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THE RELATIONSHIP BETWEEN DIFFERENT TYPES OF MARKERS AND GLUCOSINOLATES CONTENT OF PARENTAL LINES OF F_1 CMS *OGURA* HYBRIDS OF WINTER OILSEED RAPE (*BRASSICA NAPUS* L.)

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Abstract

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The protein content in dry matter of seeds of winter oilseed rape ranges from 21 to 24%, in oil-free meal from 33 to 43% and in cake mill from 28 to 34%. In order to ensure the use of rapeseed meal and expellers as feed for livestock, breeding programmes are conducted to reduce the content of antinutritional compounds in seeds. Glucosinolates in meal of oilseed rape are still one of the main antinutritive compounds, despite their low glucosinolates content in seeds of double-low open pollinated and hybrid varieties of winter oilseed rape. The breeding of rapeseed cultivars is carried out in order to reduce alkenyl glucosinolates to total elimination. The effectiveness of breeding of rapeseed genotypes with low glucosinolates content can be increased by using different types of molecular markers. Effective selection of parental forms of hybrids characterized by different glucosinolates content is the factor that has a decisive role in the advancement of this type of breeding. The application of molecular markers makes a selection process much more effective. Taking this into account, there were initiated investigations into the relationship between glucosinolates content of 18 parental lines of F_1 CMS *ogura* hybrids of winter oilseed rape and different types of markers. DNA polymorphism of parental lines was determined using RAPD, AFLP, polymerase chain reaction and isozymes. The obtained results revealed a statistically significant relationship between the markers and glucosinolates content.

Key words: glucosinolates content; hybrids; molecular markers; oilseed rape (*B. napus* L.)

Introduction

Winter oilseed rape (*Brassica napus* L.) is the most important oil plant in Poland and European Union. Seeds of oilseed rape are the source not only of oil but also of valuable proteins. The oil content in seeds ranges from 45 to 50% and protein content ranges from 21 to 24%. Defatted oilseed rape meal contains approximately 33 to 43% protein with well balanced aminoacids, considered equivalent in quality to soybean. The use of oilseed rape and other *Brassica* crops meal as protein-rich food and feed gets restricted by the presence of glucosinolates, mainly aliphatic and other antinutritive compounds. To provide for the use of oil-free meal and expellers as fodder for livestock feeding, scientific and breeding research aims to achieve the reduction of content of anti-nutritional components of *Brassica* seeds, such as: glucosinolates (aliphatic), fiber, phytates, phenolic acids, sinapic acid esters and flavonoids (Snowdon et al., 2007; Friedt and Snowdon, 2009).

Breeding of open pollinated and hybrid cultivars aims at the reduction of the level of aliphatic glucosinolates until total elimination. The effectivness of the selection of genotypes of oilseed rape with low glucosinolate content can be increased through the use of molecular markers. However, to fully exploit the potential of molecular techniques, it is necessary to search for markers associated with quantitative trait loci

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(QTLs) and to map agronomically important genes in *Brassica* genomes. Different breeding and selection programmes of *Brassica* crops successfully apply different types of DNA markers techniques. The application of molecular marker includes, but is not limited to, constructing genetic maps, genotyping alleles, localizing quantitative trait loci (QTLs), identifying varieties, evaluating genetic distance between hybrids or breeding lines and monitoring gene (Snowdon and Friedt, 2004; Bocianowski et al., 2011, 2012). In winter oilseed rape the markers associated with morphological traits, oil content, fatty acid composition and glucosinolates contents in seeds have been described (Hasan et al., 2008; Liersch et al., 2009; Federico and Federico, 2011).

Taking this into consideration, an investigation focused on the relationship between glucosinolates content of parental lines of F_1 CMS *ogura* hybrids and different types of markers has been initiated.

