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Correlations between gametophytic (pollen) and sporophytic (seed) generations for polyunsaturated fatty acids in oilseed rape *Brassica napus* L.

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Summary. Lipids were extracted from the diploid seed and haploid pollen of *Brassica napus* L. Two fractions of pollen lipids, namely the diploid-specified pollen-coat and the haploid-specified internal cytoplasmic lipids were obtained. Significant correlations exist between pollen and seed generations for linoleic (18:2) and linolenic (18:3) acids. In pollen internal storage lipids, the level of 18:3 is positively correlated and the level of 18:2 is negatively correlated with the level of 18:3 in seed lipids. Evidence is presented that strongly supports the hypothesis that lipid biosynthesis occurs within the pollen and that synthesis is specified by haploid genes. These data support the concept of pollen selection, so that selecting among living pollen grains for superior individuals has potential as a new plant breeding tool for improving seed oil quality.

Key words: Pollen selection – Linolenic acid – Linoleic acid – Oilseed breeding – Lipid biosynthesis

Introduction

Selection procedures are normally applied to the diploid progeny of controlled matings. As an alternative, however, it may be possible to select non-destructively among living haploid pollen grains for those that will produce superior progeny. Recently some claims have been made for successful pollen selection for vigour, frost hardiness, salt tolerance, herbicide resistance and heavy metal tolerance (Ottaviano et al. 1980; Windsor et al. 1987; Zamir et al. 1982; Sacher et al. 1983; Smith 1985; Searcy and Mulcahy 1985).

Pollen selection would appear to have several advantages. First, the time of selection is advanced at least half a generation. Thus, selection may be applied twice within a generation. By utilizing a technique such as fluorescence activated cell sorting (FACS), millions of individual pollen grains might be screened quickly with high selection indices, thereby drastically reducing the space and resources that are normally required in conventional plant breeding. Selection among ungerminated pollen grains avoids potentially adverse pollen/style interactions (for example see Simon and Sanford 1985) and reduced ability to achieve seed set after in vitro culture. Furthermore, the haploid nature of pollen could be exploited to select directly for both dominant and recessive genetic factors.

For pollen selection to be a feasible approach there must be a large number of post-meiotically expressed genes in pollen, with a significant proportion of these expressed in both the pollen (haploid) and crop plant (diploid) generations. Nucleic acid hybridization studies in *Tradescantia* and *Zea* have suggested that at least 85% of the genes expressed in the diploid plant may also be expressed in its haploid pollen (Willing and Mascarenhas 1984; Mascarenhas et al. 1985). In *Nicotiana*, it has been estimated that RNA and poly(A)$^+$ RNA increase approximately 7 and 20 fold, respectively, in each pollen grain after meiosis (Tupy 1982), indicating that most of the RNA is of haploid origin.

Recent studies of oilseed rape have demonstrated some interesting qualitative correlations between pollen and seed, with respect to glucosinolate content (Dungey et al. 1988) and fatty acid composition (Evans et al. 1987). In *Brassica* spp. pollen lipids accounted for 31.7% of the dry weight of mature viable pollen and comprised two lipid domains (Evans et al. 1987). The internal cytoplasmic lipids accounted for 21.9% of pollen dry weight and were presumably specified by the haploid pollen genome. The remainder, the pollen-coat lipids,

Table 1. *B. napus* cultivars analysed and their lipid properties

Cultivars	Seed oil yield	Erucic acid	Linoleic/ linolenic ratio
Chikuzen C	High	High	Low
Wesroona	Low	Low	Low
81N057-5010	Low	Low	High
81N269-7	Low	Low	High
75N314-10	Low	Low	High
81N61	Low	Low	High

were specified by the diploid parental genome and were transferred from the tapetal cells late in pollen development (Heslop-Harrison 1968). The major fatty acids of the pollen internal domain were linolenic (18:3) and palmitic (16:0), which differ markedly from the pollen-coat fatty acids, namely myristic (14:0), 16:0, stearic (18:0) acid and 18:3. No relationship for erucic (22:1) acid, a nutritionally harmful fatty acid (Vles 1974), was found between pollen internal and seed lipids, although significant variation existed in the seed lipid of cultivars analysed (Evans et al. 1987).

