results supported the initial assessment (Scheffler et al., 1992; Crawley, 1992; Crawley et al., 1993).

Transgenic oilseed rape was unable to become established and produce seed when sown to undisturbed vegetation in any of twelve experimental sites in the UK. over a two year period (Crawley, 1992.) Further, even in fertile, disturbed soil the transgenic rape had a net reproductive rate less than 1, meaning that it would become extinct without human intervention (Crawley, 1992; Crawley, et al. 1993). These findings can be summarized as:

- The ecological performance of B. napus is not affected by insertion of the kanamycin resistance gene or an herbicide tolerance gene using recombinant DNA techniques.
- There is no evidence that rapeseed is invasive of undisturbed natural habitats.
- There is no evidence that transgenic lines of rapeseed are more invasive or more persistent in disturbed habitats than their conventional counterparts.
- The net reproductive rate (replacement value (λ)) of transgenic rapeseed was less than one in the presence of interspecific plant competition (uncultivated plots) in the first year of the study.
- In the cultivated plots, λ was less than one by the second year of the study

Experimental: Since a modified oil profile may change the energy reserve of the germinating seedling, if there were a competitive advantage conferred by a change in fatty acid composition this would be expressed during seed germination and seedling establishment. If the modified oil profile conferred an advantage during seedling establishment, then such plants could potentially be more prone to invasiveness than standard canola. Experiments were designed to determine if such potential was exhibited by Laurate canola plants. Studies were conducted under both field and laboratory conditions to test for invasiveness over a range of conditions. For this petition, we report the results of germination and seedling establishment under weed- and self-competition.

Experiment 1. Field study

Fall planted canola in the southern U.S. is harvested in the late spring. The study was designed to follow the fate of seed that remains in the field following harvest. This information is summarized from a research report submitted to Calgene by Dr. Peter Kareiva and Ingrid Parker titled "Invasiveness in Oil-modified Canola: Field Study Conducted in Southeastern Georgia" included in Appendix 5.

Methods. Field grown seed of the B. napus parent variety 212/86, Laurate event 23, and four lines of canola variety A112 (parent and high stearate transgenics) were used in a randomized complete block design of cultivated and non-cultivated plots. Replicate samples of 50 seed were scattered within each plot, for a total of 600 seed for each genotype per cultivation treatment. All seed were produced under isolated field conditions to ensure genetic integrity. The site was sprinkle irrigated, soaking the soil to a 5 cm depth to prompt germination.

Results. Out of the 3,600 seed planted in cultivated soils, only 17 germinated under the harsh summer conditions at the two week census. In the weedy vegetation (uncultivated plots), germination was lower, 9 of 3,600 seed germinated. Chi-square analysis found no significant effects attributable to genotype. Observations at the 12 week monitoring point found no surviving Brassica napus plants. In contrast, common weeds, predominantly purple nutsedge, Cyperus rotundus, was established in both the cultivated and non-cultivated plots. (See the Kareiva and Parker report in Appendix 5 for a more complete list of weed species that became established in the plots). The transgenic lines were no more invasive than parental lines.

Table 15a. Canola plants surviving in the field after two and a half weeks and 12 weeks during a test for invasive potential. For each of six genetic lines, 50 seeds were scattered into twelve replicate plots in each of two treatment environments: cultivated soil and non-cultivated soil. A total of 7,200 seeds were scattered. Provided is the total number of survivors for each line in each treatment and the grand mean ± standard deviation.

	Survivors at 2 1/2 weeks		Survivors at 12 weeks	
	cultivated	non-cultivated	cultivated	non-cultivated
cv. A112	5	2	0	0
High Stearate-16	2	5	0	0
High Stearate-23	2	2	0	0
null	4	0	0	0
cv. 212/86	1	0	0	0
Laurate event 23	3	0	0	0
TOTAL	17	9	0	0
Grand Mean	2.8±1.5	1.5±2	0	0

Experiment 2. Greenhouse Study

To remove the potential effects of climatological factors (e.g.. drought stress) in the summer field study, a greenhouse study was performed concurrently with Experiment 1 using the same seedlots. The following information is summarized from a research report submitted to Calgene by Dr. Peter Kareiva and Ingrid Parker titled "Invasiveness in Oil-modified Canola: Greenhouse Study" included in Appendix 5.

Methods. Three transgenic lines of canola and one null segregant line were grown in competition with either a second individual of the same line, or of one of two parental canola varieties. Seed lots were the same as those used in experiment 1, field study. The trial included five replicates of each combination of lines, for a total of 140 plants. Seeds were planted into 4 inch pots and randomized with respect to position. Non-destructive growth measurements were conducted at 2 and 6 weeks. Insect damage (aphid density and damage from lepidopteran larvae) were censused periodically throughout the growth of the plants. Flowering and seed production are monitored every week.

Results. No differences were observed in any of the fitness characters measured. The transgenic lines were no more invasive than parental lines.

Table 15b. Fitness characteristics of Laurate canola compared to its parent measured in a greenhouse evaluation of invasive potential.

Fitness character measured	not different	different
2 wk seedling height	+	-
6 wk length of largest leaf	+	-
competition with self	+	-
competition with other canola	+	-
frequency of germination	+	-
frequency of heavy herbivore attack	+	-

Conclusions for Issue 2. Available data support a conclusion that the Laurate lines are not more likely than the parent variety to be invasive of either agricultural or nonagricultural ecosystems. Table 15c compares the elements of the PROSAMO (Crawley et al., 1993) studies with the studies undertaken using Calgene's oil-modified canola lines. Crawley found that competition from other plants (± cultivation) and preferential grazing by animals were the factors most responsible for the inability of B. napus plants to survive and reproduce outside agricultural settings. Studies completed with Laurate canola examined cultivation and competition with other plants and found no evidence of enhanced invasiveness in plants from oil-modified seeds. Studies designed to pay special attention to seed germination and seedling establishment, the phase of the life cycle where changes in seed oil should show the greatest effect, have not identified any cause for concern.

Table 15c. Comparison of studies for evaluation of invasive potential of Brassica napus transgenic lines.

Study Elements	Crawley et. al. 1993	Kareiva & Parker, 1993
genetic lines	nontransgenic: Westar, and transgenics: with *kan*^{r} & *pat*^{r} genes	nontransgenics: A112 & 212/86 and transgenics: Laurate & High Stearate
climates	3 climates, Sutherland, Berkshire, and Cornwall	3 climates, GA summer and fall, greenhouse
habitats	wet vs. dry sun vs. shade	natural south Georgia, greenhouse: no drought stress
experimental treatments * *treatments with major effects on plant demography*	± cultivation* ± vertebrate grazers* ± insect herbivores ± fungal pathogens	± cultivation ± self competition ± weed competition ± insect herbivores
characteristics determined	replacement value (λ) germination plant survival fecundity	replacement value (λ) germination plant survival fecundity
parameters measured	seedling density adult plant density mean seed production per plant	germination growth (weekly) rotting seed plants in flower seed output (number and weight)
span of study	1990-1992	May - 1993

Issue 3: Persistence of Laurate B. napus

Overview: The potential for B. napus to persist in agricultural fields has been discussed above in Section II.G. Species may be characterized as having either transient or persistent soil seed banks (Bradbeer, 1988). Cultivated B. napus forms a transient seed bank that is renewed by the shattering and/or the harvest loss of seed. In order to make a persistent seed bank the seed must exhibit considerable potential for dormancy. The ability to form persistent seed banks is common in weeds. For example, it is the characteristic of seed dormancy that contributes to the weediness of B. rapa and marks a clear distinction between wild and cultivated types and their plant pest status.

While the biochemical mechanisms of seed dormancy are not well understood, the role of dormancy in the ecology of plant populations has been the subject of study since the nineteenth-century and methods for the study of dormancy in both field and laboratory settings are well defined (Bradbeer, 1988).

For modified-oil canola, the composition of the energy source in the seed has been changed. Thus, for the Laurate canola seed, it is reasonable to test for changes in seed dormancy as an indication of potential to become a plant pest. For the purpose of this petition, of greatest importance is relative persistence of cultivated varieties and the transgenic line. However, we have included data from related wild, weedy species to serve as points of reference.

Literature: In the PROSAMO studies conducted in the UK., buried seed from transgenic oilseed lines consistently had greater mortality than the nontransgenic lines, and exposed seed were quickly eaten by small predators (Crawley, 1992.)

Seed bank decay and survival of transgenic Brassica napus has been studied under field conditions in the UK. and U.S. Seed of transgenic lines of Kanamycin resistant, Basta tolerant (cv. Westar, parent), and oil-modified High Stearate canola (cv. A112, parent) and parent cultivars (control) were tested for persistence in the seed bank. Results are summarized in Tables 16a and 16b. The percent of original buried seed found to be intact and dormant after up to 24 months of burial is presented. Eight replicates of 50 seed were buried either 2 or 15 cm (Crawley, et al. 1993) or five replicates of 100 seed were buried to a depth of 4 cm (Linder and Schmitt, 1993b; See also Mitten and Lindemann, 1993).

Although none of these studies were done with Laurate canola, we believe they are illustrative of results that would be expected for Laurate canola, based on seed dormancy experiments described immediately after this literature review section.

Table 16a. Percentage of control and transgenic canola seed intact and dormant after burial in the UK. (Crawley, et al. 1993).

average of 12 habitats in UK			
Months of burial	Nontransgenic Control	Transgenic Kanamycin^r	Transgenic Basta^{tol}
6			
12	3.6%	0.6%	0.4%
24	0.5%	0.1%	0%

Table 16b. Percentage of control and transgenic High Stearate canola seed intact and dormant after burial in the U.S. (Linder and Schmitt, 1993b).

% dormant (mean ± standard deviation)				
	California		Georgia	
Months of burial	Nontransgenic Control	Transgenic High Stearate	Nontransgenic Control	Transgenic High Stearate
6	56.6 ± 22.8	21.8 ± 12.3	3.2 ± 4.9	2.0 ± 2.5
12			1.2 ± 1.3	0.6 ± 1.3
14	18.4 ± 22.4	10.7 ± 15.9		
19			0.3 ± 0.5	2.0 ± 2.7

Results from these studies can be summarized as:

- Seed from three types of transgenic B. napus were not more persistent than the commercial nontransgenic canola varieties.

- Seed decay and exit from the seed bank was rapid. Linder and Schmitt (1993b) report half life of 1.5 months for High Stearate canola seed in California and 0.2 months in Georgia.

- Survival of the B. napus lines was not even close to comparable to the 60% one year mean survival observed for the weedy relative, Sinapis arvensis, included in the UK. study (Crawley et al., 1993).

- Neither commercial varieties of canola nor the transgenic types described in the literature review above (Basta tolerant, kanamycin resistant and High Stearate types) have the soil persistence characteristics of weeds.

Experimental: Experiments with Laurate canola were conducted under laboratory conditions to determine if Laurate canola has plant pest (e.g.. weedy) characteristics. Objectives of the studies were to evaluate dormancy potential in B. napus seed under conditions that are either conducive for germination in crop Brassicas or are known to induce dormancy in weedy Brassicas. Evaluation of baseline (true, noninduced) dormancy was also used to compare Laurate canola with weeds.

