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GLUCOSINOLATE CONTENT IN SUSCEPTIBLE AND RESISTANT CHINESE CABBAGE VARIETIES DURING DEVELOPMENT OF CLUBROOT DISEASE

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Key Word Index—*Brassica campestris* ssp. *pekinensis*; Cruciferae; Chinese cabbage; *Plasmodiophora brassicae*; clubroot disease; glucosinolates.

Abstract—The glucosinolate content in Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) during the development of clubroot disease caused by the obligate biotroph *Plasmodiophora brassicae* was investigated. Two *Plasmodiophora*-resistant and two susceptible varieties of Chinese cabbage were used and three classes of glucosinolates, aliphatic (=alkenyl), aromatic and indolic were analysed. Between the susceptible varieties 'Granat' and 'Osiris' and the resistant varieties 'Parkin' and 'Yuki' there were significant differences in glucosinolate pattern. The total glucosinolate content in roots of the two susceptible varieties was higher throughout the experimental period than in roots of the two resistant varieties. 'Osiris' showed the highest glucosinolate content of all the varieties investigated (*ca* three-fold higher than 'Granat' and *ca* five-fold higher than 'Parkin' and 'Yuki'). After infection with *P. brassicae* the indole glucosinolates increased after 14 and 20 days in roots of 'Granat' and 'Osiris', respectively, whereas there was no difference between infected and control roots in 'Parkin' and 'Yuki'. The aliphatic glucosinolates were also enhanced in infected roots of 'Granat', whereas 'Osiris' showed a very high content of aliphatic glucosinolates during the whole experimental period. Roots of 'Parkin' and 'Yuki' grown in the presence of *Plasmodiophora* spores showed an elevated concentration of aromatic glucosinolates after 14 and 30 days, respectively, which was not found in 'Granat' and 'Osiris'. Total seed glucosinolate content appeared to be correlated with the susceptibility of the Chinese cabbage varieties tested. Eight different susceptible varieties showed higher total glucosinolate contents than the two resistant varieties. Treatment of plants of the varieties 'Parkin' and 'Granat' with salicylic acid and jasmonic acid resulted in increased amounts of glucosinolates, although differences in the response were observed between the two treatments. Jasmonic acid induced mainly indole glucosinolates in the leaves, whereas salicylic acid induced indole glucosinolates also in the roots of both varieties. In the variety 'Parkin', we also observed induction of aliphatic and aromatic glucosinolates after jasmonate treatment. Although the variety 'Parkin' showed no clubroot symptoms, we were able to detect fungal structures within the roots using scanning electron microscopy. We would, therefore, rather describe this variety as tolerant not resistant to clubroot disease. The potential role of different glucosinolates in plant–pathogen interactions is discussed. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Glucosinolates are a group of secondary plant metabolites found in all species of the Brassicaceae. Over 100 glucosinolates have been described so far [1]. Glucosinolates are classified into three different classes, depending on the amino acid from which they are derived; aliphatic/alkenyl glucosinolates derived from methionine, aromatic glucosinolates derived from phenylalanine and tyrosine, and indole glucosinolates derived from tryptophan [2]. Glucosinolate content is dependent on the tissue type and age [3]. Glucosinolates and their breakdown products are thought to play a role in disease resistance against insects and fungal pathogens, but they also might be involved in host–pathogen recognition [4]. The accumulation of glucosinolates can be induced after wounding and pathogen attack [5, 6], as well as after treatment with salicylic [7] and jasmonic acids [8, 9].

Clubroot disease of the Brassicaceae is caused by the obligate biotroph *Plasmodiophora brassicae*. Infection leads to tumorous swellings of infected roots. As a result of this infection, cells are first induced to divide and, during later stages, show signs of hypertrophy [10]. The developing clubs form a strong metabolic sink on the host, although under optimum growth conditions the host may tolerate club growth without any reduction in shoot growth [11]. Earlier, it was speculated that growth hormones, e.g. cytokinins and auxins, are in some way involved in symptom develop-

ment [12, 13]. In a number of studies it was shown that synthesis of both hormones is indeed affected in infected tissue. An increased synthesis and turnover of indole-3-methylglucosinolate (glucobrassicin) in infected roots could also be demonstrated [14, 15].

