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Camalexin induction in intertribal somatic hybrids between *Camelina sativa* and rapid-cycling *Brassica oleracea*

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Abstract *Camelina sativa*, a wild relative of Brassica crops, is virtually immune to blackspot disease caused by *Alternaria brassicicola*. Intertribal somatic hybrids were produced between *C. sativa* and rapid-cycling *Brassica oleracea* as a step toward the transfer of resistance to this disease into *Brassica* vegetable crops. The plants recovered were confirmed as somatic hybrids by flow cytometry and RAPD analysis. All hybrids showed a morphology intermediate between the two parents. Rooted plants grew in soil up to 4–5 weeks, and some produced sterile flowers. Two of three hybrids tested showed a high level of resistance to *A. brassicicola*. Resistance was correlated with the induction of high levels of the phytoalexin camalexin 48 h after inoculation, as in the resistant *Camelina* fusion partner. In contrast, susceptible somatic hybrids produced much lower levels of camalexin.

Key words Intertribal somatic hybrids · *Brassica* · *Camelina sativa* · *Alternaria* · Camalexin

Introduction

The transfer of useful traits from wild relatives to phylogenetically remote crop plants through somatic hybridization is an attractive concept, though hard to achieve in practice. Toward this end, various intergeneric or intertribal somatic hybrids within the family Brassicaceae have recently been produced (Gleba and Hoffmann 1980; Bauer-Weston et al. 1993; Fahleson et al. 1994 a, b; Fosberg et al. 1994; Narasimhulu et al. 1994; Begum et al. 1995; Siemens and Sacristan 1995; Skarzhinskya et al. 1996; Hansen and Earle 1997). In some cases backcross progeny were also recovered (Fosberg et al. 1994; Begum et al. 1995; Skarzhinskaya et al. 1996).

Resistance to *Alternaria* ssp. is an important goal for crucifer breeders. Black spot disease of *Brassica* vegetables, caused by *Alternaria brassicicola*, reduces the quality of harvested products and leads to economic losses. No satisfactory source of resistance has been identified among crop *Brassica* species, nor among the species with which they readily cross. Very high resistance to *Alternaria* has been found in distantly related species (Conn et al. 1988; Zhu and Spanier 1991; Hansen and Earle 1997). One of them, *Camelina sativa* (L.) Crantz (false flax, gold of pleasure), is virtually immune (Conn et al. 1988; Tewari 1991).

The high resistance of *C. sativa* to *Alternaria* ssp. has been linked to the production of a phytoalexin with the structure 3-thiazol-2-yl-indole, commonly known as camalexin. The production of camalexin was first reported after inoculation of *C. sativa* with the fungal pathogens *Alternaria* and *Cladosporium* (Conn et al. 1988). Since then numerous studies have investigated the role of camalexin in plant defense reactions, especially in *Arabidopsis* (Glazebrook and Ausubel 1994; Rogers et al. 1996; Zhao and Last 1996; Glazebrook et al. 1997). The similarity of camalexin structure to the commercial fungicide triabendazole may explain its ability to limit the growth of pathogenic fungi (Browne et al. 1991).

Camalexin appears to be a broad-spectrum antibiotic. Tsuji et al. (1992) found it to be toxic to *Pseudomonas syringae* and *Cladosporium cucumerinum*. Rogers et al. (1996) reported that camalexin was toxic to *Fusarium oxysporum* and *Saccharomyces cerevisiae* at concentrations of 20–50 μg/ml; at higher concentrations (250–500 μg/ml) it was also toxic to numerous gram-negative and gram-positive bacteria. Camalexin

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may therefore contribute to plant defenses against multiple pathogens. In a study with phytoalexin-deficient *Arabidopsis* mutants, Glazebrook and Ausubel (1994) and Glazebrook et al. (1997) found that camalexin was not essential to combat *P. syringae* strains carrying *avr* genes, but that it did restrict the growth of virulent strains and also conferred resistance to the downy mildew *Peronospora parasitica*.

We therefore decided to use *C. sativa* (the original source of camalexin) as a donor of resistance in protoplast fusion experiments with *B. oleracea*. It was of particular interest to determine whether somatic hybrids of *C. sativa* and *B. oleracea* are resistant to *Alternaria* and whether resistance is correlated with the accumulation of camalexin. Besides being resistant to fungal pathogens, *C. sativa* is resistant to abiotic stresses and is currently receiving renewed attention as a low-input oilseed crop (Robinson 1987; Putnam et al. 1993; Schuster and Friedt 1995), traits that may also be valuable in crop *Brassicas*.