Results from these studies are summarized in Table 17. These results lead us to conclude the following:

Laurate canola will not be more persistent than standard canola cultivars grown in the U.S. Replicated experiments conducted under controlled environmental conditions demonstrate that field grown seed of the transgenic Laurate canola and standard B. napus canola varieties do not have the persistence characteristics of weeds: they have little or no baseline dormancy (up to 10 %), and they do not become dormant after exposure to temperature extremes. Seed germination and persistence characteristics of the Laurate canola are not significantly different than those of the parent canola variety, 212/86, or another variety, A112. Modifying the seed oil composition did not change the persistence characteristics of the Laurate canola seed.

Table 17. Seed Dormancy Summary. Data presented as mean percentage of seed that were dormant after the treatment specified.

Seed Line	Baseline [1] dormancy, 3 days at 25°C	High [1] Temperature Induced Dormancy, four diurnal cycles of 35/25°C	Low [2] Temperature Induced Dormancy, 10°C for 10 days	Low [3] Temperature Induced Dormancy, 5°C for 7 days	Low [3] Temperature Induced Dormancy, 1°C for 7 days
cv. 212/86 [4]	0	0	0.8 ± 2	0	1 ± 2
cv. A112 [4]	9 ± 8	2 ± 3	0.7 ± 1.5	0.6	1 ± 2
Laurate, event 23	3 ± 6	0	3	0	2 ± 2
Bc9-90 [5]	49	17 ± 7	74	23	17 ± 5

[1] Experiment 4, Table 21, except the Bc9-90 (Table 25a, Issue 7). Field grown seed.

[2] Experiment 2, Table 19. Greenhouse grown seed (except Bc9-90 was field grown).

[3] Experiment 3, Tables 20a and 20b. Field grown seed (except Bc9-90 was greenhouse grown).

[4] B. napus canola cultivars.

[5] Wild population of B. rapa.

Introduction

Germination baseline data over a wide range of temperatures was established for the canola crop using five canola cultivars (Experiment 1). Controlled environment studies evaluated responses to cues for seed germination and dormancy at 10 °C in greenhouse grown seed (Experiment 2). Additional studies were conducted to evaluate the behavior of field grown Laurate seed at low and high temperature extremes (Experiments 3 and 4).

Experiment 1 was conducted by M.S.S. Rao at the University of Georgia under the direction of Dr. Paul Raymer. Experiment 2 was conducted by R. Linder under the direction of Dr. Johanna Schmitt of Brown University. Experiments 3 and 4 were conducted at Calgene.

Experiment 1. Germination rates of canola varieties over the temperature range of canola production.

The information provided is abstracted from: Rao, M.S.S. and P.L. Raymer. 1993a. Intra-specific variation in maximum percentage and rate of germination, and seedling vigor of Brassica napus in response to temperature. (submission planned for Crop Science, manuscript provided in Appendix 5).

Methods and Materials.

Rate of germination was determined over a period of 11 days using 4 replications of 50 seed incubated at 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45°C in the dark. The number of seed germinated were counted at 4 hour intervals for the first four days and then 12 hour intervals for the next seven days. After the 11 day test, non-germinated seed were incubated for an additional 7 days at 25°C to establish seed viability. Seed was scored as germinated when the root radicle became visible.

Results and Discussion

To provide a summary of germination potential, the cumulative percent of germination is presented for each of the cultivars over the tested temperature range in Table 18a. None of the cultivars germinated at 0°, 40°, or 45°C. At 5°C there were significant differences among cultivars (LSD 5%). Incubation at 25°C of the ungerminated seed from the lower temperatures found 51 to 98% of the seed capable of germination (Table 18b). The remaining, ungerminated seed were not tested for viability, but were presumed to be viable and under a low-temperature induced dormancy. Ungerminated seed from the higher temperatures was soft and rotten and thus, no longer viable.

Data to describe the rate of germination is provided by Rao and Raymer, 1993a in the appended report (Appendix 5). To summarize their findings, germination studies of canola seed samples representing a range of commonly grown cultivars, found that all the cultivars had >90% germination within 11 days over a temperature range of 10 to 35°C. Cultivar A112 showed a lower temperature threshold than the other lines in the test. When incubated in the 5°C temperature, A112 entered the log phase of

germination by 80 hours and reached optimum germination (98%) after 190 hours (Figure 1b in the appended report (Appendix 5). The other cultivars reached optimum germination after >220 hours and their optima ranged from 67 to 86%. At the higher temperatures, the cultivars evidenced similar germination profiles (Figures 1a-1h in the appended report).

Conclusion: Results showed that the germination of canola seed samples of a range of commonly grown cultivars had 99% germination within 11 days over a temperature range of 10 to 35°C. With such rapid germination under a range of temperatures, seed bank persistence is likely to be very limited. All of the cultivars were killed by very high temperatures.

Table 18a. Cumulative percentage germination after 11 days for commonly grown canola cultivars. Data provided are the percentage of total number of seed, mean of four replications of 50 seed.

	Temperature °C									
Cultivar	0	5	10	15	20	25	30	35	40	45
A112	0	98	98	100	99	98	96	86	0	0
Bingo	0	86	98	100	100	96	100	86	0	0
Ceres	0	67	96	98	100	100	100	99	0	0
Delta	0	75	100	98	100	100	100	90	0	0
Printol	0	86	91	96	96	97	96	97	0	0

Table 18b. Cumulative percentage germination of commonly grown canola cultivars in the post-germination test. Ungerminated seed from Table 18a were incubated an additional 7 days at 25°C. Seed not capable of germination at 35°C or higher were found to be dead in the post-germination test.

	Temperature °C									
Cultivar	0	5	10	15	20	25	30	35[a]	40	45
A112	92	100	98	100	99	98	98	86	dead	dead
Bingo	98	100	98	100	100	100	100	86	dead	dead
Ceres	91	98	96	98	100	100	100	99	dead	dead
Delta	85	100	100	97	100	100	100	90	dead	dead
Printol	51	100	91	96	96	99	98	97	dead	dead

[a] Remaining ungerminated seed were dead.

Experiment 2. Cues for seed germination and dormancy. This information is summarized from a research report submitted to Calgene by C. R. Linder and J. Schmitt (1993a, included in Appendix 5). The study was designed to follow the work of Adler et al. (1993) which described strong, innate dormancy cues for wild populations of B. rapa at 10°C.

Methods. Seed of the B. napus parent variety 212/86, Laurate event 23, and a weedy population of B. rapa were subjected to a complete two-way factorial of differing light and nutrient regimes in Petri dishes. Each data point is derived from 60 seed (20 seed per replicate, 3 replicates). Linder used the same B. rapa population for this study as did Adler et al. (1993). All seed was greenhouse produced, except the B. rapa seed which was collected from a wild population in Montana.

Individual treatment factors were: Light (full light, complete darkness, simulated foliage shade); Nutrient (half-strength or one-sixteenth strength 20-19-18 supplemented with micronutrients). The study was conducted at 10°C because previous studies (Adler et al, 1993) had shown dormancy induced in a weedy B. rapa at this temperature but not in a cultivated B. rapa. Germination was censused at 12 hour intervals for the first 5 days and daily for the next 5 days. After 10 days, ungerminated, intact seeds were tested for viability: seeds were stripped of their seed coats and tested for viability using tetrazolium staining.

Tetrazolium viability assay methods. Remove the seed coat and keep the seed moist. Once all the seed coats are stripped, place naked seed in a well-plate in 1% tetrazolium solution (2,3,5-Triphenyltetrazolium Chloride, source; Sigma T8877). Cover the seed completely with tetrazolium solution and incubate the seed at 37°C in darkness for at least two hours.

Interpretation of the staining patterns requires that three areas of the seed be living: the root radicle, the cotyledons and the leaf primordia (the junction of the cotyledons and the radicle (Linder, personal communication). Seed that failed to germinate but were viable in the tetrazolium assay were dormant.

Results. Results are summarized in Table 19. The entire Linder and Schmitt report is provided in Appendix 5. Dormancy in the B. rapa seed was 50 - 93%, which is typical. Dormancy in 212/86 was 0 - 3%. Dormancy in the Laurate seed was 0 - 8%. The statistical analysis (Table 19) shows a clear difference in the dormancy of the cultivated B. napus types (212/86, A112 and Laurate canola, event 23) and the wild B. rapa, and demonstrates that Laurate canola does not have the dormancy characteristics of the weedy pest. However, since there was an indication of potential for Laurate seed to exhibit more dormancy than 212/86 under limited conditions, we conducted additional studies using field grown seed and greater replication (Experiments 3 and 4).

Conclusions. Neither the Laurate canola nor cultivar 212/86 exhibited the dormancy characteristics of weedy pests in this limited study.

Table 19. Percentage of seed dormant, dead or germinating after exposure to 10°C for 10 days. Each data point is derived from 60 seed (20 seed per replicate, 3 replicates) and presented as the mean of raw (untransformed) data. After Linder and Schmitt, 1993, figures 3, 4, and 5 (Appendix 5). Note: Proportional data were arc-sine square-root transformed before analysis by Linder and Schmitt. Please see Appendix 5 for the transformed data.

% Dormant		High Nutrient	Low Nutrient	
Line	Light	Mean *	Mean *	Combined
Laurate	Dark	6.7% C	0.0% C	3% B
	Full	0.0% C	5.0% C	
	Shade	0.0% C	8.3% C	
212/86	Dark	0.0% C	0.0% C	0.8% B
	Full	0.0% C	3.3% C	
	Shade	0.0% C	1.7% C	
B. rapa, wild	Dark	50.0% B	59.4% B	74% A
	Full	75.0% A	81.7% A	
	Shade	81.7% A	93.3% A	
ANOVA Probability >F =		0.0000	0.0000	0.0000