Until now, no data on glucosinolate contents during the development of the clubroot disease of the Brassicaceae are available. We have, therefore, used different resistant and susceptible varieties of Chinese cabbage to investigate the content of the different glucosinolates during the development of the clubroot disease in order to determine whether there is a correlation between specific glucosinolates and resistance against *P. brassicae*.

RESULTS AND DISCUSSION

Glucosinolate content in seeds of different varieties

Glucosinolates are believed to play an important role in the plant–pathogen interaction. There is evidence for the involvement of glucosinolates and their breakdown products in resistance to fungal pathogens and insects [4, 16, 17]. In our study, we wanted to compare the glucosinolate content of Chinese cabbage varieties susceptible to infection with *P. brassicae* and varieties resistant to this fungus. To exclude variations in glucosinolate content which might be species dependent, we have examined eight susceptible and two resistant varieties (unfortunately no more resistant varieties of Chinese cabbage were available) for their infection rate with *Plasmodiophora* and their seed glucosinolate content. The infection rate of the susceptible varieties ranged from 100 to 75% infection (Table 1), whereas the variety 'Yuki' showed an infection rate of only 5% and 'Parkin' no visible infection at all. Roots of 'Parkin' and 'Granat' inoculated with *Plasmodiophora* were examined by scanning electron microscopy. In 'Granat', we found, as expected, large amounts of resting spores of *Plasmodiophora* clustered in enlarged cells (Fig. 1(A)), but to our surprise we also found fungal structures in roots of 'Parkin' (Fig. 1(B)). These structures are more likely to be young plasmodia than resting spores, due to their larger size and smooth surface. The occurrence of plasmodia is dependent on the stage of development of the disease and has previously also been found in 'Granat' [18]. Resistance to clubroot has been defined as the ability of a plant genotype to limit the development of clubs on the roots after infection with *P. brassicae* [19]. However, it might be suitable to use the term 'tolerance' to differentiate between plants where no fungal invasion is taking place (resistance) and plants which are infected with the pathogen, but do not show any symptoms (tolerance). With this definition we would describe the variety 'Parkin' as tolerant to infection with *Plasmodiophora*. In earlier work, where the plants were grown in liquid culture, we were not able to demonstrate the occurrence of *Plasmodiophora* in the resistant variety 'Parkin' [18], but the culture of the plants in soil seems to provide different conditions for invasion of the roots by the fungus.

The total glucosinolate content was significantly lower in the two resistant varieties (9.31 and 8.28 μmol g^{-1} dry wt) compared with glucosinolate contents between 30 and 15 μmol g^{-1} (dry wt) for the susceptible varieties (Table 1). However, we could not detect any definite pattern as to whether one class of glucosinolates might be specifically elevated or reduced in the resistant varieties compared with the susceptible ones. It is known that, with the exception of the indole glucosinolates, only the first true leaves are able to synthesize glucosinolates [20] and, therefore, those in