In oilseed rape cultivars, significant variation exists within the seed for oil content, linoleic/linolenic acid ratio and erucic acid level (Table 1). Linoleic acid is nutritionally valuable (Ackman 1983), while linolenic acid is undesirable because its ready oxidation reduces the shelf life of the processed oil (Downey 1978). In oilseed rape 18:3 may be synthesized by the eukaryotic "18:3" and the prokaryotic "16:3" pathways (Williams et al. 1983). The main biosynthetic pathway (eukaryotic) involves desaturation, firstly of oleic (18:1) to linoleic (18:2) acid and then a further desaturation to 18:3. Oleic acid may also be utilized to form 22:1 via eicosenoic (20:1) acid, particularly in high erucic acid lines (Downey and Craig 1964). The polar or membrane lipids (e.g. phospholipids including phosphatidylcholine and glycolipids including monogalactosyldiacylglyceride) are intrinsically involved in the biosynthesis of linolenic acid (Williams et al. 1983; Browse et al. 1986). Linoleic and linolenic acid are transferred from phosphatidylcholine to form triacylglycerides (Slack 1983; Stobart et al. 1983; Stobart and Styme 1985), which are classes included in neutral or storage lipids.

In this report we describe the lipid and fatty acid analysis of pollen and seed of six cultivars (Table 1), including four high linoleic/linolenic ratio IXLIN lines (Roy and Tarr 1985). The pollen was fractionated into the coat and internal cytoplasmic domains by the differential solvent extraction method of Evans et al. (1987). The seed and pollen lipid fractions were further separated into neutral and polar lipid classes by silica gel column chromatography. The fatty acid compositions of the pollen were compared in an attempt to establish whether correlations exist that could form the basis of pollen selection for improved seed oil quality, and whether these data provide any clues as to the biosynthetic origin of pollen lipids.

Materials and methods

Plant material

Three pots (8") containing two plants for each of six cultivars of *B. napus* L. Chikuzen, Wesroona, 81N057-5010, 81N269-7, 75N314-10 and 81N61, (seeds supplied by Dr. N.N. Roy, W.A. Department of Agriculture), were grown in the University of Melbourne phytotron under controlled conditions. Temperatures were maintained at 25 °C (day) and 15 °C (night). For the first 8 weeks a 12 h day/night lighting regime was used. This was subsequently modified to a 16 h day to induce flowering. Lighting was provided by ballasted mercury lights. The relative humidity in the phytotron was 75% – 95%. Pollen was collected 11 – 16 weeks after sowing by the method of Evans et al. (1987). Seed was harvested approximately 28 days after pollination, approximately 3 weeks before maturity, to prevent sprouting in the pod because of the high humidity of the phytotron. The early harvesting would have had little effect on the fatty acid composition of the seed since Fowler and Downey (1970) showed that C18 [1] fatty acid composition was stable between 21 DAP and maturity for zero erucic acid cultivars.

Lipid extraction

The method used for analysis of the pollen and seed was modified from Evans et al. (1987). Pollen samples (90 – 210) mg were analysed in triplicate for each cultivar (excepting Chikuzen C, which gave only 1 sample). The acetone extraction of external pollen-coat lipids was modified by using a rotary evaporator (37° – 43 °C) to remove the solvent. The $CHCl_3$/MeOH (3 ml-2:1) extraction of internal cytoplasmic lipids was modified by adding an extra 2 ml of hexane to the lower phase, $CHCl_3$/Hexane-pellet (1:1), after removal of the top phase, MeOH/H_2O (1:1). Chloroform (2 ml) and hexane (4 ml) were then added to the pollen pellet for the second wash and the MeOH/H_2O phase was washed with 1 ml of $CHCl_3$. All $CHCl_3$ extracts were combined and the solvents removed by rotary evaporation.

Seed (ca. 1 g) was milled in a Krupps bench grinder and was extracted with a similar solvent/sample ratio to the pollen internals and a third hexane – $CHCl_3$ wash was included.

The pollen and seed extracts (ca. 40 mg sample for seed lipid) were separated into neutral and polar lipid classes by the procedure of Christie (1982). The lipid sample was loaded onto 1.3 g Silica (Silicar CC-4, Mallinckrodt) in a 10 mm diameter glass – teflon stop-cocked, column, in a small volume of $CHCl_3$ and eluted with 30 ml $CHCl_3$ (neutral lipids) and 30 ml MeOH (polar lipids); solvents were removed by rotary evaporation. Silica TLC (Kieselgel 60 G254, Merck) was used to observe the composition and separation of the lipid classes. The mobile phase was $CHCl_3$/MeOH/H_2O (65:25:4). Phospholipids, galactolipids and carbon-containing compounds were identified by zinzadaze, α naphthol and iodine, respectively (Christie 1983).