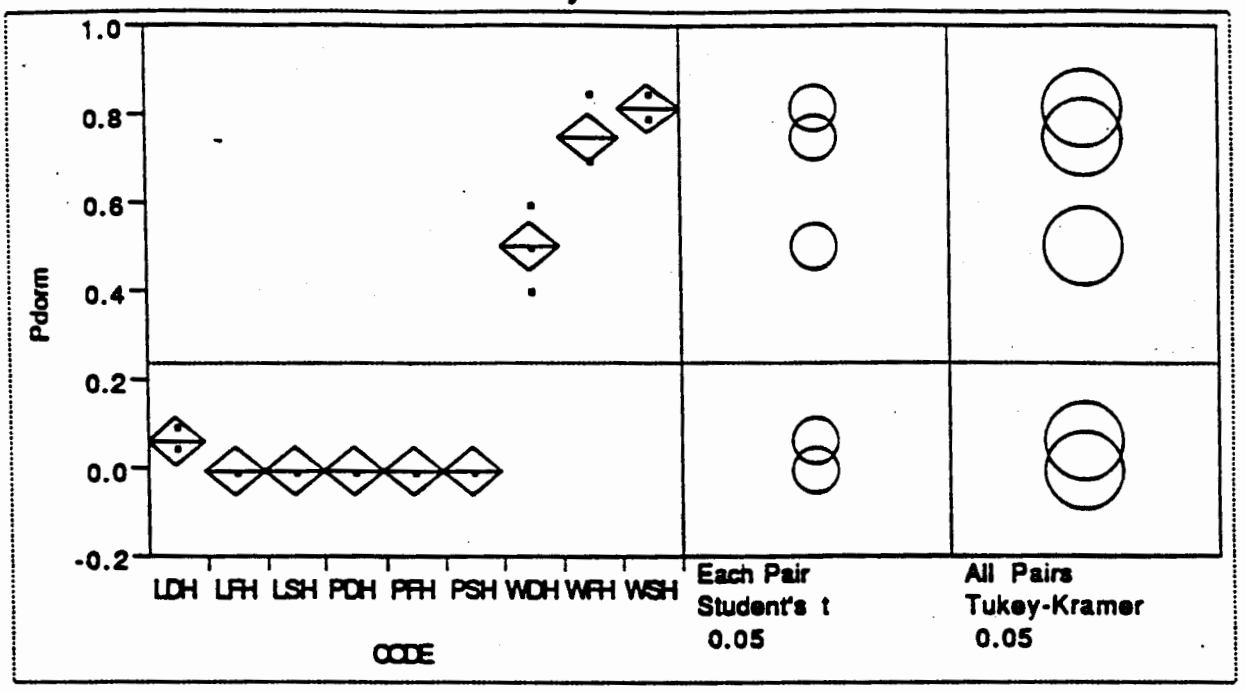
% Dead		High Nutrient	Low Nutrient	
Line	Light	Mean	Mean	
Laurate	Dark	20.0%	13.3%	10% A
	Full	8.3%	11.7%	
	Shade	1.7%	3.3%	
212/86	Dark	0.0%	1.7%	0.8% B
	Full	1.7%	0.0%	
	Shade	0.0%	1.7%	
B. rapa, wild	Dark	8.3%	3.3%	4% B
	Full	0.0%	5.0%	
	Shade	6.7%	1.7%	
ANOVA Probability >F =		0.1378	0.0672	0.0021

% Germinated		High Nutrient	Low Nutrient	
Line	Light	Mean *	Mean *	
Laurate	Dark	73.3% AB	86.7% A	98% A
	Full	91.7% A	83.3% A	
	Shade	98.3% A	88.3% A	
212/86	Dark	100.0% A	98.3% A	87% A
	Full	98.3% A	96.7% A	
	Shade	100.0% A	96.7% A	
B. rapa, wild	Dark	41.7% B	37.3% B	22% B
	Full	25.0% BC	13.3% BC	
	Shade	11.7% C	5.0% C	
ANOVA Probability >F =		0.0000	0.0000	0.0000

* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha =0.05

Table 19. Statistical analysis for the high nutrient treatments. JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.

Proportion Dormant for the high nutrient treatments
Pdorm By CODE



Means with confid. interval

Means

Summary of Fit

Rsquare	0.987106
Root Mean Square Error	0.046148
Mean of Response	0.237037
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	2.9346296	0.366829	172.2500
Error	18	0.0383333	0.002130	Prob>F
C Total	26	2.9729630		0.0000

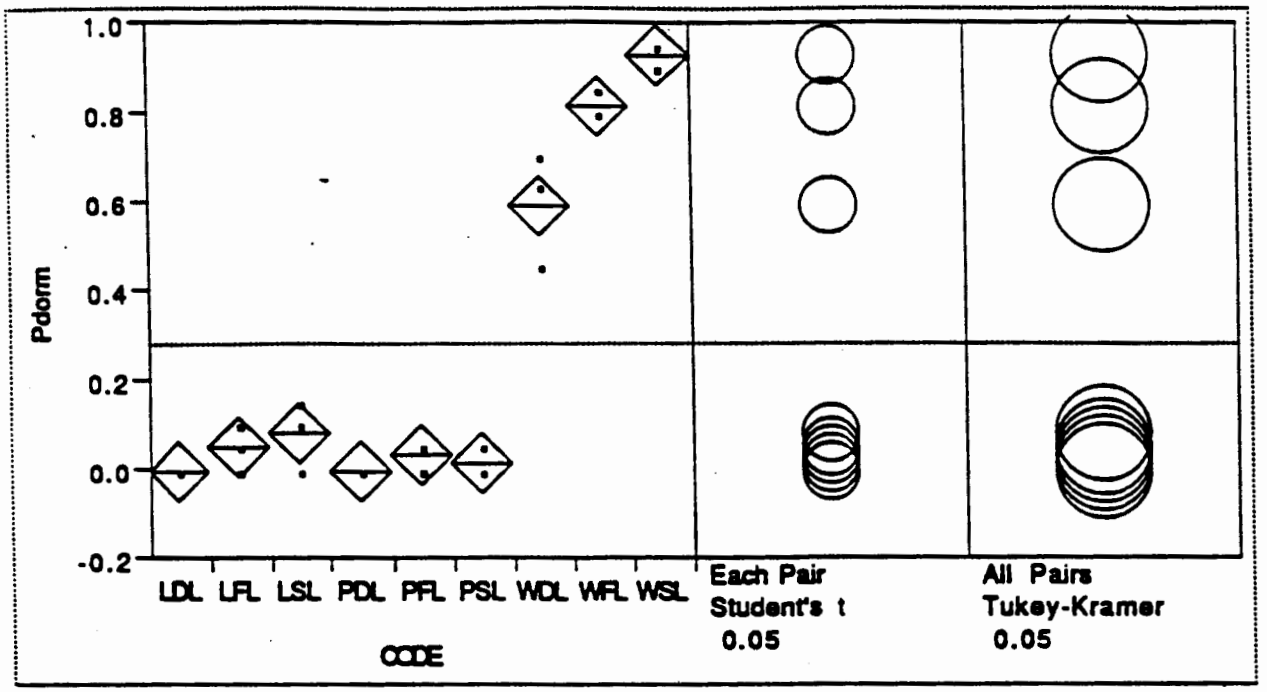
Mean Estimates

Line	Light	Level	number	Mean	Std Error
Laurate	Dark	LDH	3	0.066667	0.02664
	Full	LFH	3	0.000000	0.02664
	Shade	LSH	3	0.000000	0.02664
212/86	Dark	PDH	3	0.000000	0.02664
	Full	PFH	3	0.000000	0.02664
	Shade	PSH	3	0.000000	0.02664
B. rapa, wild	Dark	WDH	3	0.500000	0.02664
	Full	WFH	3	0.750000	0.02664
	Shade	WSH	3	0.816667	0.02664

Table 19. Statistical analysis for the low nutrient treatments. JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.

Proportion dormant for Low Nutrient Treatments

Pdorm By CODE



Means with confid. interval

Means

Summary of Fit

Rsquare	0.984386
Root Mean Square Error	0.056133
Mean of Response	0.280799
Observations (or Sum Wgts)	27

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	3.5757731	0.446972	141.8521
Error	18	0.0567175	0.003151	Prob>F
CTotal	26	3.6324905		0.0000

Mean Estimates

Line	Light	Level	number	Mean	Std Error
Laurate	Dark	LDL	3	0.000000	0.03241
	Full	LFL	3	0.050000	0.03241
	Shade	LSL	3	0.083333	0.03241
212/86	Dark	PDL	3	0.000000	0.03241
	Full	PFL	3	0.033333	0.03241
	Shade	PSL	3	0.016667	0.03241
B. rapa, wild	Dark	WDL	3	0.593860	0.03241
	Full	WFL	3	0.816667	0.03241
	Shade	WSL	3	0.933333	0.03241

Experiment 3. Seed germination and dormancy after exposure to near-freezing temperatures.

Introduction. Experiment 2 showed the potential for greenhouse grown Laurate seed to exhibit some dormancy after exposure to cold temperatures. Under conditions of commercial production, canola grown in the upper midwest will be harvested in the autumn. Seed which remains in the field and does not germinate before the onset of winter will be exposed to prolonged cold temperatures. In order to evaluate the potential for such seed to become dormant we conducted the following experiments.

Materials and Methods. The experimental temperature was 1 or 5 °C rather than the 10 °C used by Linder. Seed were incubated in distilled water, since results of Adler et al. (1993) indicate that the greatest differences between lines are exhibited in nutrient-free water. We also did not include different light treatments of the Linder study since we assumed that dropped seed would not be exposed to either foliage-type shade or full light conditions while exposed to freezing conditions in the midwest winter. Finally, we gave the cold-treated seed 5 days, rather than 2 days, to germinate at conducive temperatures, to make sure that observed "dormancy" was not an artifact of slower germination.

For low temperature dormancy testing, two studies were conducted. Seed samples were incubated first for 7 days at either 1 or 5 °C in the dark. Following a check for germination, samples were incubated at 25°C for 5 days in the dark. Seed were scored for germination and intact, non-germinated seed were tested for dormancy either via the tetrazolium viability assay or dormancy breaking treatment, GA_3 solution (200 mg/ml) in the dark at 25°C for 7 days.

Seed of the Laurate line and cultivars 212/86, A123L and A112 used in this study were harvested from Calgene's approved field plots in Michigan (1993) and 200 seed were used per treatment (8 replicates of 25 seed). Experiment 3 also included seed increased in the Calgene greenhouse of Bc9-90. All of the Michigan seed was 3-4 weeks post-harvest at the initiation of the study.

Results. Neither the commercial varieties nor the Laurate canola developed cold-induced dormancy following incubation at 1 or 5°C (Tables 20a and 20b). In no case did the B. napus seed exhibit dormancy comparable to that of wild B. rapa ($P = 0.05$).

Conclusions. Field grown seed of Laurate canola does not develop dormancy after exposure to cold temperatures. Thus, Laurate canola will not be more persistent than standard canola cultivars when produced in the upper midwestern U.S.

Table 20a. Percentage of seed that germinated, were dormant or were dead following cold treatment of 7 days at 5°C. Experiment 3, 25 seed per replication.

	number of seed per treatment	Mean % germination of cold-treated seed after 5 days at 25°C		Mean % dormant		Mean % dead
A123L	200	99	A	0.6	B	0.6
A112	200	99	A	0.6	B	0.6
Printol	100	98	A	1	B	1.2
212/86	200	99	A	0	B	1.2
Laurate, event 23	200	100	A	0	B	0
B. rapa (Bc. 9-90)	200	81	B	23	A	1.2
ANOVA Probability >F=		0.0000		0.0000		0.74928