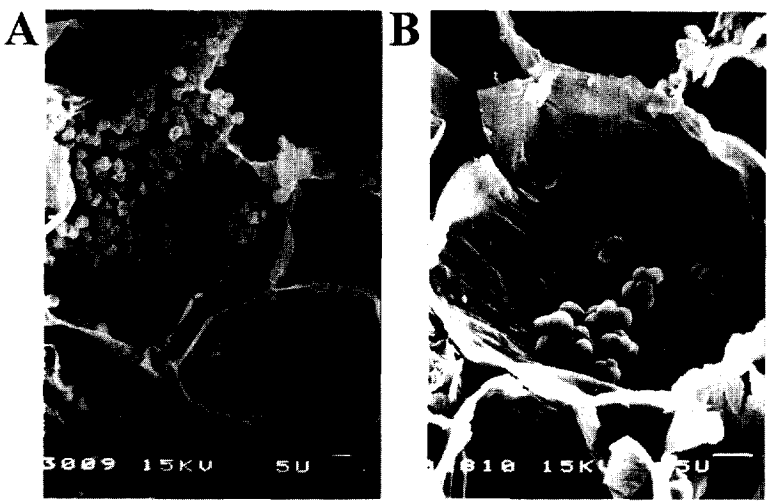

Fig. 1. Scanning electron micrographs of roots of two Chinese cabbage varieties infected with *Plasmodiophora brassicae* 6 weeks after inoculation: (A) infected roots of the susceptible variety, 'Granat', showing typical signs of hypertrophy; (B) infected roots of the resistant variety, 'Parkin', showing no clubroot symptoms. Bars represent 5 μm. The structures in 'Granat' represent resting spores, whereas in 'Parkin' young plasmodia are visible.

Table 1. Glucosinolate content in seeds of different Chinese cabbage varieties. Terms in parentheses represent percentage of each glucosinolate group

Variety	Infection (%) with *Plasmodiophora*	Glucosinolate content (μ mol g^{-1} dry wt)			
		Aliphatic	Aromatic	Indolic	Total
Nippon	100	7.70 (45)	3.94 (23)	5.44 (32)	17.08
Granat	96	6.43 (44)	4.82 (33)	3.47 (23)	14.72
Storido	95	17.80 (56)	9.57 (30)	4.66 (14)	32.03
Hong Kong	93	16.31 (75)	2.29 (10)	3.34 (15)	21.94
Sixtyres	87	12.14 (51)	4.67 (20)	6.96 (29)	23.77
Kimono	83	17.09 (56)	11.97 (39)	1.49 (5)	30.55
Okido	77	9.54 (56)	7.54 (44)	0.06 (0.4)	17.14
Osiris	75	3.60 (12)	17.29 (57)	9.42 (31)	30.31
Yuki	5	3.55 (38)	5.65 (61)	0.11 (1)	9.31
Parkin	0	3.08 (37)	2.34 (28)	2.86 (35)	8.28

the seedlings must derive from what is in the seed itself. We have, therefore, measured the activity of tryptophan oxidizing enzyme (TrpOxE), the enzyme catalysing the primary step in indole glucosinolate biosynthesis [21], which has been demonstrated to be present in seedlings and cotyledons, contrary to the flavin monooxygenases involved in the synthesis of aliphatic and aromatic glucosinolates [20]. The investigation of four susceptible and one resistant variety showed that TrpOxE activity in five-day-old seedlings of 'Parkin' (2.1 fkat mg^{-1} protein) was reduced by *ca* 50–70% compared with the susceptible varieties 'Granat' (4.3 fkat mg^{-1} protein), 'Sixtyres' (7.2 fkat mg^{-1} protein), 'Kimono' (4.4 kfat mg^{-1} protein) and 'Okido' (5.1 fkat mg^{-1} protein). This implies that the seed indole glucosinolate concentration does not reflect the content in the seedlings, whose synthesis is significantly lower in the tolerant variety. Therefore, *de novo* synthesis of indole glucosinolates might play a role during root invasion by *Plasmodiophora*.

Glucosinolates during development of disease

It has been postulated that auxins and cytokinins are involved in the development of the hypertrophy during clubroot disease in the Brassicaceae. Butcher *et al.* [22] hypothesized that the breakdown of indole glucosinolates, which leads to the release of relatively large amounts of auxin, is responsible for club root symptoms. This implies that plants lacking indole glucosinolates should not form clubs or should at least have much reduced disease symptoms.

In a previous study [18], we have investigated indole glucosinolate content in two different Chinese cabbage varieties, one susceptible and one resistant to infection with *P. brassicae* during the first two weeks after inoculation and found that the resistant variety did not have much lower levels of indole glucosinolates than the susceptible variety. However, in the susceptible variety, indole glucosinolates were induced during infection, whereas in the resistant variety we have observed no changes. This finding has been confirmed with our data on seed glucosinolate content, e.g. the susceptible variety 'Granat' has almost the same indole glucosinolate content as the resistant variety 'Parkin'.