The percentage composition of the lipid classes was estimated gravimetrically and corrected to dry weight. Pollen dry weight was estimated thermogravimetrically (Dumas et al. 1983). Seed dry weight was determined by drying ca. 1 g of meal at 110 °C overnight for each replicate.

[1] Fatty acids with 18 carbon long chain

Table 2. Normalized fatty acid compositions (%) of lipids of three cultivars of *B. napus*

Fatty acid		14:0	16:0	16:3	18:0	18:1	18:2	18:3	20:0	20:1	22:1
Lipid fractions	Cultivar										
Neutral lipids											
Seed[a]	Chikuzen C	t	3.6	–	1.6	40.2	13.3	8.5	t	13.2	17.4
	Wesroona	t	5.9	–	2.8	57.9	21.6	7.0	t	1.2	–
	81N61	t	5.5	–	1.7	53.8	31.9	2.7	t	1.4	0.1
Pollen coat[b]	Chikuzen C	28.6	26.4	t	15.6	4.7	4.1	9.7	7.7	–	–
	Wesroona	15.7	27.1	t	13.5	4.8	4.6	9.9	10.6	–	–
	81N61	23.2	29.0	t	19.4	4.7	9.3	4.7	5.3	–	–
Pollen internal[c]	Chikuzen C	t	19.7	1.7	t	2.3	7.8	59.7	t	–	–
	Wesroona	t	22.1	1.9	t	2.9	6.9	57.5	t	–	–
	81N62	t	18.5	1.8	t	2.8	33.3	35.2	t	–	–
Polar lipids											
Seed[d]	Chikuzen	t	13.7	t	t	39.8	27.0	7.9	t	2.7	t
	Wesroona	t	12.4	t	t	46.3	29.2	5.7	t	t	–
	81N61	t	11.4	t	t	39.3	39.7	3.5	t	t	–
Pollen coat[d]	Chikuzen C	–	6.0	14.0	2.5	3.6	3.0	61.4	t	t	t
	Wesroona	–	4.7	11.6	3.9	2.7	1.8	64.5	t	t	t
	81N61	–	4.0	15.2	1.6	2.7	24.0	43.0	t	t	t
Pollen internal[e]	Chikuzen C	–	30.3	t	t	1.7	8.7	54.0	–	t	t
	Wesroona	–	29.6	t	t	2.4	8.6	55.7	–	t	t
	81N61	–	27.5	t	t	2.0	35.3	29.1	–	t	t

t = trace amounts < 2.0%
[a] = trace amounts 16:1, 20:0, 20:1, 24:0, 24:1
[b] = trace amounts 12:0, 16:1, 16:3, 20:1, 22:0, 22:1, 24:0, 24:1
[c] = trace amounts 12:0, 16:1, 20:0, 20:2, 22:0, 24:0, 24:1
[d] = trace amounts 16:1, 20:0
[e] = trace amounts 12:0, 24:0, 24:1

Fatty acid analysis

Fatty acids were analysed as methyl esters and structural confirmation was obtained using GC-MS as described by Evans et al. (1987).

Statistics

Statistical analysis was carried out on a Cyber 180/990 computer using the linear regression package of Spssx (Spss, Chicago, Illinois).

Results

Seed lipid characteristics

Lipids accounted for 32.2% of seed dry weight of which neutral lipids (storage lipids, including triacylglyceride) comprised greater than 90% of the total lipid. The fatty acid composition of this fraction included oleic (18:1) and linoleic (18:2), with significant amounts of linolenic (18:3) acid, with eicosenoic (20:1) and erucic (22:1) acids occurring in the erucic acid cultivar Chikuzen C (Table 2). Polar membrane lipids, including phospholipids and galactolipids, comprised less than 10% of the lipid fraction and had a fatty acid composition similar to that of the neutral lipids, except that 20:1 and 22:1 were not abundant (Table 2).

Pollen lipid characteristics

In the present study, lipids accounted for 23.8% of the dry weight of mature viable pollen. Internal cytoplasmic lipids represented 17.5% of the pollen dry weight, whereas in the pollen-coat they represented 6.3%. Internal pollen lipids consisted of 43.1% polar lipid, while the proportion of polar lipid in the pollen-coat lipids was 10.5%. Phospholipids and galactolipids were detected by TLC in both pollen lipid domains.