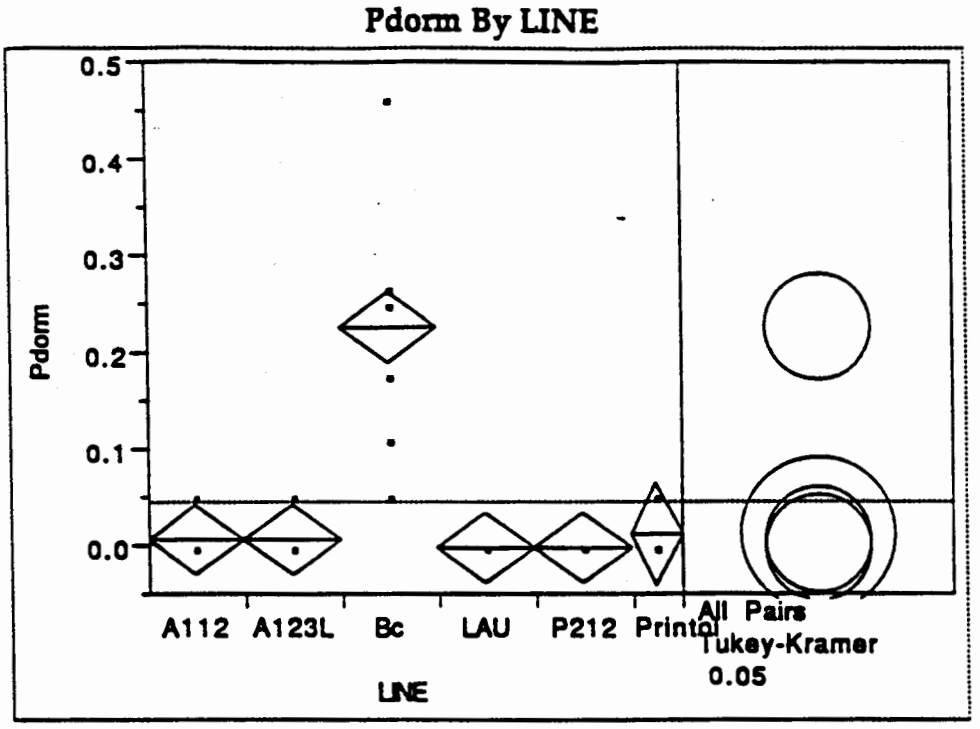
* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05

Table 20b. Percentage of seed that germinated, were dormant or were dead following cold treatment of 7 days at 1°C. Experiment 3, 25 seed per replication.

	number of seed per treatment	Mean % germination of cold-treated seed after 5 days at 25°C		Mean % dormant		Mean % dead	
A123L	100	97	A	0	B	3	AB
A112	100	89	AB	1	B	10	A
212/86	100	95	A	1	B	4	AB
Laurate, event 23	100	93	AB	2	B	5	AB
B. rapa (Bc. 9-90)	100	83	B	17	A	0	B
ANOVA Probabiltiy >F=		0.0072		0.0000		0.0348	

* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05

Table 20a. Statistical analysis for the proportion of dormant seed (5°C). JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.



Means with confid. interval

Means

Summary of Fit

Rsquare	0.745144
Root Mean Square Error	0.054164
Mean of Response	0.044916
Observations (or Sum Wgts)	44

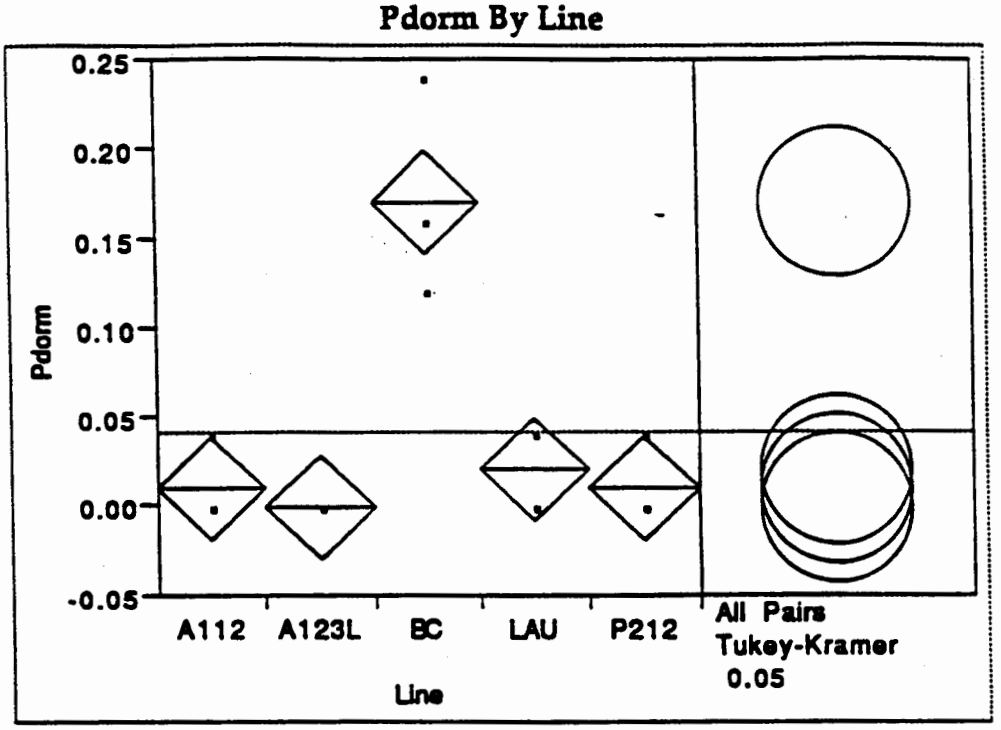
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	0.32594724	0.065189	22.2208
Error	38	0.11148101	0.002934	Prob>F
C Total	43	0.43742825		0.0000

Mean Estimates

Line	Level	number	Mean	Std Error
A112	A112	8	0.006579	0.01915
A123L	A123L	8	0.006579	0.01915
Bc9-90	Bc	8	0.227302	0.01915
Laurate	LAU	8	0.000000	0.01915
212/86	P212	8	0.000000	0.01915
Printol	Printol	4	0.013158	0.02708

Table 20b. Statistical analysis for the proportion of dormant seed (1°C). JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.



Means with confid. interval

Means

Summary of Fit

Rsquare	0.877014
Root Mean Square Error	0.027809
Mean of Response	0.042
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	0.08272000	0.020680	26.7414
Error	15	0.01160000	0.000773	Prob>F
C Total	19	0.09432000		0.0000

Mean Estimates

Level	number	Mean	Std Error
A112	4	0.010000	0.01390
A123L	4	0.000000	0.01390
BC	4	0.170000	0.01390
LAU	4	0.020000	0.01390
P212	4	0.010000	0.01390

Experiment 4. Effect of high temperature on seed germination and dormancy. A high temperature study using diurnal fluctuations performed at Calgene was designed predict the dormancy potential of spring harvested seed produced in the southeastern U.S. We consulted with Linder on the study design, who recommended using diurnal fluctuations of high (e.g.. 35 °C) and conducive temperatures (e.g.. 25 °C).

Materials and Methods. Standard germination test; 4 reps of 25 seed each (100 seed total per treatment), in distilled water. Seed were harvested from Georgia and California field plots (1993) and the seed was 12 to 16 weeks post-harvest at the time of the study. Treatments (4 replicates of 25 seed) consist of 0, 1, 2 or 4 days of exposure to diurnal temperature cycles of 35/25 °C, 8/16 hours in the dark. After temperature treatment, seed were moved to 25°C in the dark for germination. Following 3 days at 25°C, germinated seed were counted and intact, non-germinated seed were tested for viability using a tetrazolium assay.

Results. The seed exhibits some baseline dormancy (as expected). Dormancy is not increased by exposure to high temperatures (Table 21). No significant differences between transgenic and nontransgenic seed were noted.

Conclusions. Field grown seed of Laurate canola does not develop dormancy after exposure to high temperatures. Thus, Laurate canola will not be more persistent than standard canola cultivars when grown in the southeastern U.S.

Table 21. High temperature dormancy test. Percentage of seed germinating, dormant (viable in tetrazolium test), or dead (either non-viable in tetrazolium test or rotten) after exposure to diurnal temperature fluctuations. Each data point represents 100 seed (4 replications of 25) and the data are reported as mean±standard deviation. Baseline germination and dormancy are reported as 0 days in the diurnal treatment. Means comparison across the varieties showed no significant differences.

	Days of exposure to 35°C/ 25°C diurnal cycle	Mean % germination after exposure to diurnal cycles	Mean % of seed dormant	Mean % of seed that were dead
A112	0	91%±8	9%±8	0%
	1	98%±4	3%±6	0%
	2	98%±4	2%±4	0%
	4	97%±4	2%±3	1%±2
Laurate, event 23	0	97%±6	3%±6	0%
	1	96%±3	3%±4	1%±2
	2	97%±6	2%±4	1%±2
	4	94%±5	0%	6%±5
212/86	0	100%	0%	0%
	1	92%±4	0%	5%±4
	2	100%	0%	0%
	4	92%±10	0%	8%±10
ANOVA Probability >F= for all heat treatments combined		0.4069	0.6757	0.0564

Issue 4: Potential selective advantage of the introduced genetic construct

Literature: Modifications of oil composition achieved through classical breeding, such as the dramatic reduction of the percentage of erucic acid and concomitant increase in oleic acid associated with the development of canola (Table 22a), have not significantly altered agronomic characteristics of the crop. B. Stefansson, developer of the first registered canola variety, writes "In many years of developing and using varieties of rapeseed with low erucic acid content in the oil we have not been able to detect any other characteristic associated with high or low erucic acid content in the oil" (B. Stefansson, personal communication, Appendix 5).

We have seen no reports in the scientific literature that express concern that a modified seed oil profile in traditionally bred varieties of B. napus might make it weedy. Similarly, there is nothing in the literature to indicate there are any efforts or concern to prevent the movement of genes from high oleic acid (i.e.. low erucic acid) rapeseed into related Brassica species even though such relatives do not possess a canola-type fatty acid composition (Appelqvist, 1970; Kumar and Tsunoda, 1980).

Within the plant family Brassicaceae, fatty acid composition in seed varies widely: for example, concentration of erucic acid varies from 0 to 53 % and concentration of polyunsaturated fatty acids varies from 18 - 75% in the example species we examined. The same range of variation exists when the example species are divided into pestiferous and nonpestiferous groupings (Table 22b and 22c). There is no demonstrable relationship between seed fatty acid composition and plant pest status, rather, they are clearly independent. Thus, there is ample reason to expect that further alteration of the fatty acid composition in B. napus will have literally no effect on its lack of plant pest characteristics. By the same token, if the 12:0 ACP thioesterase gene were to move from Laurate canola into a weed species and be expressed, the resultant change in fatty acid profile would be expected to have no influence on the weediness or competitiveness of the recipient plant.

Table 22a. Fatty acid profiles (weight %) of high erucic acid rapeseed (HEAR) and canola oils. Major compositional differences are highlighted.

	16:0	18:0	18:1	18:2	18:3	20:1	22:1
HEAR	3.2	1.2	23.0	13.8	7.1	11.1	38.9
Canola	3.9	1.8	64.5	18.1	9.1	1.3	0.1

Table 22b. Comparison of fatty acid composition in seed among examples of pestiferous and nonpestiferous species of the family Brassicaceae that occur in the United States (Appelqvist, 1970; Hilditch and Williams, 1964; Kumar and Tsunoda, 1980).