In the present study, we have used another technique for the determination of glucosinolates. Previous results have been obtained by degrading the indole glucosinolate fraction by myrosinase and measuring the release of SCN^- ions. We have now shown that the different classes of glucosinolates are differentially induced during the development of the clubroot disease (Fig. 2). This phenomenon has been reported for the infection of oilseed rape leaves with the fungus *Alternaria brassicae* [5]. A detailed time-course study on the glucosinolate content during this infection indicated that all classes of glucosinolates responded in young leaves, but at different times after infection, and that there was no increase in aliphatic glucosinolates in older leaves.

The total glucosinolate content was slightly lower in control roots of 'Parkin' and 'Yuki' (resistant) than in 'Granat'. The variety 'Osiris' exhibited the highest glucosinolate content (*ca* three-fold higher than 'Granat' and four-fold higher than 'Parkin' and 'Yuki'), which is mostly due to the high concentration of

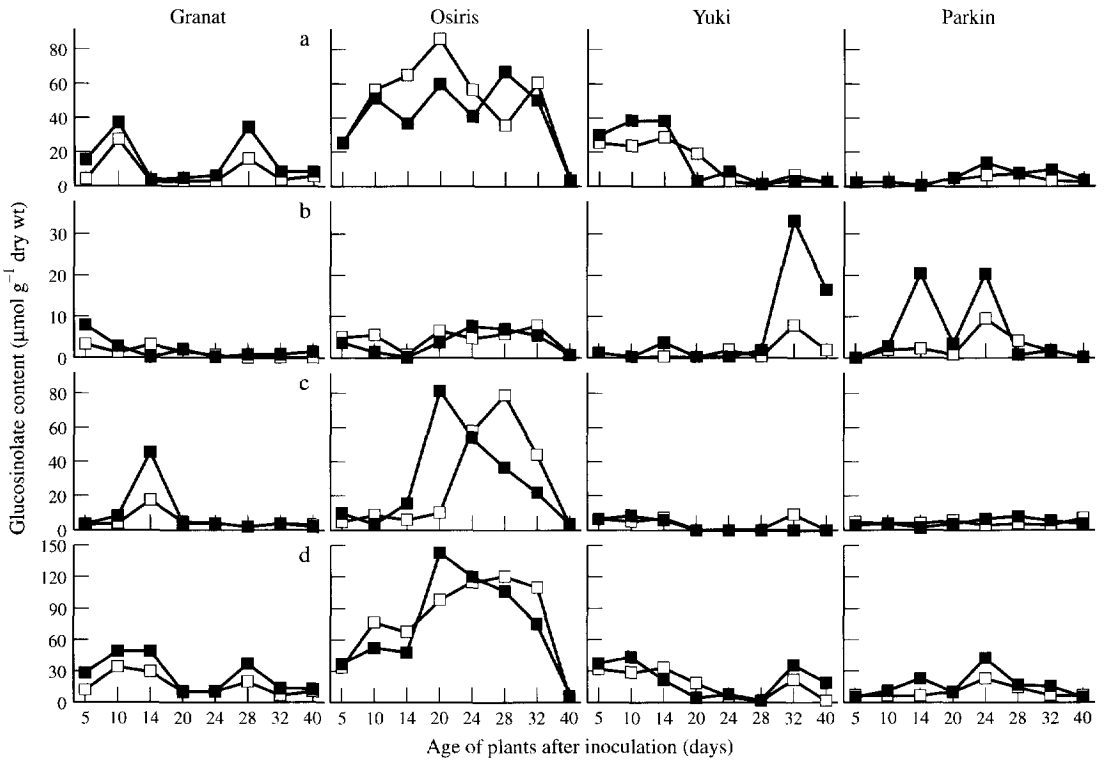

Fig. 2. Glucosinolate content in roots of two resistant ('Parkin' and 'Yuki') and two susceptible ('Granat' and 'Osiris') varieties of Chinese cabbage to infection with *Plasmodiophora brassicae*. Glucosinolates are divided into (a) aliphatic, (b) aromatic and (c) indolic compounds. Total glucosinolate content is given under (d). Seeds were directly germinated on soil containing *Plasmodiophora* spores; thus, the time-points represent days after sowing. (□) Control roots; (■) infected roots.

