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Genotypical Differences in Total Sulfur Contents and Cysteine Desulfhydrase Activities in *Brassica napus* L.

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Summary

BURANDT P., PAPENBROCK J., SCHMIDT A., BLOEM E., HANEKLAUS S. & SCHNUG E. 2001. Genotypical differences in total sulfur contents and cysteine desulfhydrase activities in *Brassica napus* L. – Phyton (Horn, Austria) 41 (1): 75–86, with 3 figures. – English with German summary.

The sulfur supply of agricultural crops plays an important role in their defence strategies against pathogens. The mechanisms of sulfur induced resistance (SIR) are, however, not yet known. The working hypothesis was the involvement of volatile sulfur compounds in SIR, especially the evolution of H_2S. In total, ten *Brassica* lines (no. 1-10) of winter oilseed rape showing a broad variation with view to their genetic pedigree were selected from a set of about 100 genotypes, and leaf samples were taken just before flowering. The activities of a H_2S releasing L-cysteine desulfhydrase-like enzyme (EC 4.4.1.-) and of *O*-acetylserine(thiol)lyase (OAS-TL, EC 4.2.99.8) were measured, and the total sulfur content was determined by X-ray spectroscopy. The higher the sulfur content in plants, the lower was the L-cysteine desulfhydrase ac-

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tivity and the higher was the OAS-TL activity. This is the first time that a correlation was found between enzymatic H_2S evolution from L-cysteine and the total sulfur content which is an indicator for the sulfur nutritional status of the crops. The lines showing the strongest significant differences were selected for further investigations.

Zusammenfassung

BURANDT P., PAPENBROCK J., SCHMIDT A., BLOEM E., HANEKLAUS S. & SCHNUG E. 2001. Genotypische Unterschiede im Gesamtschwefelgehalt und in Cysteindesulfhydrase-Enzymaktivitaeten in *Brassica napus* L. – Phyton (Horn, Austria) 41 (1): 75–86, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Schwefelversorgung von landwirtschaftlichen Kulturpflanzen ist von großer Bedeutung für deren Resistenz gegenüber pilzlichen Krankheitserregern. Die physiologischen Prozesse dieser durch Schwefel induzierten Resistenz sind jedoch noch weitgehend unerforscht. Ein möglicher Mechanismus, der in dieser Arbeit untersucht wurde, ist die Freisetzung von gasförmigen schwefelhaltigen Stoffwechselprodukten, insbesondere H_2S, die zu einer erhöhten Resistenz gegenüber pilzlichen Pathogenen führt.

Aus insgesamt 100 Winterrapslinien mit einer großen genetischen Variabilität wurden für die Untersuchungen zehn verschiedene *Brassica napus* Linien ausgewählt, die sich besonders stark unterschieden. Blattmaterial wurde auf die Aktivitäten des H_2S-freisetzenden Enzyms L-Cystein-Desulfhydrase (EC 4.4.1.-) und der *O*-acetyl-L-serin(thiol)lyase (OAS-TL, EC 4.2.99.8) untersucht und mit dem Gesamtschwefelgehalt der Pflanzen verrechnet. Je höher der Schwefelgehalt in den Pflanzen war, desto niedriger war die L-Cystein-Desulfhydrase-Aktivität und desto höher die OAS-TL-Aktivität.

Die Ergebnisse sind als erster Hinweis darauf zu werten, daß eine Beziehung zwischen der enzymatischen Bildung von H_2S aus L-Cystein und dem Gesamtschwefel-Gehalt besteht, was darauf hindeutet, daß die pflanzliche Resistenz und die Schwefelversorgung miteinander korreliert sind. Die *Brassica*-Linien mit den größten signifikanten Unterschieden wurden für weitere Versuche ausgewählt.