The fatty acid compositions of the pollen internal and pollen-coat lipid domains were quite distinct. The major fatty acids of both neutral and polar lipids of the internal domain were 16:0, 18:2 and 18:3. The ratio of 18:2/18:3 was slightly greater and 16:0 was more abundant in the internal polar lipids than in the internal neutral lipids (Table 2). In contrast, the main fatty acids of the pollen-coat neutral lipids were myristic (14:0) acid, 16:0 and stearic (18:0) acid, whereas the pollen coat polar lipids were more unsaturated and had longer car-

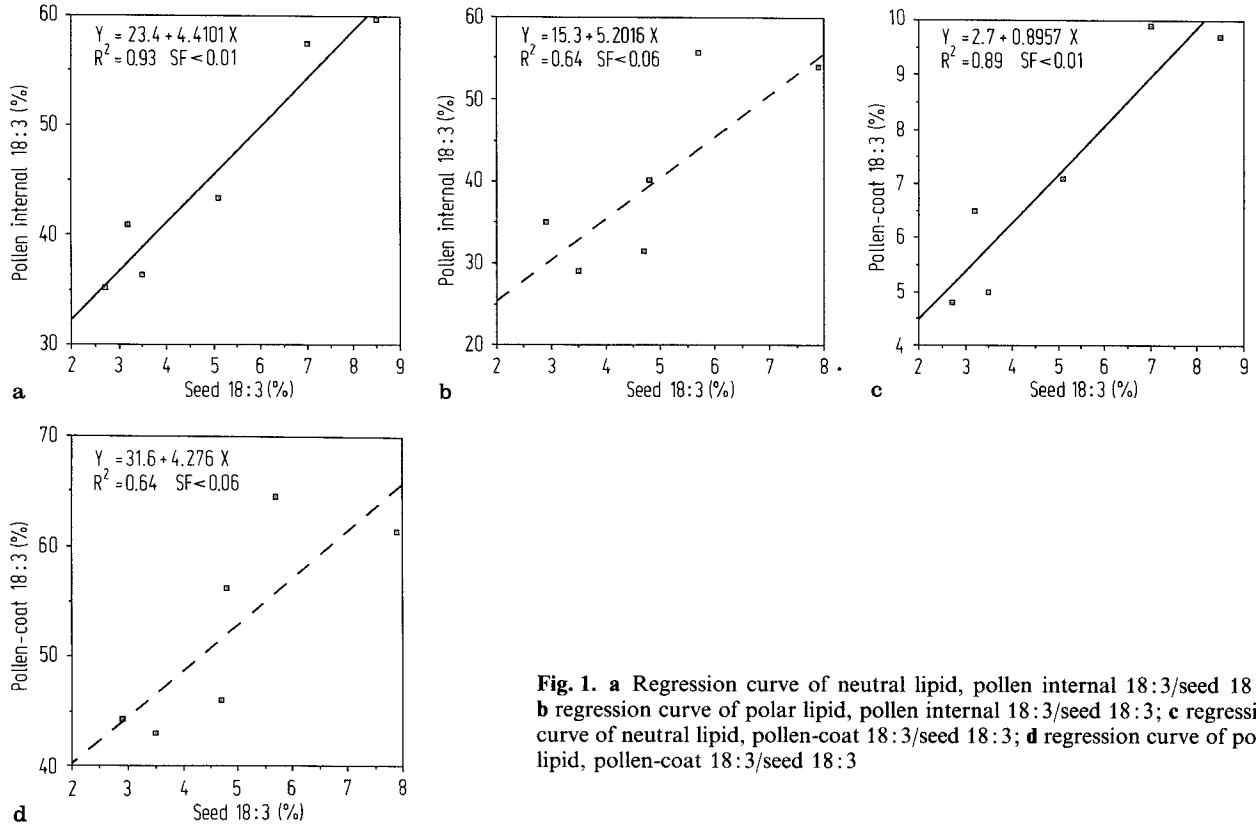

Fig. 1. **a** Regression curve of neutral lipid, pollen internal 18:3/seed 18:3; **b** regression curve of polar lipid, pollen internal 18:3/seed 18:3; **c** regression curve of neutral lipid, pollen-coat 18:3/seed 18:3; **d** regression curve of polar lipid, pollen-coat 18:3/seed 18:3