aliphatic glucosinolates. Infected roots of 'Granat' showed an additional peak in the aliphatic glucosinolates at day 28 after inoculation with *Plasmodiophora* (hypertrophy clearly visible), which is mainly due to an increase in glucoberteroin, and a prominent peak of indole glucosinolates 14 days after inoculation. Compared with the controls, the fraction of neo-glucobrassicin was mainly enhanced. In contrast, the aromatic glucosinolate fraction was only slightly enhanced after infection during early time points (five days). The infected roots of the variety 'Osiris' showed a peak of indole glucosinolates after 20 days and in healthy roots after 28 days, both again due to enhancement of neo-glucobrassicin. Bennett *et al.* [23] have shown that the predominant indole glucosinolate in young leaves of 35-day-old Chinese cabbage plants is neo-glucobrassicin, which we have confirmed now for the roots of the same cabbage variety. In the resistant varieties 'Parkin' and 'Yuki', we did not observe changes in aliphatic or indolic glucosinolates after infection, whereas aromatic glucosinolates were markedly enhanced 14 and 24 days after inoculation in 'Parkin' (mainly glucotropaeolin) and 14 and 30 days after inoculation in 'Yuki' (mainly gluconasturtiin). The life-cycle of the pathogen can be divided into two distinct phases: the primary phase, which is confirmed to the root hairs of the infected plants and which takes *ca* 14 days under laboratory conditions, and the secondary phase, which occurs in the cortex and stele of hypocotyl and roots, leading to tissue proliferation [10]. Primary infection is observed in resistant *Brassica* species as well as in some non-Brassicas, whereas secondary infection and, thus, symptom development, is restricted to susceptible varieties of the Brassicaceae [24, 25]. The increase in aromatic glucosinolates after 14 days might be involved in the defence reaction of the resistant variety 'Parkin' against the pathogen. A second peak in aromatic glucosinolates in 'Parkin' was observed after 24 days, but a smaller peak at this time-point was also observed in control tissue. Interestingly, the number of individual glucosinolates determined during this experiment was also lower in the two resistant varieties compared with the two susceptible (data not shown). Whether this correlation has any significance for the defence reaction cannot be answered at the moment.

Glucosinolate accumulation can also be induced by insect attack [16, 26] and fungal infection [5]. Such induction does not lead to similar increases in the content of all glucosinolates in the tissue, but rather to the increase of certain compounds during the development of a disease. Mechanical wounding or feeding by flea beetles (*Phyllotreta cruciferae*) induce a systemic increase in the amounts of indole glucosinolates in cotyledons and leaves of oilseed rape (*Brassica napus*

and *B. juncea*), but do not affect aliphatic or aromatic glucosinolates [6].