C. sativa and *B. oleracea* are both members of the *Cruciferae* (*Brassicaceae*) family, but belong to different tribes. Sexual crosses between them are not feasible. Narasimhulu et al. (1994) obtained somatic hybrids between *C. sativa* and *B. carinata*, but failed to achieve normal rooting or to establish plants in soil. In the present paper we describe the production of intertribal somatic hybrids between *C. sativa* (tribe *Sisymbrieae*) and rapid-cycling *B. oleracea* (tribe *Brassiceae*) that are resistant to *A. brassicicola*. The resistance of detached leaves to inoculation with spores of this pathogen was correlated with the induction of camalexin.

Materials and methods

Plant materials

Seeds of *C. sativa* L. "Boha" (from Dr. Zubr, The Royal Veterinary and Agricultural University of Denmark), rapid-cycling (r.c.) *B. oleracea* (Crucifer Genetics Cooperative #3-1), and *B. oleracea* ssp. *italica* broccoli cv "Shogun" (Sakata Seeds Co., Japan) were used for the protoplast experiments. Seeds were sterilized for 5 min with 70% ethanol, then for 15 min in 10% Clorox, and rinsed three times in sterile water before transfer to Magenta boxes. Seeds were germinated on LS medium (Linsmaier and Skoog 1965) with 1% sucrose and no growth regulators (LS-0). The plants were grown on the same medium at 25°C under cool white fluorescent lights (60 μE $m^{-2}s^{-1}$) with a 16-h photoperiod.

Protoplast isolation, fusion and culture

Leaves of 1-month-old plants grown in vitro were initially used. *Camelina* plants did not respond well to in vitro conditions; therefore, in the second experiment the *Camelina* protoplasts were isolated from leaves of 2-month-old plants grown in the greenhouse. Leaves were sterilized for 30 s with 70% ethanol, 10 min with 10% Clorox, rinsed three times with sterile water, and pre-cultured for 2 days in liquid LS medium containing 1 mg/l of BA. Protoplasts were isolated as described (Hansen and Earle 1994).

Protoplasts of r.c. *B. oleracea* were treated with 3 mM of iodoacetate for 15 min immediately before isolation to prevent division of unfused cells. Protoplasts of both partners were mixed 1 : 1 and placed on the bottom of plastic Petri dishes. Fusion was induced with polyethylene glycol (Sigareva and Earle 1997). After fusion, protoplasts were cultured in the dark in liquid medium B (Pelletier et al. 1983) and then transferred to Millipore filters over a feeder layer of *B. napus* cells (Walters and Earle 1990). Calli were transferred sequentially from medium B to C to E and to F (Pelletier et al. 1983). Shoots that regenerated from calli on medium E or F were excised and placed onto LS-0 medium, solidified with either 6 g/l of Phytagar (Gibco BRL) or 2.2 g/l of Gelrite (Chemical Dynamics Corp.). Some plants were transferred into soil mix and hardened (Sigareva and Earle 1997).

Nuclear DNA content

The nuclear DNA content of leaves of parental plants and regenerated hybrids was determined by flow cytometry (Arumuganathan and Earle 1991). Nuclei from rice (cv Taipei 309, 2C = 0.87 pg) were used as an internal standard. Samples were analyzed on an EPICS PROFILE cell cytometer (Coulter Electronics, Hialeah, Fla.).

RAPD analysis

Genomic DNA was extracted from young leaves according to Hu and Quiros (1991). Ten-base oligonucleotide primers (Operon Technologies, Alameda, Calif.) were used to test parental and fusion-derived plants. Amplification conditions were those of Hu and Quiros (1991): 2 min 30 s of denaturation at 94°C followed by 45 cycles of 92°C/30 s, 35°C/1 min, and 72° C/1 min. PCR products were separated on a 2% agarose gel and photographed with Polaroid 667 film.