Introduction

The clean air acts have led to a drastic decrease of SO_2 emissions in western Europe (DÄMMGEN & al. 1998) and macroscopic sulfur (S) deficiency has become the most widespread nutrient disorder since then (SCHNUG & HANEKLAUS 1998). Besides there being a decrease in yields and negative impacts on crop quality, a higher susceptibility of *Brassica* crops against certain diseases has been observed (SCHNUG & al. 1995a). Scoring the infestation of different oilseed rape genotypes with *Pyrenopeziza brassicae* in 2000 revealed that the susceptibility of the crop towards this disease was closely related to the natural S supply, such that the highest infestation could be observed consistently at the site with the lowest S deposition. Field trials of SCHNUG & al. 1995a and BOURBOS & al. 2000 showed that S fertilization efficiently controlled the infection of oilseed rape with *Pyrenopeziza brassicae* and grapes with mildew, respectively.

The physiological background for the natural resistance of crops against certain pests and diseases is thought to be related to increased H_2S emissions of the plants which are highly toxic and thus may act as a fungicide (SEKIYA & al. 1982, SCHRÖDER 1993, BEAUCHAMP & al. 1984). The influence of the plant S supply on the amount of H_2S released by oilseed rape is not yet known, but in the case of other secondary S compounds, such as glutathione and glucosinolates such relationships were found (HANEKLAUS & al. 1999, SCHNUG & al. 1995b). Before testing the influence of the S supply on the release of H_2S, genotypic differences and ranges of the plant S status and enzymatic activities need to be elaborated. Therefore it was the aim of these investigations to examine various genotypes of different pedigrees in a S-rich environment with view to genotypic differences in the total S content and the activity of the H_2S releasing enzymes like cysteine desulfhydrases. L-cysteine desulfhydrase proteins catalyse the degradation of cysteine to pyruvate, ammonium and H_2S or to alanine and H_2S (RENNENBERG & al. 1987, ZHENG & al. 1993). Former experiments have led to the assumption that *O*-acetylserine(thiol)lyase (OAS-TL) acting as a catalyst for the synthesis of cysteine from *O*-acetylserine and H_2S might also be involved in the release of H_2S (SCHMIDT 1977a, b). Therefore, both enzymes might contribute to the evolution of H_2S.

Materials and Methods

Ten different winter rape (*Brassica napus*) genotypes (line 1-10) which were grown at the plant breeding station of DSV (Deutsche Saatveredelung at Thuele; 8°42.0' E; 51°36.0' N) were investigated out of a set of about 100 genotypes. The S demand of the crop was fully acknowledged by a S fertilization of 100 kg S ha^{-1} at the start of the vegetation period. Plant material was collected from 10 lines on the 15th of April in 1999 with 4 repetitions just before flowering. Younger, fully developed leaves of the upper third of the oilseed rape crop were taken. Samples were both frozen in liquid nitrogen and dried in a ventilated oven at 80 °C until constancy of weight, they were then fine-ground (< 0.12 mm) using a RETSCH ultra-centrifugal mill for further analysis.

For the determination of the total S contents by X-ray fluorescence spectroscopy powdered parts were prepared, mixing 1.1 g of plant material with 4.4 g of HOECHST wax C (SCHNUG & HANEKLAUS 1999).

The cDNA sequence encoding the mature plastid OAS-TL protein (Acc. number X81698, HESSE & al. 1999) was amplified by PCR using red *Taq* polymerase (Sigma, Germany). Primers used were as follows: primer 17 (5'-GCG GAT CCG CTG TAT CTA TCA A-3') with a *Bam*HI restriction site and primer 18 (5'-TAT GTC GAC TCA AAG CTC GGG CTG-3') with a *Sal*I restriction site. The PCR product was cloned into the pQE-30 vector behind a 6x His-tag coding sequence (Qiagen, Germany). The resulting *pQE-CS B* plasmid was transformed in the *E. coli* strain XL1-Blue. After growth at 37 °C to an OD_{600} of 0.8 in Luria-Bertani medium containing ampicillin (100 µg/ml) induction was carried out for 3 h with 1 mM IPTG. Cell lysis was obtained by adding 1 mg/ml lysozyme and vigorous pottering. The recombinant protein

was purified under non-denaturing conditions by nickel-affinity chromatography according to manufacturer's instructions (Qiagen). To remove salt and imidazole combined fractions were dialyzed with excessive amounts of 20 mM Tris/HCl, pH 8.0 at 4 °C overnight. About 35 mg purified protein was obtained from one liter IPTG induced *E. coli* culture.