Some clear correlations have been reported between glucosinolate content of plants and insect feeding or herbivore damage. Malik *et al.* [27] reported a significant negative correlation between the glucosinolate content of 27 species and the fecundity of aphids feeding on them. Glen *et al.* [4] demonstrated that a slug species was deterred from feeding on the emerging cotyledons of oilseed rape in direct proportion to the glucosinolate content of the seedlings. On the contrary, it was demonstrated that it is the presence of glucosinolates which induces feeding by several insect species that specialize on Brassicaceae [28, 29]. Studies on cabbage stem flea beetles showed that this species would only feed on glucosinolate-containing plants, both Brassicas and non-Brassicas [30]. After inoculation with *P. brassicae*, we found symptoms of clubroot (=hypertrophy of the roots) only in varieties which showed high glucosinolate levels, especially indole glucosinolates ('Granat' and 'Osiris'). However, we have confirmed by SEM that the fungus also occurred in the resistant variety 'Parkin' (see Fig. 1), which did not show any symptoms of clubroot. This is an indication that the indole glucosinolate and may be also the auxin content of the respective plants may influence symptom development of clubroot disease. The absence of these compounds does not confer resistance to the plant, but rather prevents club development. However, evidence from the literature concerning this point is conflicting. While Butcher *et al.* [12] and Chong *et al.* [31, 32] found correlations between low indole glucosinolate content of the plants and resistance to clubroot, Mullin *et al.* [33] could not confirm these results. Previous results [18] have shown that indole glucosinolates were only elevated under conditions where club formation was observed. Invasion of roots of the susceptible variety 'Granat' in liquid culture did not lead to clubroot symptoms; the indole glucosinolate content was not increased compared with the control.

Our study has shown that in healthy roots the indole glucosinolate content of the susceptible and resistant varieties did not differ very much, but that after infection with *Plasmodiophora* the indole glucosinolate content was increased in the two susceptible varieties, whereas the two resistant varieties did not show such an increase. Measurements of TrpOxE in infected and healthy roots of the two varieties 'Granat' and 'Parkin' at a development stage where symptoms were clearly visible showed that enzyme activity in 'Parkin' was not enhanced in roots, which were grown in the presence of *Plasmodiophora* spores compared with control roots (5.70 and 5.38 fkat mg^{-1} protein, respectively), whereas in 'Granat' we detected significantly higher TrpOxE activity in infected (13.47 fkat mg^{-1} protein) than in control roots (7.38 fkat mg^{-1} protein). Together with the results on TrpOxE activity in seedlings we postulate that *de novo* synthesis of indole glucosinolates plays a role in the initial infection process, as well as in later stages of infection for clubroot symptom development.

Glucosinolates formed during treatment with salicylic and jasmonic acids

Two plant growth regulators, which are involved in signal transduction and/or modulation of resistance in plants (jasmonic [34] and salicylic acids [35]), are active in the induction of glucosinolates [5, 8]. Salicylic acid (SA) belongs to a group of plant phenolics, which have been found in many plant species [36]. There is evidence that SA plays an important role during metabolic events leading to systemic acquired resistance in plants [37] and to the accumulation of pathogenesis-related proteins [38].

SA is an endogenous compound in Chinese cabbage as demonstrated by GC–mass spectrometry [39]. Determinations of endogenous SA concentrations after inoculation of plants with *Plasmodiophora* has shown that after two weeks no SA could be detected, whereas six-week-old plants showed an increase in SA of *ca* three- and four-fold in roots and leaves, respectively, compared with control tissues [39]. Plants treated for two and six weeks with 5 mM SA after infection with *Plasmodiophora* showed a reduction in infection rate of *ca* 50 and 80%, respectively [39].

Jasmonic acid and its methyl ester are ubiquitous plant compounds with inhibitory and promotory effects on many physiological processes, including the triggering of various biosynthetic pathways associated with reactions to wounding, herbivory and infection [40]. Jasmonic acid and methyl jasmonate induced large increases in the concentration of indole glucosinolates (up to 20-fold) in cotyledons and leaves of oilseed rape and mustard (*B. juncea*) [8], whereas aliphatic and aromatic glucosinolates were unaffected. However, the response was slightly different between *B. rapa* and *B. juncea* concerning the individual indole glucosinolate enhanced. Similar results have been reported by Doughty *et al.* [5]. The proportion of indole glucosinolates after treatment with methyl jasmonate changed from 20% of the total glucosinolate content to *ca* 90% of the total in treated leaves of oilseed rape. Sprayed plants also accumulated 2-hydroxy-3-butenylglucosinolate (progoitrin) and 4-pentenylglucosinolate (glucobrassicanapin), but these compounds were also observed in controls which were sprayed with the surfactant, Triton X-100, alone. On the other hand, treatment with SA resulted in an increase mainly in 2-phenylethylglucosinolate (gluconasturtiin), whereas other glucosinolates were little affected [7]. The responses of oilseed rape after treatment with either jasmonic acid or SA is more specific than that observed after fungal infection [5]. Our results showed that the leaves of two Chinese cabbage varieties ('Parkin' and 'Granat') treated with jasmonic acid contained high concentrations of indole glucosinolates (both glucobrassicin and neo-glucobrassicin were enhanced; *ca* five-fold higher in 'Granat' and *ca* three-fold higher in 'Parkin' compared with controls) (Fig. 3).