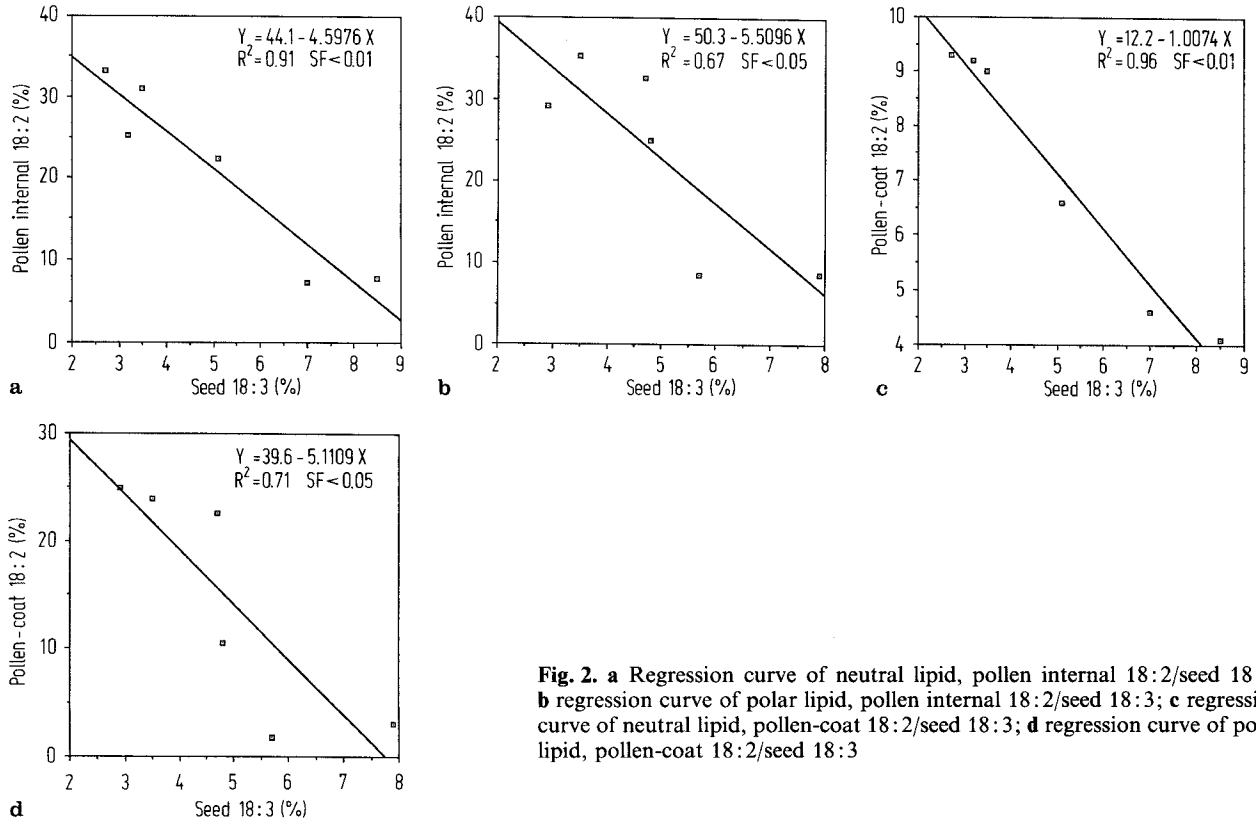

Fig. 2. **a** Regression curve of neutral lipid, pollen internal 18:2/seed 18:3; **b** regression curve of polar lipid, pollen internal 18:2/seed 18:3; **c** regression curve of neutral lipid, pollen-coat 18:2/seed 18:3; **d** regression curve of polar lipid, pollen-coat 18:2/seed 18:3