Species	Fatty acid composition, % by weight of oil						
	Total Saturates[1]	18:0	18:1	18:2	18:3	18:2 + 18:3	22:1
Nonpestiferous species							
Brassica carinata	3.9 - 6.1	1	6.3 -11.8	11 - 19	7 - 16.7	18 - 35	36 - 53
Brassica napus (HEAR)	4.4	1.2	23	13.8	7	21	39
Brassica oleracea	7	2	8	20	15	35	36
Lepidium sativum	11	2	21	10	32	42	9
Lepidium virginicum	9	2	17	6	31	37	19
Matthiola bicornis	9	3	14	12	63	75	0
Raphanus sativus	8	1.7	25	15	12	27	25
Pestiferous species							
Brassica nigra	5.7	1.7	9	13	16.5	30	46
Brassica rapa (turnip)	3 - 5.7	1 - 1.5	11 - 33	13 - 26	8 - 13	23 - 39	12-47
Brassica tournefortii	4.7	1.0	8.8	12	13.5	25	47.8
Capsella bursa pastoris	15	6	11	18	35	53	0
Diplotaxis muralis	14	2.8	10.6	20	30	50	19
Hirschfeldia incana	4.4	1.0	9	12	17	29	41.5
Lepidium densiflorum	8	2	18	5	42	47	14
Lepidium latifolium	7	2	15	34	36	70	0
Malcolmia africana	12	2.0	14	15	58	73	0
Sinapis alba	5	1.3	16.7	7.7	10.5	18	53
Sinapis arvensis	5.8	1.5	11.5	14	16	30	34
Thlaspi arvense	4	0.6	10.7	18.1	23.1	41	30.6

1 16:0 plus 18:0. Some of the species may also contain small amounts of another saturated fatty acid, myristate (14:0).

Table 22c. Summary of comparison of fatty acid composition in seed among examples of pestiferous and nonpestiferous species of the family Brassicaceae that occur in the United States.

	Range of fatty acid composition, % by weight of oil						
	Total Saturates[1]	18:0	18:1	18:2	18:3	18:2 + 18:3[2]	22:1
Nonpestiferous species	4 - 11	1 - 3	6 - 25	6 - 20	7 - 63	18 - 75	0 - 53
Pestiferous species	3 - 15	1 - 6	9 - 33	5 - 34	8 - 58	18 - 73	0 - 53

1 16:0 plus 18:0. Some of the species may also contain small amounts of another saturated fatty acid, myristate (14:0).

2 Polyunsaturated fatty acids

The environmental risks of potential transfer of the 12:0 ACP thioesterase gene from Laurate canola to related weedy species appear to be minimal or absent. Further, with the use of good agricultural practices such as properly controlled seed production techniques, appropriate isolation distances for maintenance of oil quality, and crop rotation, the transfer should be relatively rare. These control standards are the same as those used for classically-bred rapeseed, including varieties with disease resistance traits or other traits that could confer a selective advantage if transferred to a weedy species.

At the USDA APHIS Workshop on Safeguards for Planned Introductions of Transgenic Oilseed Crucifers (USDA, 1990a) there was much discussion of genes that might have positive, neutral or detrimental effects within natural populations of crucifers. The general consensus (adapted from USDA, 1990a), presented as a continuum diagram, was :

Selective Advantage	Neutral	Selective Disadvantage
Stress tolerance	Marker genes (kan, gus)	Male Sterility
Disease Resistance	Lipids	
Insect Resistance	Seed Storage Proteins	
Low Glucosinolates[1]		
Herbicide resistance		

[1]Others thought this to be of selective disadvantage

In this scheme, transgenic canola containing a Brassica gene that confers an altered oil profile (lipids) is in the neutral or perhaps the selective disadvantage category. Thus, plants receiving the 12:0 ACP thioesterase gene are not expected to be more competitive, and may be somewhat less competitive than their parents. Based on the analysis derived from the information in Tables 22b and 22c (above) we expect that the effect would be neutral.

It is also important to remember that it is common in traditional plant breeding to introduce traits which may be expected to confer a selective advantage such as stress tolerance or disease resistance. These breeding practices are considered acceptable, and we have not seen them questioned as constituting any type of a hazard. There may be several reasons for this:

- Breeders continue to use wild germplasm as a source of desirable agronomic traits (Warwick, 1993). Weeds tend to be more resistant to various stresses and diseases than do crops. For example, in a survey of 18 species of wild crucifers, all were resistant to blackleg disease (Salisbury, 1988). Since the wild species already are resistant, gene flow from the crop back to the weed would not confer a new advantage.

- If gene flow to weedy relatives and incorporation of genes conferring a selective advantage were likely then there should be evidence of gene flow in the opposite direction as well (i.e. from the weed to the crop). B. napus has neither become drought tolerant from growing in proximity to B. juncea nor resistant to blackleg from growing in proximity to B. nigra (or many other species).

- Breeders and seed producers are well aware of what precautions must be taken to preserve a particular oil profile in B. napus. If gene flow from weedy species such as Sinapis arvensis, or B. nigra was at all prevalent, it would immediately result in unacceptably elevated (unlawful) levels of erucic acid in the crop (fatty acid composition is determined by the genotype of the embryo, not the maternal parent). Since producers isolate the crop from B. rapa, B. juncea and high erucic varieties of B. napus only, gene flow from other weedy species must be rare and have little effect.

Opinions expressed by foreign government agencies concerning the relative environmental risks of conducting contained field trials with transgenic oil-modified B. napus and transgenic B. napus with other types of modifications.

B. napus canola is an important crop in Canada and Europe, as discussed in Section II.B. Governments in producing nations have expended considerable thought and funding on research in the crop. The evolution of their regulations reflects the current status of experience and perspective concerning risks associated with contained field trials.

Canada: A section from the Summary of the Consultative Committee on Plant Biotechnology, Agriculture Canada, "Proposed Changes for 1992" (Appendix 6) is included to provide background information. In an official proposal of the Plant Products Division, gene-based potential risks associated with the small scale release of transgenic plants are reviewed. Plant material which has been modified to include components such as the gene marker nptII (AKA *kan*^{r}), genes native to the species being altered, and genes producing nutritional/compositional changes would be placed in a low risk category, and regulatory oversight requirements would be reduced. In contrast, plant material modified to include traits such as novel pesticide tolerance, or novel insect and pest tolerance would be placed into a category requiring closer regulatory scrutiny.

United Kingdom: Transgenic oil-modified B. napus was recently classified as a "low hazard GMO" by the Department of the Environment and Advisory Committee on Release into the Environment in the United Kingdom. Transgenic B. napus lines carrying the kanamycin resistance gene and having modified oil composition qualify for the newly instituted "Fast track procedures" for field trials since "they do not possess inherent characteristics that pose a risk of damage to the environment and therefore there is no requirement to take special control measures" (UK DOE/ACRE 1993).

Experimental: Selective advantage was evaluated under both field and laboratory conditions.

Experiment 1. Seedling emergence from various depths. Experiments conducted by R. Linder at Brown University (Linder and Schmitt, 1993. Appendix 5).

Materials and Methods. Greenhouse grown seed of Laurate canola (event 23) and the parental line 212/86 were germinated in half-strength nutrient solution in the dark at 20 °C. After germination, they were planted at 10, 4, 0.5 and 0 cm depth (15 replicates per seed type per depth) and incubated in a greenhouse at 20 °C day (16 hr) and 17 °C night. Seedling emergence was recorded daily. Seedling vigor was evaluated by measuring biomass and calculating relative growth rates. If the Laurate canola were to possess a selective advantage it should be demonstrated during the seedling phase when it must rely on its altered seed storage lipid reserves for growth.

Results. Emergence. None of the seeds emerged from 10 cm, and all of the seeds emerged from 0 and 0.5 cm. The laurate canola germinated more slowly than the parent line, indicating that it would have a competitive disadvantage[29]. Low nutrient conditions (meant to simulate a nonagricultural setting) increased the germination delay of the laurate canola.

Results. Seedling vigor. Biomass of the Laurate seedlings was significantly less than the parent at 2 weeks, but equivalent to the parent at 4 weeks post-emergence. Between 2 and 4 weeks post-emergence the growth rate for the Laurate canola was greater than the parent 212/86, but not greater than the growth rate of another canola cultivar, A112.

Discussion. The emergence results seem to indicate that the Laurate canola would establish more slowly than the parental control. On the other hand Laurate canola seedlings became vigorous. If, under field conditions, the Laurate canola demonstrated increased growth rate, earlier bolting to shade competitors, and/or earlier seed set, this could indicate a potential for competitive advantage. Linder and Schmitt advised that further studies be done to determine the significance of their seedling vigor results.

Calgene has addressed the concerns of Linder and Schmitt by

1. Documenting the phenology of Laurate canola during several field seasons and demonstrating that Laurate canola does not bolt or mature more rapidly than its parent, 212/86 (Table 13b & Appendix 1).
2. Having competition studies performed with Laurate canola (see Issue 2, Invasiveness, above) which demonstrate that Laurate canola is not more likely to establish than is the parent 212/86.

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[29] We have not seen evidence of slower germination of the Laurate seed in field trials. However, since the seed used in the Linder and Schmitt studies had slow emergence, we felt it necessary to alter the experimental design of seed dormancy studies performed at Calgene, to give the seed 5 days to germinate after cold treatment. See Issue 3, Experiment 3.

Field experimentation.

Replicated field trials were conducted in California, Georgia and Michigan with the Laurate canola lines, as described earlier in this document (Section IV). Traits such as seed germination, seed yield, seedling vigor, bloom dates and maturity dates were measured in the parental line, other cultivars and transgenic lines. 212/86 and Laurate canola are both late maturing varieties. The transgenic lines did not exhibit increased tolerance to stress, disease or insects. The agronomic performance parameters measured for the transgenic lines (including seedling vigor) were within the range of expected values for the canola crop (Appendix 1).

Conclusion: Based on combined data from Calgene's field studies, growth chamber and field studies conducted by Kareiva and Parker (Issue 2) and the growth chamber study of Linder and Schmitt there is no indication that Laurate canola would have a selective or competitive advantage over standard canola. These results provide support for the views widely held by qualified experts that oil modification genes do not confer a selective advantage to oilseed Brassica. Finally, these results are as expected, since there is no demonstrable relationship between seed fatty acid content and plant pest status in the family Brassicaceae.

Issue 5: Pollen movement to nontransgenic B. napus

Introduction. The issue of pollen movement has provoked a great deal of recent research. However, it should be noted that pollen movement and successful outcrossing are not of environmental concern *per se*, but only if the introduced genes make the recipient weedy or invasive (Karieva et al., 1994). We have demonstrated above that Laurate canola is neither weedy nor invasive and that seed fatty acid composition is not related to plant pest characteristics. Thus, we believe that pollen movement, as discussed in Issues 5, 6 and 7 herein, is of secondary importance to this Petition.

Literature: The Laurate genetic construct could potentially move into wild or naturalized species of Brassica by production of hybrid progeny from crosses with the Laurate canola as either the pollen or maternal parent. The most likely path for movement of the construct into naturalized species would be through pollen from the Laurate canola. If the Laurate canola were the maternal parent, the vast majority of the seed from commercial fields would be rendered nonviable by crushing for oil production after harvest. Seed production is carried out under very strict isolation conditions, so the risk of producing hybrid seed in seed production fields is small. Hybrid seed from commercial canola fields that escaped harvest and remained in the field could germinate in subsequent years, but would likely be controlled by cultivation or herbicide application to the new crop (non-canola due to rotational practices) .

As discussed above (Section II.E.2), outcrossing percentages in both transgenic and nontransgenic B. napus measured by many different researchers under field conditions are low, and outcrossing rates decrease with distance. Still, it should be assumed that unless plants are isolated by very great distances, some outcrossing may still occur (Kareiva et al., 1994). We are not aware of any concern that oil modified canola would exhibit greater rates of outcrossing than existing varieties.

Related studies with transgenic High Stearate Canola

Outcrossing studies with Laurate canola have not been performed. The studies described below are included since they were conducted with a similar transgenic, oil-modified B. napus developed by Calgene that contains:

- a gene for oil-modification under control of the napin promoter (as does Laurate canola) and
- the kanamycin resistance gene under control of the 35s promoter (as does Laurate canola).