Analysis of resistance to *A. brassicicola*

A. brassicicola was grown on potato dextrose agar (39 g/l) at 25°C with a 16-h photoperiod (50–60 μE $m^{-2}s^{-1}$). Two to three weeks after subculturing, conidia were washed off the plates with 10 ml of distilled water. To screen for susceptibility to *A. brassicicola*, detached leaves were placed in glass Petri dishes containing Phytagar (6.6 g/l, pH 5.8). Plates were sealed with Parafilm to retain moisture and incubated for 5 days. Drops (15 μl) of conidial suspensions (5×10^4 conidia/ml) were placed on leaves of *C. sativa*, r.c. *B. oleracea*, and three somatic hybrids. Drops of distilled water were placed on leaves as controls.

Disease symptoms were evaluated 5 days after inoculation using the visual rating scale established by King (1994). Disease severity ranged from 1 to 10, where 1 = no disease, 2 = few small flecks, 3 = small flecks but no large lesions, 4 = small flecks and a few lesions, 5 to 9 = increasing number and size of lesions, and 10 = dead. Plants with ratings of 3 or less were classified as having resistance, a rating of 4 indicates slight or borderline resistance, and ratings of 5 and above were considered to have increasing degrees of susceptibility,

Camalexin determination

Camalexin was extracted according to the procedure of Glazebrook and Ausubel (1994). For each sample 50 mg of fresh or frozen leaf tissue were weighed and heated in 80% methanol for 20 min. Tissue was removed, and methanol was evaporated under reduced pressure in a centrifugal evaporator (Jouan, Inc., Winchester, Va.). The

aqueous residue was extracted with two 100-µl aliquots of chloroform, which were combined and completely dried in a centrifugal evaporator. The residue was dissolved in 15 µl of chloroform, loaded on silica thin-layer chromatography (TLC) plates (J.T. Baker/VWR Scientific), and developed for 1 h in 9 : 1 (v/v) chloroform/methanol. Camalexin was visualized by its fluorescence under a hand-held long-wave ultraviolet lamp (365 nm). The silica with camalexin was scraped off the plate, and camalexin was extracted into 2 ml of 100% methanol. Fluorescence was measured with a Perkin-Elmer (Branchburg, N.J.) MPF-44B spectrofluorometer set at 315-nm excitation and 385-nm emission. Camalexin concentration was determined by comparison with a standard curve obtained by using synthetic camalexin kindly provided by Dr. Jane Glazebrook (University of Maryland, College Park, Md.). For each data point, the assay was performed on three samples, and the results reported as the mean and standard deviation.

Results and discussion

Recovery of fusion products

We produced intertribal somatic hybrids between *C. sativa* and r.c. *B. oleracea* as a first step to possible introgression of agronomically important traits into crop *Brassicas.*

Fusion with r.c. B. oleracea

In the first fusion experiment using in vitro grown *Camelina* plants, we recovered 150 calli (Table 1), all of which were transferred to medium F. After 3 weeks, one callus showed signs of differentiation and produced a shoot from one side and a root from the other. This was probably a somatic hybrid since no shoots were ever produced from control *Camelina* colonies. The shoot was cut off from the fusion-derived callus and multiplied on medium F. Many new shoots were obtained and transferred to LS-0 medium. On medium solidified with Gelrite, the shoots appeared vitrified. Culture on medium solidified with 6 g/l of Phytagar normalized the morphology of the shoots. Many shoots flowered in Magenta boxes but did not root.

In the second experiment, using greenhouse-grown *Camelina* plants, we recovered 1008 colonies, the majority of which were probably non-regenerating *Camelina* colonies (Table 1). On medium F, 11 calli produced multiple shoots, which were excised and transferred to fresh medium. Nine calli produced multiple normal-looking shoots, which could be cultured on LS-0 medium.

Rooting was a problem for these hybrids. The same phenomenon was observed by Narasimhulu et al. (1994), who were unable to root their *C. sativa* + *B. carinata* somatic hybrids. Difficulty in rooting of the somatic hybrids may be due to the fact that the *C. sativa* itself rooted poorly in vitro, rather than to the hybridization itself.