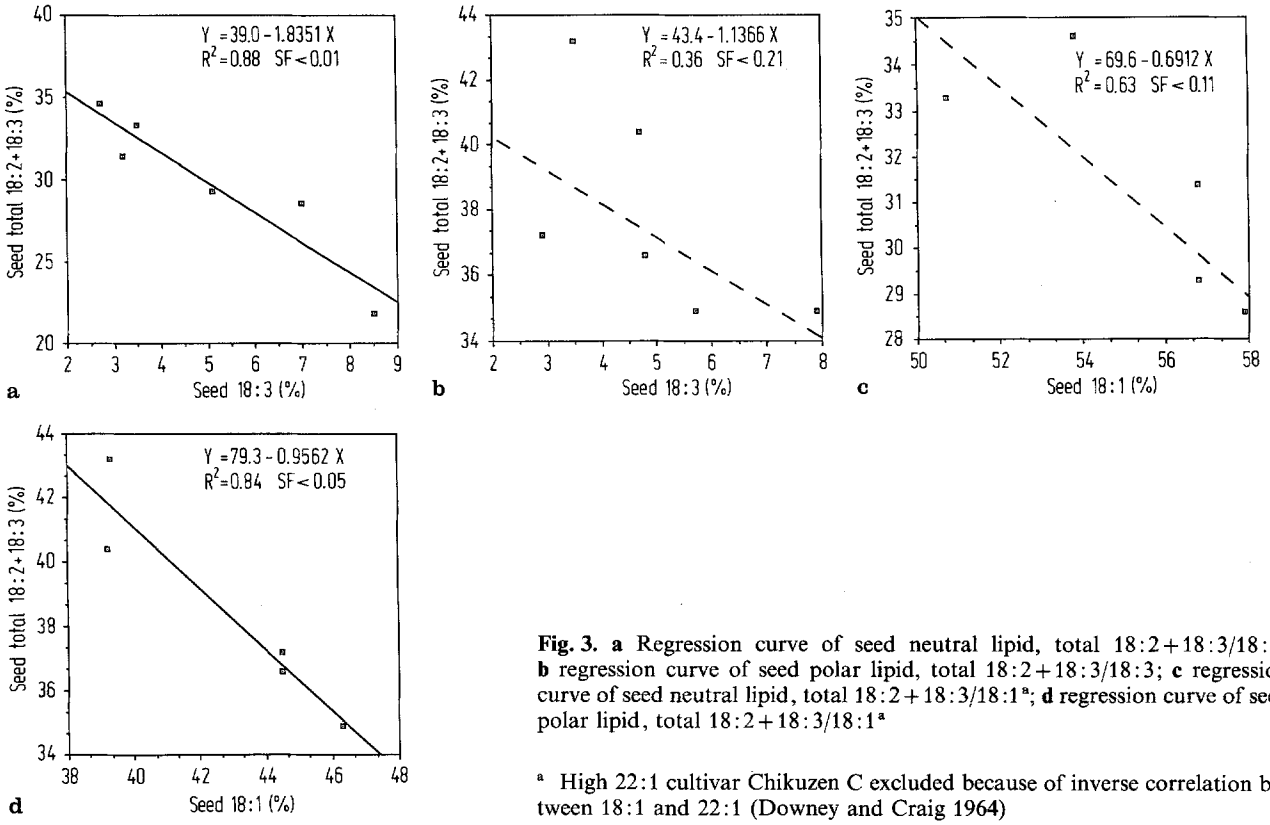

Fig. 3. **a** Regression curve of seed neutral lipid, total 18:2+18:3/18:3; **b** regression curve of seed polar lipid, total 18:2+18:3/18:3; **c** regression curve of seed neutral lipid, total 18:2+18:3/18:1[a]; **d** regression curve of seed polar lipid, total 18:2+18:3/18:1[a]

[a] High 22:1 cultivar Chikuzen C excluded because of inverse correlation between 18:1 and 22:1 (Downey and Craig 1964)

bon chains (16:3, 18:2 and 18:3). Pollen-coat polar lipids also had a much lower ratio than the internal lipids (18:2/18:3).

Relationships between pollen and seed fatty acid composition

The level of 18:3 in the pollen internal neutral lipids was significantly and positively correlated with the level of 18:3 in seed neutral lipids (Fig. 1A). A similar relationship for 18:3 was shown by seed and pollen internal polar lipids (Fig. 1B). Table 2 shows that the range of intercultivar variation present in the seed neutral and polar lipids was effectively amplified more than fourfold in the pollen internal neutral and polar lipids (difference between highest and lowest cultivar 18:3 level, seed 5.8 and 4.4% to pollen internal 25.4 and 26.6%, respectively). In addition, the level of 18:3 in the pollen-coat positively correlated with the level of 18:3 in the seed in both neutral and polar lipids (Fig. 1C, D), although the levels of 18:3 differed considerably between the two pollen-coat fractions. Furthermore, a significant inverse relationship existed between seed and pollen-coat and internal lipids for 18:3 and 18:2 in both neutral and polar lipids (Fig. 2A–D).