Enzyme activities were measured with crude plant extracts and with purified recombinant OAS-TL protein. The frozen *Brassica* leaves were mortared to a fine powder. 100 mg of the powder was suspended in 1 ml cold 20 mM Tris/HCl, pH 7.5, and centrifuged to remove cell debris. The supernatant was used in enzyme assays. Affinity purified protein was added to the assay in indicated amounts. The assay for measuring OAS-TL activity contained in a total volume of 1 ml: 5 mM *O*-acetylserine, 5 mM Na_2S, 3.3 mM dithiothreitol, 100 mM Tris/HCl, pH 7.5, and enzyme extract (modified from SCHMIDT 1990). The reaction was initiated by the addition of Na_2S and incubated for 30 min at 37 °C, after which the reaction was terminated by adding 1 ml acidic ninhydrin reagent (0.8 % ninhydrin (w/v) in 1:4 conc. HCl:HOAc) to determine the cysteine concentration (GAITONDE 1967). The samples were heated at 100 °C for 10 min to allow color development and cooled on ice. Finally 2 ml ethanol were added to stabilize the color complex. The absorbance at 560 nm was measured using a standard curve for quantification.

L-cysteine desulfhydrase activity was measured by the release of sulfide from cysteine in a total volume of 1 ml: 2.5 mM dithiothreitol, 0.8 mM L-cysteine, 100 mM Tris/HCl, pH 9.0, and enzyme extract. After 15 min at 30 °C the reaction was terminated by adding 100 µl of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µl 20 mM N, N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 M HCl (SIEGEL 1965). The formation of methylene blue was determined at 670 nm in a spectral photometer and quantified using a Na_2S standard curve.

For each of the four samples collected per line the assays were repeated three times on independent days. In general the standard deviations for the determination of sulfide from cysteine of these independent repeats were rather high. Currently, these technical problems are under investigation to improve reproducibility of this particular enzyme assay. Therefore the statistical analysis was done by comparing relative values for both enzyme activities. For the L-cysteine desulfhydrase activity 100% was 9.34 nmol (mg protein x min)$^{-1}$, for the OAS-TL activity 100% was 132.84 nmol (mg protein x min)$^{-1}$. Protein estimation was done according to BRADFORD 1976 using bovine serum albumine as a protein standard.

The ANOVA procedure and t-test were used to determine which means were significantly different from others at the 5% significance level employing the CoHort software package (SIMONS 1995).

The valuation of the fungizide infestation and the ELISA test was done by the DSV. The **E**nzyme **L**inked **I**mmuno **S**orbent **A**ssay (ELISA) is an immunological test which can be used to detect and measure either the antigen or the amount of bound antibody.

Results

Differences in the total S content and L-cysteine desulfhydrase and *O*-acetylserine(thiol)lyase activity between 10 winter oilseed rape genotypes are summarized in table 1. Statistical analysis of the combined re-

Table 1.
Differences in the relative L-cysteine desulfhydrase and *O*-acetylserine(thiol)lyase activity and total sulfur content of genotypes of different pedigrees (t-test).

Genotype 1999	Total S [mg g^{-1}]	L-cysteine desulfhydrase [nmol (mg protein × min)$^{-1}$]		*O*-acetylserine(thiol)lyase [nmol (mg protein × min)$^{-1}$]	
		EA[1]	rel. [%][2]	EA[1]	rel. [%][2]
1	7,08 [a 3]	6,02	82,1 [b]	169,431 [a]	127,1 [a]
2	5,93 [bc]	6,97	95,1 [ab]	143,497 [ab]	107,6 [ab]
3	5,76 [bcd]	6,99	95,4 [ab]	125,522 [b]	94,8 [b]
4	5,01 [d]	7,77	106,0 [a]	137,926 [ab]	103,5 [ab]
5	5,43 [cd]	7,52	102,6 [a]	134,681 [ab]	101,5 [ab]
6	5,62 [bcd]	7,79	106,3 [a]	135,761 [ab]	101,1 [ab]
7	5,90 [bc]	7,41	101,0 [ab]	112,360 [b]	84,6 [b]
8	6,36 [ab]	7,78	106,2 [a]	123,600 [b]	93,1 [b]
9	5,97 [bc]	7,26	99,1 [a]	120,014 [b]	90,5 [b]
10	5,62 [bcd]	7,75	105,2 [a]	127,077 [b]	96,0 [b]
LSD5 %	0,84		14,8		15,7