Contrary to the previously described results, we also found an increase in the aromatic glucosinolates in the leaves of 'Parkin' (glucotropaeolin), but not in

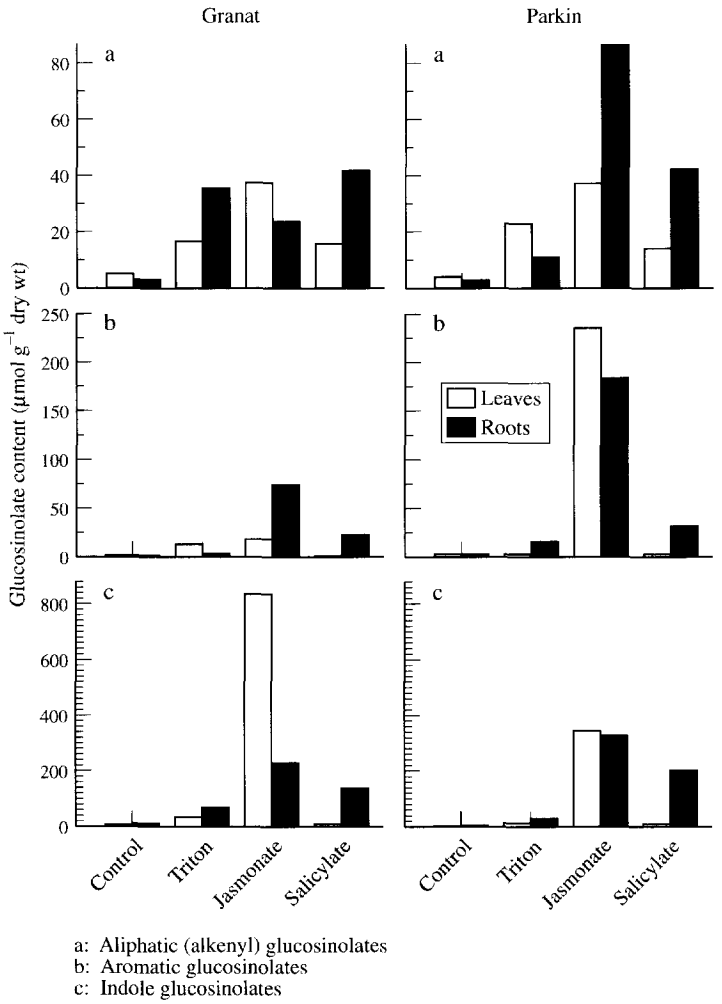

Fig. 3. Effect of 5 mM jasmonic and salicylic acids on glucosinolate content of two Chinese cabbage varieties (one resistant, 'Parkin', one susceptible, 'Granat'). Three-week-old plants were sprayed with a solution of either acid in 0.2% Triton X-100 every day for one week. As controls, plants were either grown without any treatment or sprayed with 0.2% Triton X-100 alone. (□) Leaves; (■) roots. (a) Aliphatic, (b) aromatic and (c) indole glucosinolates.