As indicated in the conclusions (below), we think that results from outcrossing studies with transgenic High Stearate canola are predictive of the behavior of Laurate canola since there is no reason to expect that Laurate canola would behave differently than High Stearate canola: both types of transgenics have tissue specific expression (seed) of an oil-modification gene

that should not affect pollen composition[30], size or weight and thus should not influence pollen movement by wind or bees. Also, the High Stearate studies were conducted in the United States. Interestingly, results for transgenic High Stearate canola are comparable to published results from studies with other types of transgenic and nontransgenic canola (compare Tables 7a, 7b and 7c in section II.E.2, and see also conclusions at the end of results for this experiment) even though these other experiments were conducted in distant geographical areas (Canada, UK and France).

Outcrossing in transgenic High Stearate canola: The information provided is abstracted from Morris et al. (1993. Appendix 5).

Introduction

Gene flow studies with transgenic High Stearate canola were conducted in the field under USDA/APHIS permits 91-168-01 and 91-205-01 in collaboration with Drs. William Morris (Duke University), Peter Kareiva (University of Washington) and Paul Raymer (University of Georgia). Seed samples for outcross analysis and honeybee behavior data were collected in the Spring of 1992 at the Baker County site in Georgia and the Yolo County site in California. Outcrossing rate was measured using a seed germination assay to detect resistance to the antibiotic, kanamycin. In addition, studies were conducted to examine the efficiency of various combinations of trap beds and bare isolation zones. Such a design allows the evaluation of not only isolation distance, but the value of plant material (barren or trap crop) in the isolation strategy.

Methods and Materials

The trials were planted in November 1991, plants flowered in March and April 1992 and seeds were harvested in May 1992.

Field Plot Plan The basic design for the yield trials treated thirty entries in randomized complete block design with four replicated ranges. Each range consisted of a fifteen plots arranged in a two tier fashion. The entire field was then 15 plots by 8 plots. Plantings were placed on pre-formed beds in California. Depending upon the equipment used, the bed width was either 5 (CA) or 6 (GA) feet wide. Plot length was 5 feet. The plots were surrounded on all four sides by a non-transgenic border 15 feet wide. The border ranges on the North and South section of the trial were expanded by 25 ft to accommodate four barren zones of either 5, 10, 15 or 20 ft. Each zone was enclosed along the North-South axis by a barrier fence or "bee raceway" constructed of shade cloth, 6 ft high. The barrier fence was in place at the beginning of flowering. Figure 1 of Morris et al., 1993 (Appendix 5) illustrates the modification of the basic plan to accommodate a pollen containment study carried out at the Baker County, GA and Yolo County, CA sites.

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[30] Fatty acid composition of pollen from Laurate canola is addressed in Table 14c, section VI.A.

Pollinator observations Domesticated honey bees, Apis mellifera, were the predominant visitor to canola flowers. Their behavior was observed at each of the two sites by Dr. Morris and his assistants. The relative frequency of visits of honeybees was determined by performing focal plant observations. At each of six locations at each site, the number of visits in a 10 minute period by each pollinator taxon to each of 8 focal plants was counted. Observations were repeated at several times daily over 8 days (CA) or 3 days (GA) at the peak of flowering.

The effectiveness of barren zones were quantified by following bees leaving the transgenic plots. A total of 783 bees at the California trial and 222 bees in Georgia were followed.

Harvest of seed Seed was collected from the north and south beds, the three outer beds and the trap beds in the trap crop experiment, to see how effective the trap beds and/or isolation zones were in reducing gene escape. For the 3 beds at the end of each row in the trap bed experiments, four evenly spaced plants were collected from every other row in each bed (16 plants per bed). From the 8 non-transgenic trap beds, four evenly spaced plants were collected from every other row in each bed (16 plants per bed, three beds per set).

In addition, seed was collected from the 5x15 ft border beds on the east and west edges of the plot to estimated gene dispersal over distance in a continuous bed. Seed was collected from one plant from each of the 7 rows in the bed at 5 distances (0, 1, 2, 10 and 15 ft) from the edge of the bed that faces the transgenics.

In all seed from 1,246 plants were collected at each site. Samples in Georgia were collected and threshed at the field site by Dr. Paul Raymer with assistance from Ameri-Can agronomist, Greg Mitchell. Seed harvest in California was accomplished by Dr. Bill Morris and his assistants. Field threshing and cleaning of the California seed was completed by Sarah Jones, of Calgene. Dr. Morris handled all the blind coding of seed envelopes prior to delivery to the Calgene laboratory for analysis.

Screening seed for Kanamycin resistance. This assay (a blind study) was performed at Calgene. From each envelope, a sample of 200 seed was surfaced sterilized and germinated under aseptic conditions in the presence of 200 mg/l G418, the kanamycin analog (Geneticin, Sigma). Seedlings were scored following 7 days in germination conditions; 20°C, 16 hour photoperiod. The differences were very clear, either growing and green (transgene present) or yellow cotyledons and no development past the cotyledonary stage (transgene not present).

Results and Discussion

The data from continuous border beds show that pollen transfer and successful pollinations decline rapidly as distance increases from the transgenic plots. Between adjacent plants, the outcrossing frequency was 1.9% in California and 3.4% in Georgia. At the 15 ft (4.5 M) distance, the outcrossing frequency had declined to 0.5% in CA and 0.6% in GA (Table 23).

Observed bee visitations in the open fields showed no preference for transgenic or non-transgenic lines.

Outcrossing rates obtained from Calgene's transgenic High Stearate canola field plots in two locations in the U.S. are comparable to published levels for nontransgenic canola, i.e. the introduced genetic construct does not increase outcrossing frequency. The High Stearate canola plants exhibit the same pollination characteristics as the unmodified parents, and as distance from the transgenic plots increases, pollen transfer and successful pollination drops rapidly (Table 23). Outcrossing rates measured in the U.S. are comparable to those measured in the UK., Canada and France with other genotypes of transgenic B. napus as discussed in section II.E above.

Conclusions

From these studies we conclude that the magnitude of outcrossing in Laurate canola can be sufficiently estimated from existing data gathered using other genotypes of B. napus, including those modified using recombinant DNA techniques (High Stearate, Basta tolerant and kanamycin resistant types). This position is supported by the work of Kareiva and Morris (1993). They report that results of gene flow studies at transgenic cotton trials were not significantly different between sites in 5 states. Further, results from their studies matched published results from a completely independent study with a different variety, different introduced genetic construct and different plot design and sampling methodology.

Table 23. Outcrossing rates measured in transgenic High Stearate canola[1] (adapted from Morris et al., 1993)

Location	Outcrossing %	Distance (M)
California	1.9	0.0
	1.0	0.3
	0.8	0.6
	0.7	3
	0.5	4.5
Georgia	3.4	0.0
	1.5	0.3
	1.2	0.6
	0.5	3
	0.6	4.5

[1] High Stearate canola is not the subject of this petition. However, we believe that these data are predictive of results that would be obtained for Laurate canola in similar experiments.

Issue 6: Outcrossing to wild relatives

Literature: As discussed above (Section II.E.2) Brassica napus can form fertile hybrids under field conditions in the U.S. with other B. napus, B. rapa, or B. juncea but not with B. nigra or Sinapis arvensis (Table 7). Hybrids can be formed with difficulty under greenhouse conditions between B. napus and B. carinata, but B. carinata is rarely cultivated and not naturalized: thus, we do not consider this to be a risk. Production of field hybrids with B. adpressa (Hirschfeldia incana) or Raphanus raphanistrum is extremely unlikely. Reports of hybrids formed with other species all used manual pollination methods and most also required intervention by man with such techniques as embryo rescue or bud emasculation (Table 9a; USDA, 1992).

B. rapa and B. juncea are the only B. napus relatives growing in the U.S. likely to form hybrids with the Laurate canola. These species do occur in some U.S. canola growing regions. However, B. rapa is much more prevalent and more likely to grow in proximity to the transformed canola than is B. juncea. In addition, hybrids with B. rapa are more likely because B. rapa is self-incompatible, whereas B. juncea is self-compatible. As discussed in Section II.E.2.b. above, although hybrids can be formed between B. rapa and B. napus, their fertility is low, fertility is not enhanced by backcrossing into B. rapa, and hybrids tend to revert to the B. napus form. Crosses between these species are least successful when B. napus is the pollen parent.

Experimental: Generation of crop/weed hybrids.
Materials and Methods.

Pollen from a Laurate canola line (event 23) and the parental B. napus line, 212/86, was used in open flower pollinations of a wild B. rapa population (denoted Bc9-90). The source of the wild population was seed families collected from the Bitterroot Valley, Montana, by Randy Linder (Brown University). All plants were grown in pots in a temperature controlled greenhouse (16 hour days, 18-24 °C day, 15-18 °C night temperatures). Self-incompatibility was confirmed in the Bc9-90 plants. Siliques were allowed to mature before seeds were collected, dried and counted.

Crossing methodology.

Mature flower pollinations are accomplished by hand emasculation of the floral bud the day before anthesis or early in the day of anthesis. Undehisced anthers are removed with forceps. The stigma surface is dusted with pollen from either a fresh flower from the pollen donor or stored pollen on a bee-stick (Williams, 1990). Each raceme is tagged using a 1 x 1 cm paper tag with light weight string and marked with the pollen source and date of pollination. All floral buds are removed that were not subject to the hand pollination (Downey et al., 1980).

Results.

Table 24 shows results. Low numbers of seed were set per pollination, (6 - 8) compared to the 20 - 30 seed set with same species crosses. The number of seed set on the B. rapa plants after pollination by Laurate canola and two commercial varieties in this test was within the range expected based on reports from the literature summarized in Table 9a (Section II.E.4).

Conclusions. Results using two cultivated B. napus varieties and one Laurate line as pollen parents in crosses with B. rapa were comparable to published values (Table 9a: 0.5 - 9 hybrids per pollination). Production of B. rapa X Laurate canola hybrids by manual pollination in a greenhouse occurred at a low frequency and should occur at a much lower frequency under field conditions.

Table 24. Generation of hybrids with B. rapa as the female parent and B. napus as the pollen donor.

Brassica rapa female	*Brassica napus* male	number of flower pollinations	number of seed obtained	seed per pollination[a]
Literature summary (Table 9a)	Literature summary (Table 9a)			0.5 - 9
Bc 9-90	cv. A112	55	409	7.4
Bc 9-90	cv. 212/86	58	333	6
Bc 9-90	Laurate canola event 23	175	1451	8

[a] Same species crosses yield 20 - 30 seed per pollination.

Issue 7: Fate of B. rapa X Laurate canola hybrids

Literature: If any hybrids were formed under field conditions, these would not be expected to be more weedy than existing populations of either species. First, the much reduced fertility of the hybrids limits their population growth (Bing, 1991; Salam and Downey, 1978). Second, the hybrids tend to be less competitive. In European studies, laboratory produced hybrids did not survive in the field (Cherfas, 1991). Third, hybrids tend to revert to the cultivated form, i.e. to B. napus rather than to the more weedy, B. rapa form (Bing, 1991; Salam and Downey, 1978). Finally, the oil modification phenotype is very unlikely to confer any selective advantage or plant pest characteristics (USDA, 1990a; Tables 22b and 22c in Issue 4, above).