[1] estimated activity (the enzyme activity was estimated on the basis of the mean values of the 12 measurements of in total 10 genotypes)

[2] relative values are mean values of triple measurements with the mean value being calculated separately for each measurement and including four repetitions in the field

[3] different letters indicate statistically significant differences between means at the 5 % probability level

lative values of enzyme activities revealed significant differences between certain lines (Table 1). It could be demonstrated that lines 4, 5, 6, 8, 9 and 10 showed significantly higher desulfhydrase activities when compared with line 1. Though based on relative values genotypical differences between lines could be shown.

The highest S content was 7.08 mg S/g dry weight, this was shown by genotype 1, which is above the critical nutrient threshold of 6.5 mg S/g dry weight (SCHNUG & HANEKLAUS 1998). It was also genotype 1 which had the lowest L-cysteine desulfhydrase and highest *O*-acetylserine(thiol)lyase activity (Table 1). In comparison genotype 4 showed the lowest S content and almost the highest L-cysteine desulfhydrase activity. A significant inverse relationship between total S content and L-cysteine desulfhydrase activity was found ($r = 0.729$; $p < 0.05$) while none could be verified for the enzyme *O*-acetylserine(thiol)lyase. An increasing *O*-acetylserine(thiol)lyase activity was related to a decreasing L-cysteine desulfhydrase activity ($r = 0.675$; $p < 0.05$).

It was demonstrated before, that cysteine desulfhydrases contain pyridoxal phosphate as a co-factor and form aminoacrylate as an intermediate (TAI & COOK 2000). In plants and bacteria another group of enzymes, the

OAS-TLs, occurs that also contains pyridoxal phosphate as a co-factor and are involved in cysteine biosynthesis while forming aminoacrylate as an intermediary product (reviewed in TAI & COOK 2000). To follow former observations suggesting H_2S-release as a side reaction from the cysteine synthase reaction the plastid isoform of OAS-TL was expressed in *E. coli* and purified to homogeneity by Ni^{2+}-affinity chromatography. The recombinant homogeneous OAS-TL protein catalysed the formation of cysteine from *O*-acetylserine and H_2S but also the formation of H_2S from cysteine. In a molar ratio the enzyme formed about 25 times more cysteine than H_2S per mg protein during the same incubation time (Figure 1). To be able to quantify and distinguish between the H_2S-release, catalysed by cysteine desulfhydrase activities on the one hand and the release of H_2S formed in the side reaction of OAS-TL enzymes on the other, the cysteine synthase activity levels were determined in crude protein extracts of all *Brassica* lines. The steady-state OAS-TL activities of lines 3, 7, 8, 9 and 10 differed significantly from line 1 (Table 1). However, as a general tendency, lines showing a high desulfhydrase activity had a low OAS-TL activity and vice versa, e.g. lines 1, 8, 9, and 10. For visualization the relative values of both activity assays from all lines investigated were compared (Figure 2). The relative ratios of cysteine to sulfide formed differ in the selected *Brassica* lines.

Scoring of fungal infestation with *Phoma* and *Verticillium* shortly before harvest in the field (developmental stage 83-85; STRAUSS & al. 1994) yielded no statistically significant differences between the genotypes. In comparison, the ELISA value for infestation rates with *Verticillium* decreased with increasing S status of the crop, thus indicating a higher natural resistance (Figure 3).