'Granat'. Salicylate treatment even decreased the glucosinolate content in leaves of both varieties. In roots of 'Granat', the indole glucosinolates (neo-glucobrassicin, followed by glucobrassicin) were also enhanced, but only by *ca* two-fold, whereas in 'Parkin' there were no differences between roots and shoots after jasmonate treatment. After SA treatment, we have also recorded elevated indole glucosinolate levels in the roots of both varieties (mainly neo-glucobrassicin in 'Parkin' and 4-methoxyglucobrassicin in 'Granat') and also an increase in the aromatic glucosinolates (gluconasturtiin, in both varieties). Roots of 'Parkin' also showed an increase in aliphatic (mainly gluconapin) and aromatic glucosinolates (gluconasturtiin) after treatment with both substances. The control treatment (Triton X-100 + 0.5% ethanol) also increased the glucosinolate content compared with water controls, but the differences after jasmonate and salicylate treatment were much more prominent (Fig. 3). We have demonstrated that not only leaves, as in previous studies [5, 7, 8], but also roots of Chinese cabbage respond to treatment with jasmonate and salicylate by altering the endogenous glucosinolate content and pattern. We have shown that the pattern is different in a resistant and a susceptible Chinese cabbage variety.

EXPERIMENTAL

Plant material. Seeds of different Chinese cabbage (*B. campestris* ssp. *pekinensis*) varieties were obtained from commercial sources. Granat as from J. Fuchs, Samenhandlung, Frankfurt, Germany; Osiris, Hong Kong and Sixtyres from Juliwa, Julius Wagner GmbH, Heidelberg, Germany; Storido, Kimono and Okido from Nickerson-Zwaan, Netherlands; Nippon from Sperli, Sperling & Co., Germany; and Parkin and Yuki from S & G Samen GmbH, Kleve, Germany.

Infection procedure. Plants were grown from seeds cultivated in soil at 23° with 90% humidity [18]. Inoculation with *P. brassicae* was performed as

described elsewhere [41], using a field isolate. Following infection, plants were further cultivated in soil at 23°, 60% humidity and a light–dark regime of 16 hr/8 hr light intensity (280 μmol m^{-2} sec^{-1}), as previously described [18]. Roots of infected and control plants were harvested between 5 and 40 days after inoculation.

Treatments with jasmonic and salicylic acids. Chinese cabbage seeds were sown on to soil and cultivated as described above. After 3 weeks of culture, seedlings were sprayed with a 5 mM soln of either SA (Merck) or jasmonic acid (Sigma) in an aq. soln containing 0.2% Triton X-100. Controls were sprayed with Triton X-100 only. Spraying was performed every 2nd day for 8 days. The last treatment was the day prior to harvest of plants.

Glucosinolate extraction and analysis. Plants were harvested, washed thoroughly and dried with filter paper. Tissue was then immersed in liquid N_2, freeze-dried, milled and stored at −20° prior to glucosinolate extraction. For each extraction, 200 mg dry wt powdered material was used. Seeds were directly ground and extracted for glucosinolates. Individual glucosinolates were extracted and quantified according to ref. [3]. Glucosinolates were desulphated with arylsulphatase (Sigma) on a DEAE Sephadex A25 column and desulphoglucosinolates eluted with H_2O for HPLC. Desulphoglucosinolates were sepd on a reverse-phase C18 column with MeCN (A) and H_2O (B) as solvent. The gradient programme was 5 min A–B (1:99), 15 min gradient to A–B (23:77), 5 min A–B (23:77), 15 min gradient to A–B (100:0), equilibrating for 5 min to A–B (1:99). Detection of glucosinolates was at 229 nm; allyglucosinolate (sinigrin, Sigma) was used as int. standard. All values presented are the means of 3 different measurements.

Measurement of TrpOxE. The enzyme converting tryptophan into indole-3-acetaldoxime (IAOX) was determined according to ref. [21] using 6.6 kBq (3.6 μmol) of L-^{3}H-tryptophan (Hartmann Analytik, Germany) as substrate. Protein concn was 0.5 mg in the total assay volume of 500 μl. All assays were run in triplicate.

Scanning electron microscopy. Infected roots were harvested and washed with dist. H_2O. Tissue was frozen in liquid N_2 and subsequently freeze-dried. Sections of *ca* 1–2 mm were cut from the clubs, placed on a carbon grid and sputtered for 5 min with gold.

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