Experimental: Germination and dormancy potential of hand-crossed hybrids[31] and parents was evaluated at temperature extremes. All hybrid seed lots were greenhouse produced. Examination of hybrids for potential dormancy at high (35°C) temperatures was completed at Calgene (Experiment 1). Linder and Schmitt completed dormancy and germination cues at 10°C (Experiment 2). Seed were limited so that not all of the hybrid types could be included in every experiment.

Experiment 1. Germination and dormancy of hybrids at high temperature.

Methods. Seed were sampled into four replicates of 25 and placed on filter paper moistened with deionized water in germination dishes (100 x 15 mm Petri dishes). Incubation began with 2 days at 35°C in the dark. Subsequently, the samples were incubated at 25°C for 2 days and germination scored. Samples of seed that had not been submitted to the 35°C incubation were placed in the 25°C to serve as baseline data for these seed lots. Hybrids were given a total of 5 days at 25°C to compensate for slow germination. Seed were scored for germination and intact, non-germinated seed were tested for dormancy via the tetrazolium viability assay. Methods were developed in collaboration with Dr. Paul Raymer, University of Georgia. The sources of seed for the parent cultivar, 212/86 and cv. A112 were the same seed lots used in the low temperature study described in Issue 3 Table 19a.

Results. The wild B. rapa seed (Bc9-90) exhibits a baseline dormancy of 49% (Table 25a), however the B. rapa X Laurate B. napus hybrids have little or no dormancy (3%). Exposure to high temperature (35°C) breaks the dormancy of the wild B. rapa seed but did not change the germination profile of the hybrids (Table 25b).

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[31] Produced as described in Issue 6, above.

Table 25a. Baseline germination and dormancy of the hybrid and control seed lots[32] used in the high temperature study. Data provided are the percentage of total number of seed, mean of four replications of 25 seed.

	germination 2 days at 25 °C	additional germination 5 days at 25 °C	dormant[a]	dead[b]
A112	100 A*	0 B	0 C	0 C
212/86	98 A	0 B	1 C	1 C
Bc9-90 (wild B. rapa)[c]	45 C	0 B	49 A	6 C
Tobin[d] x Laurate, event 23	36 C	2 B	0 C	61 B
Bc9-90 x Laurate event 23	69 B	11 A	3 C	17 C
Bc9-90 x A112	3 D	0 B	13 B	84 A
ANOVA Probability >F=	0.0000	0.0000	0.0000	0.0000

* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05

a viable seed in tetrazolium assay

b non-viable in tetrazolium assay

c 8% of Bc9-90 seed was lost in the tetrazolium assay

d cultivated variety of B. napus rapeseed

Table 25b. High temperature induced and enforced dormancy in the hybrid and control seed lots following two days at 35°C. Data provided are the percentage of total number of seed, mean ± standard deviation of four replications of 25 seed.

	germination after 35 °C for 2 days	germination after 25 °C for 3 days[a]	dormant[b]	dead[c]
A112	0 B*	65 AB	13 A	22 B
212/86	0 B	96 A	2 B	2 C
Bc9-90 (wild B. rapa)	0 B	83 AB	17 A	0 C
Tobin x Laurate event 23	3 A	10 C	0 B	87 A
Bc9-90x Laurate event 23	2 A	81 AB	3 B	14 BC
Bc9-90xA112	0 B	2 C	5 B	93 A
ANOVA Probability >F=	0.0074	0.0000	0.0000	0.0000

* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05

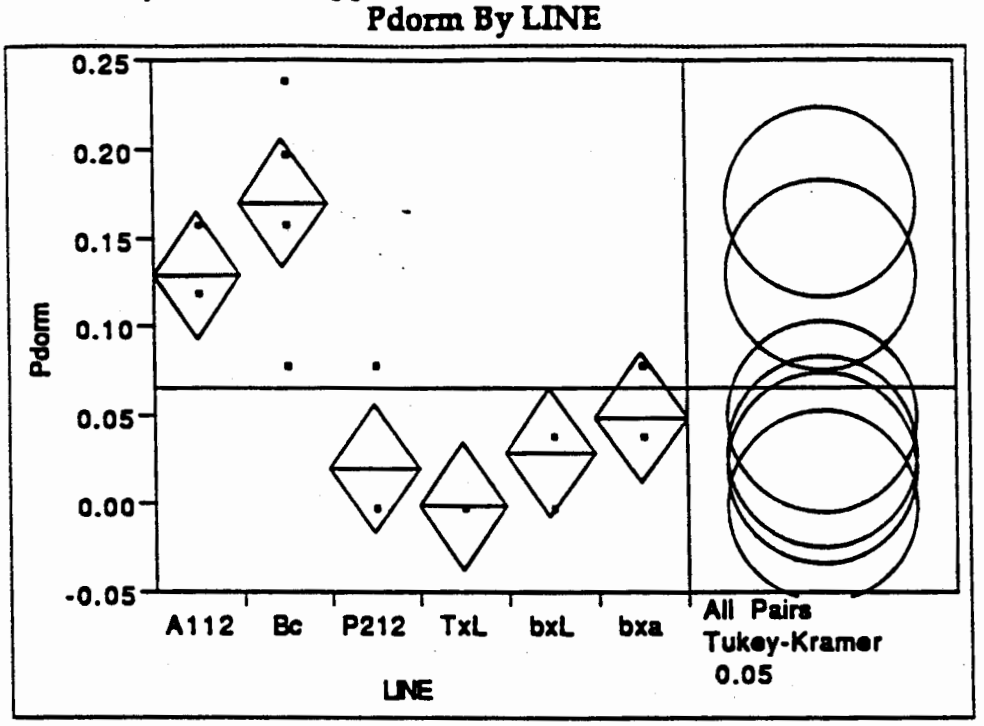
a = intact seed germinating following return to favorable conditions; 3 days at 25 °C.

b = viable seed in tetrazolium assay

c = non-viable in tetrazolium assay

32 Bc9-90 x 212/86 seed were used up in the Linder & Schmitt study (Experiment 2 below) and thus were not available for use in this high temperature study.

Table 25b continued. Statistical analysis of the proportion dormant of the high temperature treated seed. JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.



Means with confid. interval

Means

Summary of Fit

Rsquare	0.803738
Root Mean Square Error	0.035277
Mean of Response	0.066667
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	0.09173333	0.018347	14.7429
Error	18	0.02240000	0.001244	Prob>F
CTotal	23	0.11413333		0.0000

Mean Estimates

Lines	Level	number	Mean	Std Error
A112	A112	4	0.130000	0.01764
212/86	Bc	4	0.170000	0.01764
Bc9-90 (wild B. rapa)	P212	4	0.020000	0.01764
Tobin x Laurate event 23	TxL	4	0.000000	0.01764
Bc9-90x Laurate event 23	bxL	4	0.030000	0.01764
Bc9-90xA112	bxa	4	0.050000	0.01764

Experiment 2. Germination and dormancy of hybrids at low temperature. The information provided is from: Linder and Schmitt. 1993. A High Laurate Transgene's Effects On Seed Dormancy and Germination Cuing, and Seedling Emergence and Vigor. Experiment 1. (Report provided in Appendix 5).

Materials and Methods. Hybrids formed at Calgene (see Issue 6, above) were used in controlled experiments to evaluate seed germination and dormancy under conditions known to reveal the dormancy potential in a wild population of B. rapa, BC9-90. Seeds used were BC9-90, BC9-90 X Laurate F_1, and BC9-90 X 212/86 F_1. Seed were imbibed and incubated for 10 days at 10 °C in the dark, in full light, or in shade. Germination was censused, and nongerminated seed were moved to 20 °C. After 2 days at 20 °C germination was censused again, and nongerminated seed were tested for viability by tetrazolium staining. Growth rate of seedlings was also evaluated.

Results. The wild B. rapa population exhibited 50% - 93% dormancy (Tables 26a-c). In contrast, the hybrid control exhibited $\leq$13 % dormancy and the B. rapa X Laurate hybrid seed exhibited $\leq$13 % dormancy. Neither kind of hybrid had dormancy characteristics comparable to the weed, B. rapa ($P < 0.01$). These results indicate that the Laurate hybrids would be unlikely to persist in the seed bank, and would have high rates of seedling mortality due to germination at inopportune times.

The rate of germination of the hybrid seeds was insensitive to standard germination cues such as nutrient levels and light intensity (Figure 2 of Linder and Schmitt, 1993).

Discussion. One would have expected a strong maternal effect from BC9-90 to confer intermediate levels of dormancy on the hybrids (Adler et al., 1993), but this effect was absent. Since seed dormancy is an important part of the survival strategy of weedy B. rapa, it is clear that introgression of genetic material from Laurate canola into B. rapa would make it less, rather than more, able to persist.

Conclusion. The B. rapa X Laurate canola hybrids exhibited no selective or competitive advantage relative to B. rapa X control canola hybrids, and were less likely to persist than the wild B. rapa.

Table 26a. Average percentages of dormant, dead and germinated seeds from the laurate hybrid and controls after 10 days incubation at 10°C.

% Dormant [a]		High Nutrient	Low Nutrient
Line	Light	Mean *	Mean
B. rapa, wild	Dark	50.0% B	59.4% B
	Full	75.0% A	81.7% A
	Shade	81.7% A	93.3% A
Laurate Hybrid [c]	Dark	3.2% C	1.7% C
	Full	6.7% C	1.7% C
	Shade	13.3% C	3.3% C
Hybrid Control [d]	Shade	7.5% C	12.5% C
	Full	10.0% C	2.5% C
	Dark	2.5% C	2.4% C
ANOVA Probability >F =		0.000	0.000

* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05

% Dead [b]		High Nutrient	Low Nutrient
Line	Light	Mean	Mean
B. rapa, wild	Dark	8.3%	3.3%
	Full	0.0%	5.0%
	Shade	6.7%	1.7%
Laurate Hybrid	Dark	5.0%	1.6%
	Full	1.7%	1.7%
	Shade	3.3%	1.7%
Hybrid Control	Dark	2.5%	2.5%
	Full	2.5%	5.0%
	Shade	0.0%	2.5%
ANOVA Probability >F =		0.2678	0.9196