Discussion

Sulfur (S) metabolism and plant resistance against pests and diseases are related but the underlying mechanisms and possible advantages of improved S fertiliser strategies are still unknown. Recently, it has been observed that throughout Europe certain diseases (e.g. light leaf spot in oilseed rape) occur more frequently especially in areas with low S inputs (THOMAS 1994). The S metabolism provides several efficient mechanisms by which plants are able to tackle abiotic and biotic stress. Mechanisms involved in the response to plant pathogens include the production of S containing compounds during the secondary metabolism of the agriculturally important *Brassica* species, resulting in the release of volatile S compounds (GRUNDON & ASHER 1986, SIMAN & JANSSON 1976, TOLLSTEN & BERGSTRÖM 1988, SCHRÖDER 1993), glutathione (LAMOUREUX & RUSNESS 1993), the production of S rich proteins (BOHLMANN 1993) and localised deposition of elemental S (COOPER & al. 1996, RESENDE & al. 1996).

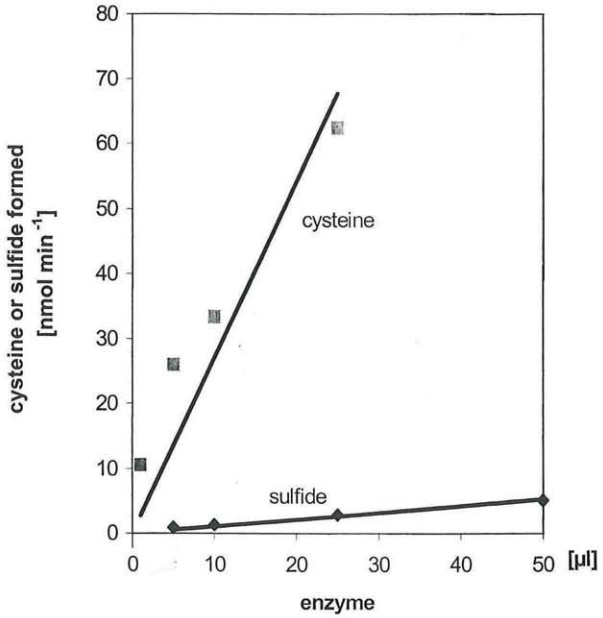

Fig. 1. L-cysteine desulfhydrase and OAS-TL activities of the recombinant purified OAS-TL protein using increasing amounts (1 to 50 µl) of enzyme.

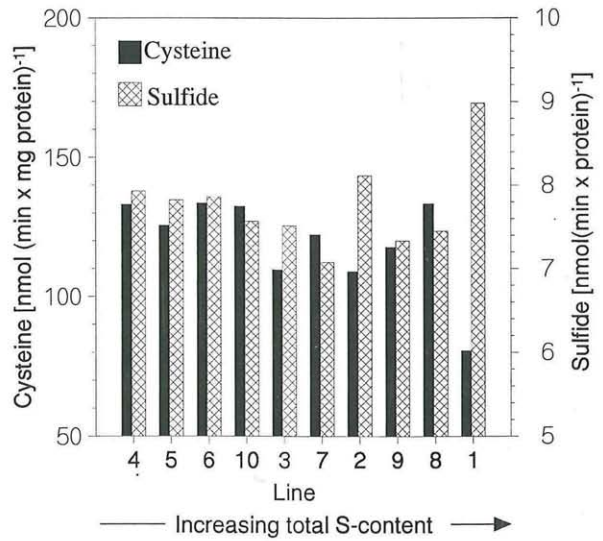

Fig. 2. The ratios of L-cysteine desulfhydrase activity to OAS-TL activity in the selected *Brassica* varieties were depicted.