In seed neutral lipids, the combined level of 18:2+18:3 increased as the level of 18:3 decreased (Fig. 3A). In the seed polar lipids a similar but non-significant trend may be recognised (Fig. 3B). Within both types of seed lipid, the level of 18:1 was negatively correlated with the level of 18:2+18:3 (Fig. 3C, D). However, in the neutral and polar of both pollen-coat and internal lipids, the level of 18:3 (Table 2) did not significantly affect the combined 18:2+18:3 level.

Discussion

Inter-generation correlations

In the pollen generation of oilseed rape, the level of 18:3 in both the neutral (storage) and polar (membrane) lipids was positively correlated with the level of 18:3 in the seed generation. Furthermore, the range of variation between cultivars in the 18:3 level in seed was effectively amplified more than fourfold in the pollen internal domain. In contrast, the levels of 18:2 in the two generations were negatively correlated.

In the seed, the decrease in 18:3 was associated with more than a corresponding increase in 18:2 at the expense of 18:1. However, in both pollen-coat and internal neutral and polar lipids the combined level of 18:2 and

18:3 was unchanged, although the level of 18:3 varies considerably, particularly in the pollen internal lipids. This suggests that in seed lipid biosynthesis, 18:3 could act as an allosteric inhibitor to the 18:3 desaturation pathway, but not in the pollen-coat neutral lipid or internal lipid domains. Consequently, as the level of 18:2 is inversely correlated with the level of 18:3 in seed and also in both pollen-coat and internal domains, it appears that the proteins undertaking the desaturation from 18:1 to 18:3 are specified by the same gene loci in seed, pollen and the tapetum, but the genes regulating the overall pathway are different in each tissue and generation. Thus, the variation in 18:3 between cultivars is probably due to the different alleles that specify the proteins for the 18:2 to 18:3 desaturation.

Pollen-coat and internal lipid domain differences

Lipids of the pollen-coat and internal domains combined, account for almost a quarter (23.8%) of the dry weight of mature pollen. However, both domains differed considerably in their lipid and fatty acid compositions. The pollen internal domain constituted the major proportion of pollen lipid (73.5%) in which membrane (polar) lipids were more abundant than in the pollen-coat (43.1% compared to 10.5% of lipid, respectively). The predominant fatty acids of the pollen internal domain were 16:0, 18:2 and 18:3 in both polar and neutral lipids, although levels were slightly different. In contrast, the major fatty acids of the pollen-coat neutral (14:0, 16:0 and 18:0) and polar (16:3, 18:2 and 18:3) lipids differed considerably from each other and the internal domain.

The appearance of phospholipids in the acetone extracted pollen coat was unexpected because, unlike galactolipids, they are insoluble in acetone (Christie 1982). Since the fatty acid composition of the pollen-coat polar lipids was different to that of the internal polar lipids and galactolipids are mainly restricted to plastid membranes (Douce and Joyard 1980; Whittaker 1986), it is unlikely that these lipids originated from partial extraction of the pollen internals. An explanation may be that we detected the exinic outer layer, a membrane-like structure (Gaude and Dumas 1984). This may have been sloughed off into suspension during extraction. Alternatively, *Brassica* pollen-coats may contain not only lipids but also cytoplasmic remnants, including mitochondria and membrane fragments (Dickinson 1973; Dickinson and Lewis 1973), so that the polar lipids may in part or full result from these tapetal remains.

Lipid biosynthesis

The role of the tapetum is to provide nutritive substances to the developing pollen (Vasil 1967; Mascarenhas 1975; Reznickova and Willemse 1981a). In the locular fluid of the anther, carbohydrates (Dickinson and Bell 1976; Reznickova and Willemse 1981 b) and acetate (basic lipid substrate; Reznickova 1975; cited by Reznickova and Dickinson 1982) have been detected and are probably transferred to the developing pollen. Reznickova and Willemse (1981 b) found "channel-like" structures in the pollen coat of *Lilium*, which may indicate enzymatic digestion, releasing substrates for lipid and starch accumulation within the pollen grain. In *Muscari*, Gabara (1977) found by autoradiography that isotopes of acetate and palmitate are significantly incorporated into the pollen cytoplasm. Although free palmitate can be utilized by the prokaryotic "16:3" pathway of biosynthesis (Thompson et al. 1986), little 16:3 is accumulated within the internal pollen domain of *Brassica*. Therefore, acetate is probably the major tapetal hydrolytic product transported through the pollen grain wall for fatty acid and subsequent lipid biosynthesis.