Rooting of shoots from two vigorous hybrid lines (H5 and H8) was compared on standard LS-0 medium and LS-0 medium supplemented with 1 or 10 mg/l of IBA or 0.1% charcoal (Table 2). Of the H5 plants, 20% rooted on unsupplemented LS-0 medium. No H8 shoots produced roots on this medium. The addition of charcoal did not improve rooting. On medium with 1 mg/l of IBA, 30% of H5 shoots and 10% of H8 shoots produced roots. On medium with 10 mg/l of IBA, more shoots formed roots (40% of H5 and about 20% of H8), but these were multiple roots coming from the callus at the base of the shoot. When rooted plantlets were transferred to soil, those with multiple callus-derived roots did not survive. Plants that produced one or more strongly branching roots grew in soil up to 4–5 weeks, and some reached the flowering stage.

Maintaining the plants in soil was difficult. Even the plants that survived through flowering grew poorly and had sterile flowers. Attempts to pollinate them with pollen from r.c. *B. oleracea* were unsuccessful. It has previously been observed that intertribal hybrids are harder to grow to mature plants in soil than closer phylogenetic combinations (Waara and Glimelius 1995). Some symmetric and asymmetric intertribal hybrids were described to have male and female fertility (Fosberg et al. 1994; Skarzhinskaya et al. 1996); however, asymmetric hybrids obtained via irradiation pretreatment of *B. napus* + *L. fendleri* hybrids had better fertility than symmetric hybrids (Skarzhinskaya et al. 1996). Flow cytometry and RAPD analysis indicated that our plants were symmetric hybrids (see below).

Fusion with broccoli cv "Shogun"

C. sativa was also fused with broccoli in the hope of obtaining more vigorous somatic hybrids. More than

Table 1 Recovery of somatic hybrids after fusion of *B. oleracea* with *C. sativa*

Brassica fusion partner	Protoplasts fused	Calli obtained (% of protoplasts fused)	Calli with shoots (% of calli)
r.c. *B. oleracea*	3×10^5	150 (0.05%)	1 (0.7%)
r.c. *B. oleracea*	1×10^6	1008 (0.1%)	11 (1.1%)
"Shogun" broccoli	2.5×10^6	4036 (0.2%)	7 (0.2%)

Table 2 Rooting of somatic hybrid lines H5 and H8 on different media

Line	Rooted plantlets (% of 40 shoots tested)			
	LS-0	LS + IBA (1 mg/l)	LS + IBA (10 mg/l)[a]	LS-0 + 0.1% Charcoal
H5	8 (20%)	12 (30%)	15 (37.5%)	3 (7.5%)
H8	0	4 (10%)	7 (17.5%)	0

[a] Plants with multiple callus-derived roots formed on this medium survived poorly in soil

4000 colonies were recovered, but only seven developed shoots (Table 1). The majority of the colonies probably came from unfused *Camelina* cells, but those that formed shoots were probably fusion products (since control *Camelina* colonies were non-regenerable). The shoots recovered were similar to those obtained in the previous fusions, so there appeared to be no specific advantage to the use of broccoli in place of r.c. *B. oleracea* as a fusion partner.

Analysis of *C. sativa* + r.c. *B. oleracea* fusion products

Hybridity of the plants recovered from this fusion combination was confirmed by RAPD analysis (Fig. 3). Primer OPA-09 showed consistent polymorphism between the parents: bands of approximately 520 and 1030 bp were present only in r.c. *B. oleracea*, while bands of about 396 and 344 bp were present only in *C. sativa*. Five independent hybrids (one from the first experiment and four from the second) were analyzed. All showed the sum of the parental bands.

Flow cytometry supported the identification of the regenerates as somatic hybrids. Seed-grown *C. sativa* has a nuclear DNA content of 1.53 pg/nucleus (three plants tested), while the DNA content of seed-grown r. c. *B. oleracea* is 1.3 pg/nucleus (Table 3). The plant recovered from the first fusion experiment had a DNA content of 2.8 pg, close to the sum of the parental genomes. Six hybrids from the second fusion experiment had DNA contents of 2.7–2.9 pg, suggesting that these were also symmetric somatic hybrids. The two plants with a DNA content of 4.1 and 4.6 pg probably developed from the fusion of more than two protoplasts.

The vegetative and floral morphology of hybrid plants was intermediate between the two parents (Fig. 1). *C. sativa* leaves are lanceolate with trichomes on the margins and surface of the leaves. R.c. *B. oleracea* leaves are oval and waxy with serrated margins. Leaves of the hybrids were lanceolate with trichomes and serrated margins (Fig. 2 A). *Camelina* plants have yellow flowers smaller than those of *B. oleracea*. Their ovary forms a round capsule while r.c. *B. oleracea* flowers have long pistils. Flowers of the hybrid plants were small, like those of *Camelina*, but had elongated pistils, resembling *B. oleracea* flowers. (Fig. 2 B).