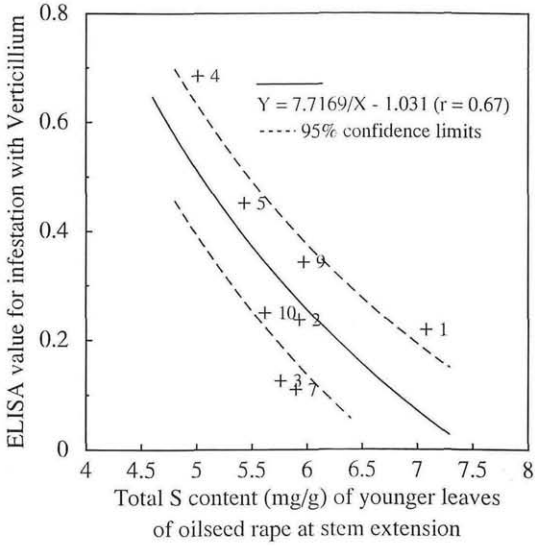

Fig. 3. Relationship between total S content of younger, fully differentiated leaves of different oilseed rape genotypes at stem extension and infestation rates with *Verticillium* (according to the ELISA test) before harvest.

Some aspects of S metabolism are not fully understood. For example it is not known if the H_2S emission takes place before or after the cysteine synthesis or whether the H_2S emission is genetically controlled or directly correlated with the S supply.

The results of these investigations indicate that the capability of different *Brassica napus* lines to use the plant available soil S is genetically controlled, this is because the total S content of 10 lines varied between 5.0 and 7.1 mg g^{-1} S. All lines received a uniform high S fertilization but the lines showed a high variability in their capacity to form H_2S. Higher plants probably contain several enzymes which are involved in H_2S-release. As a general tendency one can conclude from the results of the enzyme analysis that the higher the S content in the plants was, the lower the L-cysteine desulfhydrase enzyme activity was and the higher the OAS-TL activity was. Therefore a genetic influence on the activity of the investigated enzymes L-cysteine desulfhydrase and OAS-TL is probable.

The experiments showed that beside the enzyme activity, the molar ratio between cysteine and H_2S-formation differed in the investigated 10 lines, too. However, with respect to the biochemistry of the cofactor pyridoxal phosphate, the molar ratio of cysteine and H_2S formed by the OAS-TL proteins was expected to be equal. Therefore one has to conclude that in addition to H_2S-release by OAS-TL proteins desulfhydrases play a

major role in the evolution of H_2S from *Brassica* plants or probably from higher plants in general. For future investigations on the enzymological basis of H_2S-release one has to keep in mind the involvement of different enzyme reactions and therefore different enzyme families. Recently, it was also demonstrated in yeast, that the simple hypothesis that variation in OAS-TL activity is correlated with H_2S production and release is not supported by any data (SPIROPOULOS & BISSON 2000).

The results which are presented in this paper are a first evidence that the evolution of gaseous S compounds and therefore the S nutrition plays an important role in plant resistance against fungal infections. It was shown that the S nutrition controlled the infection of oilseed rape with *Pyrenopeziza brassicae* and grapes with mildew (SCHNUG & al. 1995a, BOURBOS & al. 2000) and the results of this paper indicate that the infestation of oilseed rape with *Verticillium* is also influenced by the S nutrition of the crop. In contrast to *Pyrenopeziza brassicae* which infect the plant via wind or precipitation of conidiospores on outer surfaces, *Verticillium dahliae* is a fungi which survives in the soil and infects the plant via the roots (PAUL 1992). Therefore both fungii are very different in their life cycle and it is yet not known how the S metabolism influences the infestation with fungii. The results which are presented in Figure 3 show a relationship between the S nutrition of oilseed rape plants and the infection with *Verticillium*: with higher total S content in the leaves the plants show less infestation with *Verticillium*. H_2S evolution from the crops seems more likely to influence fungii like *Pyrenopeziza* or *Phoma* which infect the plant via leaf infestation.

No significant relationship between scoring in the field and the results from the ELISA test for infestation with *Phoma* and *Verticillium* was found which indicates that one major problem in finding causal relationships between S status, enzyme activities and fungal infestations may be the choice of an appropriate method for the evaluation of the plant health status.

And last but not least further investigations are necessary with the same genetic lines, but different S nutritional status. In the presented investigations all plants received the same S fertilisation and differences in the S status and S metabolism depended exclusively on genetic differences. Therefore it can be expected that trials with different amounts of S fertilisation will deliver more information about causal relationships between S nutrition and plant resistance mechanisms.

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