The significant level of 16:3 in the pollen-coat polar lipids indicates that in formation of these lipids, fatty acid synthesis by prokaryotic "16:3" desaturation pathway (Williams et al. 1983; Browse et al. 1986) is significant, although it has a minor effect on 18:3 levels. However, with the pollen internal lipids, as with the seed, 16:3 is a minor component. It is therefore likely that the main pathway contributing trienoic fatty acids to the seed, pollen internal and pollen-coat neutral lipids is the eukaryotic "18:3" pathway.

The biosynthetic machinery involved in lipid and fatty acid synthesis (Cresti et al. 1983, *Oenothera*) and the protein required for neutral and phospholipid synthesis (Whipple and Mascarenhas 1978) have been observed within the pollen of *Tradescantia*. Furthermore, another plant storage compound, starch, has been demonstrated to be synthesized in an in vitro culture of isolated (free of tapetal influence) pollen microspores of *Nicotiana tabacum* L. (Kyo and Harada 1986). Consequently, lipids are probably synthesized de novo by the pollen rather than by direct transfer of lipids into the pollen from the pollen-coat. The observed differences in fatty acid composition of the pollen-coat and internal lipids and the similarity between pollen internal polar and neutral lipids lend support to this hypothesis.

Origin of proteins for lipid biosynthesis

There is a marked decline in the ribosome population in pollen just prior to meiosis (eg. *Lilium*; Mackenzie et al. 1967 and *Cosmos*; Knox et al. 1970). In *Lilium*, poly $(A)^+$ RNA (Bird et al. 1982; Porter et al. 1983) and dedifferentiation of the plastids and mitochondria (Dickinson and Heslop-Harrison 1970, 1977) have also been shown to accompany the phase change between sporophytic and gametophytic generations. In developing *Hyoscya-*

mus niger and *N. tabacum* microspores, maximum RNA synthesis occurred shortly after the microspore mitotic division and decreased steadily to pollen maturity (Reynolds and Raghavan 1982; Tupy et al. 1986). During this period, Tupy (1982) found that the pollen grain volume increased about two times and the amount of total RNA seven times, whereas the amount of poly $(A)^+$ RNA increased about 20 times and its estimated size increased from 700 to 2,100 nucleotides. In *Tradescantia* and *Zea*, it has been suggested that at least 85% of the nucleotides are present in both the diploid plant and its haploid pollen (Willing and Mascarenhas 1984; Mascarenhas et al. 1985). Since neither DNA nor RNA are transferred from the tapetum (Taylor and McMaster 1954; Moss and Heslop-Harrison 1967), RNA must be restored to the cytoplasm by the expression of mitochondrial and plastid DNA and the haploid nuclear genome, indicating that most RNA is of haploid origin.

Previous studies have shown that a large number of proteins in pollen in a range of plant species are produced as the result of gametophytic expression, for example, rice (Parnell 1921), maize (Brink and MacGilivray 1924; Demerec 1924; Schwartz and Osterman 1976; Sari Gorla et al. 1983), *Cucurbita* (Mulcahy et al. 1979; Gay et al. 1986), *Clarkia dudlyana* (Weeden and Gottlieb 1979) and *Brassica campestris* (Singh and Knox 1984). This demonstrates that pollen is capable of transcribing its DNA into mRNA and further translating these messages into active enzymes that could be utilized to synthesize lipids.

Potential for pollen selection

The results of this study indicate that the proteins undertaking the desaturation pathway from 18:2 to 18:3 are specified by the same genes, although overall the pathway is regulated by different genes in the two generations. This suggests that the regulation of the fatty acid composition of pollen internal lipids is controlled by the pollen, and that these lipids are specified by the expression of haploid genes within the pollen itself, thus fulfilling the prerequisite for pollen selection of parallel gene expression in both plant generations.

The strong correlation between seed and pollen internal lipids for an 18:2/18:3 ratio is a character for which pollen selection techniques could be developed. The pollen coat may be removed without affecting the viability of pollen (Iwanami and Nakamura 1972) and the three methylene interrupted bonds of 18:3 make it almost unique within the internal lipid domain. Already a specific destructive colorimetric test has been developed and used in seed of linseed to select low 18:3 mutants (Green and Marshall 1984). The test in its present form could presumably be modified for pollen to identify potentially low 18:3 plants within a population by utilizing the amplification of 18:3 variation in pollen internal lipids. Therefore, pollen selection for lipid quality has the potential to be developed as a plant breeding tool.

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