Table 3 Analysis of r.c. *B. oleracea* + *C. sativa* hybrids

Line	DNA content (pg/nucleus)	RAPD bands	Interpretation
r.c. *B. oleracea*	1.3	B[b]	Parent
C. sativa	1.53	C[c]	Parent
H[a]	2.8	B + C	Somatic hybrid
H1	3.0	B + C	Somatic hybrid
H2	4.1	nt[d]	nd[e]
H4	2.7	B + C	Somatic hybrid
H5	2.9	B + C	Somatic hybrid
H6	4.6	nt	nd
H8	2.8	B + C	Somatic hybrid
H10	2.8	B + C	Somatic hybrid
H11	2.7	B + C	Somatic hybrid

[a] Somatic hybrid obtained in the first fusion experiment
[b] *Brassica*
[c] *Camelina*
[d] Not tested
[e] Not determined

Tests of resistance to *A. brassicicola*

A major objective of the fusion efforts was the transfer of resistance to *Alternaria*, since no source of high resistance to this fungus is known among crop *Brassica* species. It was therefore interesting to compare the resistance of somatic hybrids with that of the parental lines. Our tests confirmed that *C. sativa* is indeed highly resistant to *A. brassicicola*; however, responses of leaves from young (1 month old) and older (3 months) plants were different. Leaves of 3-month-old plants showed no signs of disease when inoculated with either 5×10^4 or 3.5×10^5 conidia/ml. Leaves of 1-month-old plants developed necrotic spots 48 hours after inoculation, without further symptoms of disease. Leaves of the r. c. *B. oleracea* fusion partner developed symptoms when inoculated with either 5×10^4 or 3.5×10^5 conidia/ml. *B. oleracea* was therefore rated as susceptible, with ratings from 6 to 9 (Fig. 4). Leaves of three somatic hybrids were tested with 5×10^4 conidia/ml when they were 1- and 3-months old. Two (H5 and H1) showed high resistance (rated 2 or 3), while one (H8) showed intermediate resistance (rated 4).

Determination of camalexin levels

Infection with *A. brassicae* was previously shown to induce camalexin production in *C. sativa* (Conn et al. 1988). To test whether *A. brassicicola* also induced camalexin, we inoculated leaves of *C. sativa*, two somatic hybrids (with high and intermediate levels of resistance respectively), and r.c. *B. oleracea*, with conidia of *A. brassicicola*. The concentration of conidia in the inoculum was an important factor in the induction of camalexin in *C. sativa*. Inoculation with 5×10^4 conidia per ml gave inconsistent results; camalexin was induced in some samples, but not in others. Moreover the magnitude of induction was low. Inoculation with 3.5×10^5 conidia/ml consistently induced camalexin, but the maximum level of induction was only 3.5 μg/g of leaf tissue. In contrast, after inoculation with 10^6 conidia/ml, camalexin accumulation reached a maximum of 41 μg/g of leaf tissue (Fig. 5). The latter concentration was therefore used in further tests.

Another important factor in camalexin induction was the age of the *C. sativa* plants. Leaves from