No difference in treatments

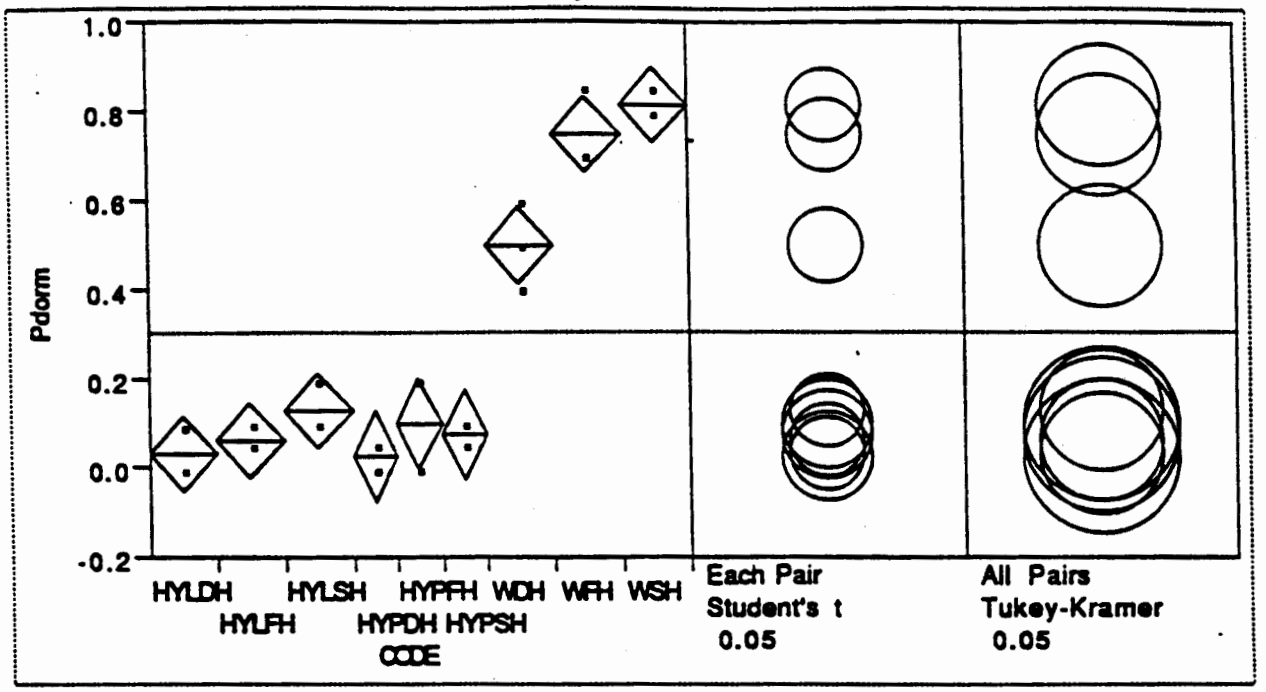
% Germinated		High Nutrient	Low Nutrient
Line	Light	Mean *	Mean
B. rapa, wild	Dark	41.7% A	37.3% A
	Full	25.0% AB	13.3% A
	Shade	11.7% AB	5.0% B
Laurate Hybrid	Dark	91.8% C	96.7% C
	Full	91.7% C	96.7% C
	Shade	83.3% C	95.0% C
Hybrid Control	Dark	95.0% C	97.6% C
	Full	90.0% C	95.0% C
	Shade	92.5% C	85.0% C
ANOVA Probability >F =		0.0000	0.0000

* Means Comparisons for all pairs using Tukey-Kramer HSD, Alpha=0.05

a Seed that failed to germinate and were viable in the tetrazolium assay
b Seed that failed to germinate and were nonviable in the tetrazolium assay
c B. rapa Bc9-90 X laurate canola, event 23
d B. rapa Bc9-90 X B. napus cultivar 212/86.

Table 26b. Statistical analysis for the high nutrient treatments. JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.

Proportion Dormant for the High Nutrient Treatments
Pdorm By CODE



Means with confid. interval

Means

Summary of Fit

Rsquare	0.969314
Root Mean Square Error	0.07002
Mean of Response	0.303967
Observations (or Sum Wgts)	24

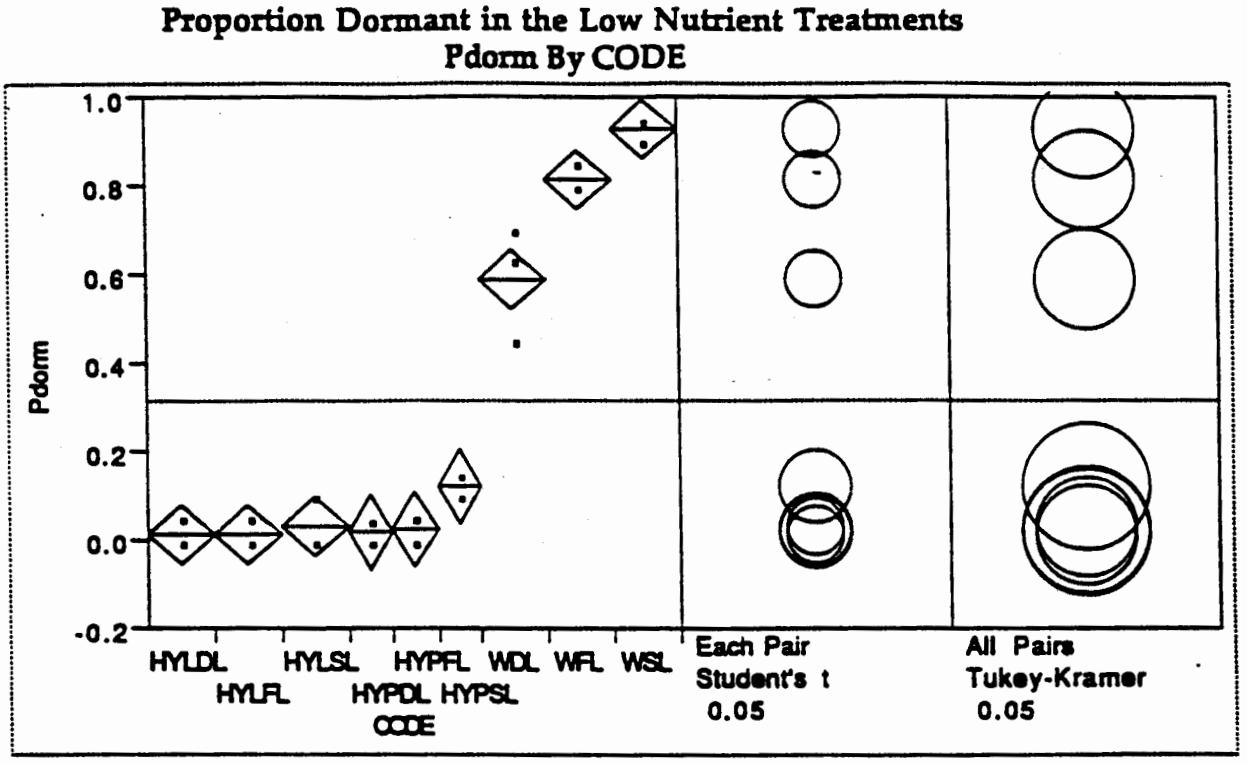
Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	2.3230234	0.290378	59.2269
Error	15	0.0735420	0.004903	Prob>F
C Total	23	2.3965654		0.0000

Mean Estimates

Line	Light	Level	number	Mean	Std Error
Laurate Hybrid	Dark	HYLDH	3	0.031733	0.04043
Laurate Hybrid	Full	HYLFH	3	0.066667	0.04043
Laurate Hybrid	Shade	HYLSH	3	0.133333	0.04043
Hybrid Control	Dark	HYPDH	2	0.025000	0.04951
Hybrid Control	Full	HYPFH	2	0.100000	0.04951
Hybrid Control	Shade	HYPSH	2	0.075000	0.04951
B.rapa, wild	Dark	WDH	3	0.500000	0.04043
B.rapa, wild	Full	WFH	3	0.750000	0.04043
B.rapa, wild	Shade	WSH	3	0.816667	0.04043

Table 26c. Statistical analysis for the low nutrient treatments. JMP® User's Guide, 1989. Version 2. SAS Institute Inc. Gary, NC, USA. pp.263-279.



Means with confid. interval

Means

Summary of Fit

Rsquare	0.985048
Root Mean Square Error	0.057938
Mean of Response	0.3158
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	3.3173210	0.414665	123.5301
Error	15	0.0503519	0.003357	Prob>F
C Total	23	3.3676730		0.0000

Mean Estimates

Line	Light	Level	number	Mean	Std Error
Laurate Hybrid	Dark	HYLDL	3	0.016667	0.03345
Laurate Hybrid	Full	HYLFL	3	0.016667	0.03345
Laurate Hybrid	Shade	HYLSL	3	0.033333	0.03345
Hybrid Control	Dark	HYPDL	2	0.023800	0.04097
Hybrid Control	Full	HYPFL	2	0.025000	0.04097
Hybrid Control	Shade	HYPSL	2	0.125000	0.04097
B.rapa, wild	Dark	WDL	3	0.593867	0.03345
B.rapa, wild	Full	WFL	3	0.816667	0.03345
B.rapa, wild	Shade	WSL	3	0.933333	0.03345

Table 27. Laurate hybrids seed dormancy summary. Baseline and induced dormancy in parental lines and weed X crop hybrids. Data are combined from several studies and presented as the percentage of seed that were dormant. See also descriptions of individual studies in text above.

Seed type	Baseline[33] dormancy (25 °C)	Heat induced[34] dormancy (35 °C)	Cold induced[35] dormancy (10 °C)
A112 (B. napus)	0 - 9	13	0 - 0.67
212/86 (B. napus)	1	2	0 - 2.5
BC9-90 (B. rapa)	45	17	50 - 93
BC9-90 X Laurate	3	3	1.7 - 13
BC9-90 X 212/86[36]			2.4 - 12.5
BC9-90 X A112	13	5	

[33] Percentage of seed dormant after incubation in distilled water in the dark for 5 days at 25 °C. Based on 100 seed (4 replicates of 25 seed).

[34] Percentage of seed dormant after incubation in distilled water in the dark for 2 days at 35 °C followed by incubation in the dark for 3 days at 25 °C. Based on 100 seed (4 replicates of 25 seed).

[35] Range of percentage of seed dormant after incubation in a two-way factorial design of light conditions and nutrients for 10 days at 10 °C followed by incubation for 2 days at 25 °C. Based on 40 hybrid seed per treatment (2 replicates of 20 seed) or 60 parental seed lines per treatment (3 replicates of 20 seed).

[36] Referred to as hybrid control by Linder and Schmitt (1993). These hybrid seed were used up in the Linder and Schmitt low temperature study and thus were not available for the baseline dormancy or high temperature studies conducted at Calgene.

VII. Statement of Grounds Unfavorable

Results of Linder and Schmitt (1993a) using greenhouse produced seed of Laurate canola indicated a potential for the Laurate canola to have more seed dormancy and increased seedling vigor relative to the parent, 212/86, under some environmental conditions. Linder and Schmitt did not believe that their studies were definitive. Rather their results led them to propose that further studies should be done to determine if the Laurate canola is likely to be either more competitive or more persistent than standard canola varieties. The Linder and Schmitt results were not corroborated by further experiments conducted using field grown seed and so we have concluded that Laurate canola is neither more persistent nor more competitive than standard varieties. We have discussed the results of our follow-up studies with Linder and Schmitt and they agree that studies with field grown seed are more relevant to the risk assessment. Finally, Calgene's experience with Laurate canola in field trials demonstrates that it does not have the persistence or invasive characteristics of weeds.

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IX. Appendices.

Appendix 1. Field trial results from field tests in the U.S.

Appendix 2. DNA sequence data

Appendix 3. Analysis of Laurate canola lines for DNA sequences outside the T-DNA borders

Appendix 4. Biosynthesis of fatty acids in plants

Appendix 5. Research reports, theses, regulatory submissions and preprints

Appendix 6. U.S., UK and Canadian government documents

Appendix 7. List of Preparers

Appendix 8. Literature references

Appendix 9. Weedy or naturalized members of the family Brassicaceae found in the United States