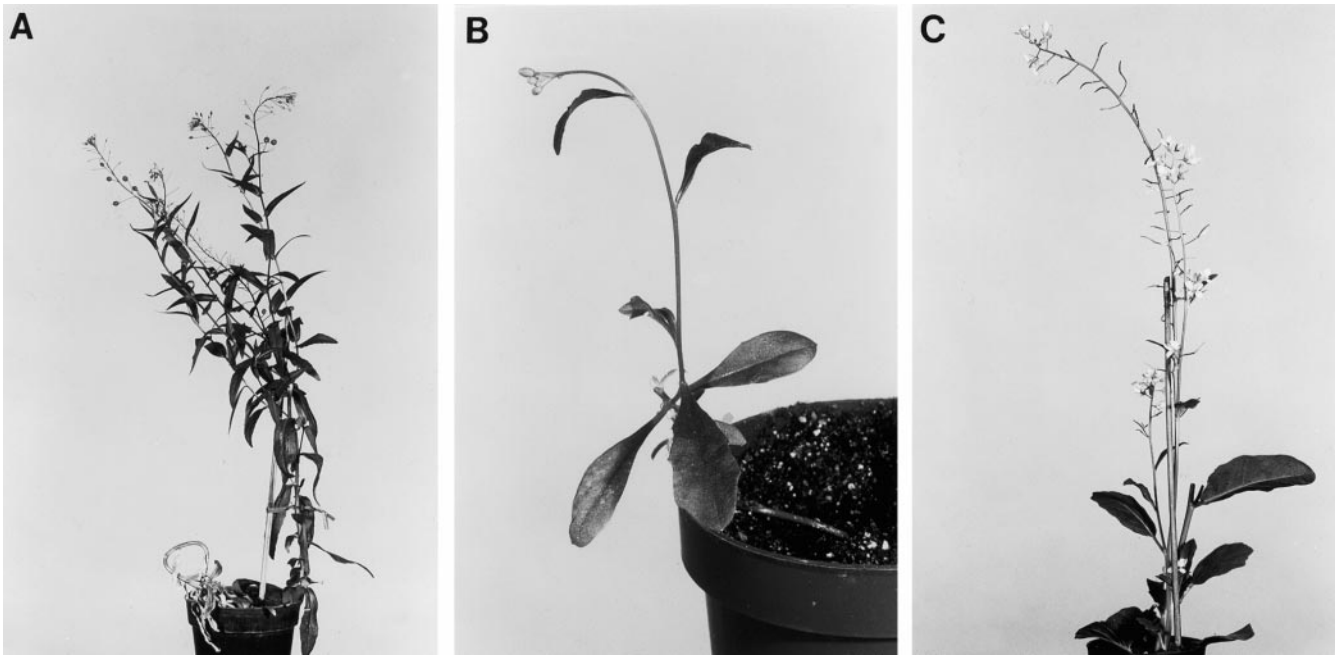

Fig. 1A–C Plants of the fusion partners and their somatic hybrid in soil: (**A**) *C. sativa*; (**B**) somatic hybrid; (**C**) r.c. *B. oleracea*. The parental plants are 30–45 cm high. The somatic hybrid is 10–15 cm high

Fig. 2 (**A**) Leaves and **(B)** ovaries of (from right to left): *C. sativa*; somatic hybrid; r.c. *B. oleracea*

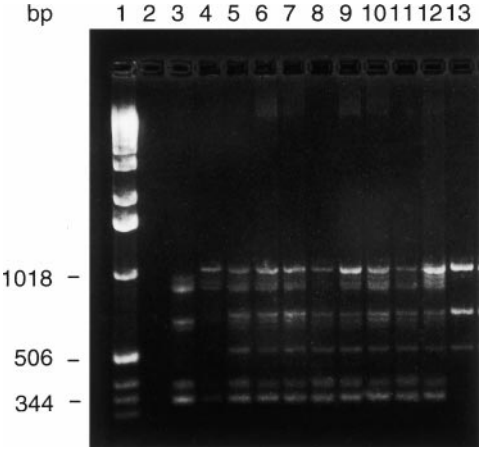

Fig. 3 RAPD banding pattern generated by primer OPA-9. *Lane 1*: 1-kb ladder; *lane 2*: blank; *lane 3*: *C. sativa*; *lanes 4–12*: somatic hybrids; *lane 13:* r.c. *B. oleracea*

1-month-old plants developed necrotic spots characteristic of a hypersensitive response approximately 24–48 h post-inoculation. This usually coincided with the time of camalexin induction. In contrast, leaves of 3-month-old *Camelina* plants, which showed no signs of necrosis after inoculation with *Alternaria*, never produced camalexin, at least not at detectable levels.

Twenty four hours after inoculation, camalexin levels in *Camelina* leaves were only slightly higher than in control r.c. *B. oleracea* leaves, where camalexin induction was never observed (Fig. 5). After 48 h *Camelina* leaves contained 27.1 $\pm$ 1.6 μg of camalexin/g of leaf tissue. Induction of camalexin coincided in time with the formation of necrotic spots. Sixty six hours post-inoculation the camalexin concentration reached 37.8 $\pm$ 3.6 μg/g of leaf tissue.

Induction of camalexin in a somatic hybrid resistant to *A. brassicicola* (H5) reached high levels (21.5 $\pm$ 5.1 μg/g of leaf tissue) 48 h post-inoculation and decreased

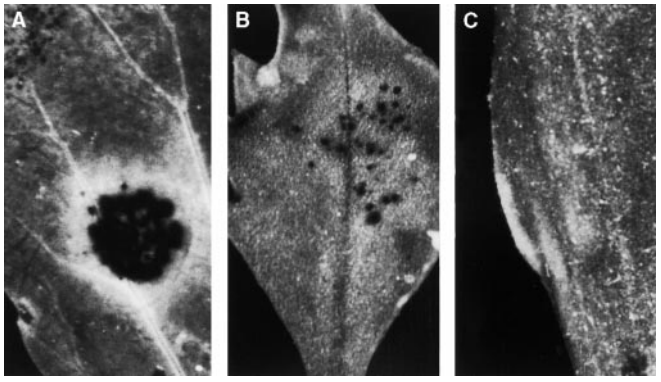

Fig. 4 Responses of fusion partners and somatic hybrids 5 days after inoculation with conidia of *A. brassicicola*: (**A**) *B. oleracea*; (**B**) somatic hybrid; (**C**) *C. sativa*

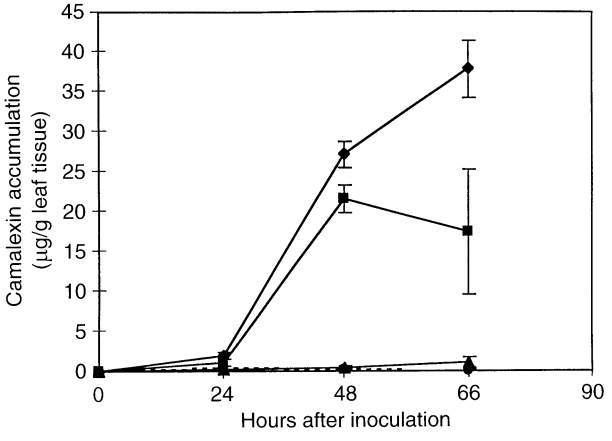

Fig. 5 Accumulation of camalexin in fusion partners and somatic hybrids. ◆—◆ *C. sativa*; ■—■ somatic hybrid H5; ▲—▲ somatic hybrid H8; ●- - -● *B. oleracea*. Plants were inoculated with 15 μl of an *A. brassicicola* suspension (10^6 conidia/ml). At the times indicated approximately 50 mg leaf tissue were excised, and camalexin was determined as described in the Materials and methods. Each point represents the mean and standard deviation of three replicate samples

to 17.4 ± 8 μg/g by 66 h (Fig. 5). In a more susceptible hybrid (H8), camalexin induction was only 1.1 ± 0.7 μg/g 66 h after inoculation, barely higher than in the negative control. Nevertheless, even for line H8, it was possible to observe camalexin spots on TLC plates (Fig. 6), and in some samples the value reached a maximum concentration of 4 μg/g 48 h post-inoculation.

The extent to which camalexin produced by *C. sativa* contributes to its resistance to *A. brassicicola* remains to be determined. In any case, the fact that some of our intertribal somatic hybrids are resistant to *Alternaria* and produce camalexin after inoculation suggests that they can express defense reactions and biochemical pathways similar to the *Camelina* fusion partner. Recovery and analysis of progeny of the somatic hybrids

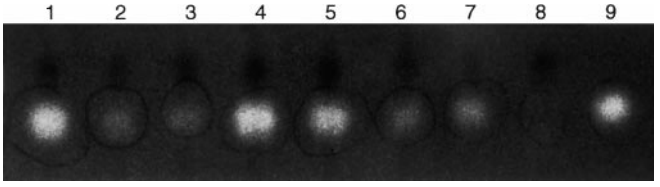

Fig. 6 Thin-layer chromatography analysis of camalexin: *Lane 1*: *C. sativa* 48 h after inoculation; *lanes 2–3*: somatic hybrid H8 48 h after inoculation; *lanes 4–5*: somatic hybrid H5 48 h after inoculation: *lanes 6–7*: H5 24 h after inoculation; *lane 8*: *B. oleracea* 48 h after inoculation; *lane 9*: camalexin standard

should help clarify the relationship between resistance and the induction of camalexin, and may eventually contribute to the production of improved *Brassica* crops.

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