Materials and Methods

Plant material and field trials

The plant material was developed at Plant Breeding and Acclimatization Institute in Poznan and Plant Breeding Company Ltd Strzelce – Borowo Division. Eighteen parental lines of winter oilseed rape F_1 CMS *ogura* hybrids (eight CMS *ogura* lines and 10 paternal lines – six restorers and four without restorer gene) were used as plant material in this project. Field trials were performed in four replications of completely randomized block design, during the crop seasons of 2002-2003 and 2003-2004. After the harvest the glucosinolate contents in seeds of 18 parental lines and F_1 hybrids were measured. The analyses of total glucosinolate content and glucosinolates composition were performed by gas chromatography of silyl derivatives of desulfoglucosinalates (Michalski et al., 1995). The investigated parental lines of F_1 hybrids were significantly different with regard to total glucosinolate contents and composition (Figure 1A-1F).

In order to determine the association between different types of markers and glucosinolate composition of parental lines of F_1 hybrids, the analyses of random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and isozyme markers were performed.

RAPD, AFLP, PCR analysis and isozyme

Genomic DNA was extracted from young leaves of 10 plants using a modified CTAB procedure according to Doyle and Doyle (1990). The DNA samples were analysed using 57 arbitrary 10-bp-long oligonucleotides as primers (Operon Technologies, USA) according to the basic procedure of the RAPD reaction described by Williams et al. (1990). AFLP analysis was performed using standard methods in accordance with the manufacturer's instructions (Gibco RRL., AFLP Analysis Reagent Kit, AFLP Analysis System I) and as previously described by Vos et al. (1995). DNA (150 ng) was doubled-digested with *Eco*RI and *Mse*I restriction enzymes, and then ligated with adaptors (AFLP Core Reagent Kit and AFLP Starter Primer Kit Gibco BRL Life Technologies Inc.). The selective nucleotides for the AFLP primers used in this study included five *Eco*RI and seven *Mse*I primer in 23 primer combinations. All PCR reactions were performed in an Eppendorf Mastercycler Gradient Thermal cycler. The analyses of isozymes in starch gel electrophoresis were conducted according to methods developed by Schields et al. (1983) and Vallejos (1983). Five isozyme systems were tested, including: isocitrate dehydrogenase (IDH, EC 1.1.1.42), leucine aminopeptidase (LAP, EC 3.4.11.1), malate dehydrogenase (MDH, EC 1.1.1.37), phospho-glucoisomerase (PGI, EC 5.3.1.9), 6 phosphogluconate dehydrogenase (6 PGD, EC 1.1.1.44).

Statistical analysis

The association between molecular markers and glucosinolates was estimated using regression analysis. The marker observations were tested as a independent variables and considered in individual models. We used the critical significance level equal to 0.01, resulting from a Bonferroni correction, for each regression model (Bocianowski and Selidler-Łożykowska, 2012). All analyses were performed with the procedure in GenStat v. 10.1 (GenStat, 2007).

Results and Discussion

A total of 225 (33.7% of all markers studied) RAPD, 354 (59.3%) AFLP markers and 18 (3%) isozyme patterns were screened for polymorphism and searched for the relationship between molecular markers and glucosinolate content of parental lines of F_1 CMS *ogura* hybrids. Of particular interest are the markers listed in Tables 1-6. The association of three types of markers with particular glucosinolates content in seeds of parental lines of winter oilseed rape hybrids is exhibited by gluconapine (Table 1), glucobrassicanapine (Table 2), progoitrine (Table 3), 4-hydroxybrassicanapine (Table 4), total glucosinolates (Table 5) and total alkenyl glucosinolate content (Table 6).

26 RAPD (12) and AFLP (14) markers were correlated with alkenyl glucosinolate- gluconapine. The nine bands of RAPD represented an increase of this glucosinolate while the eight AFLP and three RAPD markers showed a decrease in gluconapine (Table 1). The percentage of total phenotypic variability explained by particular markers ranged from 31.1% to 38.2% (Table 1).

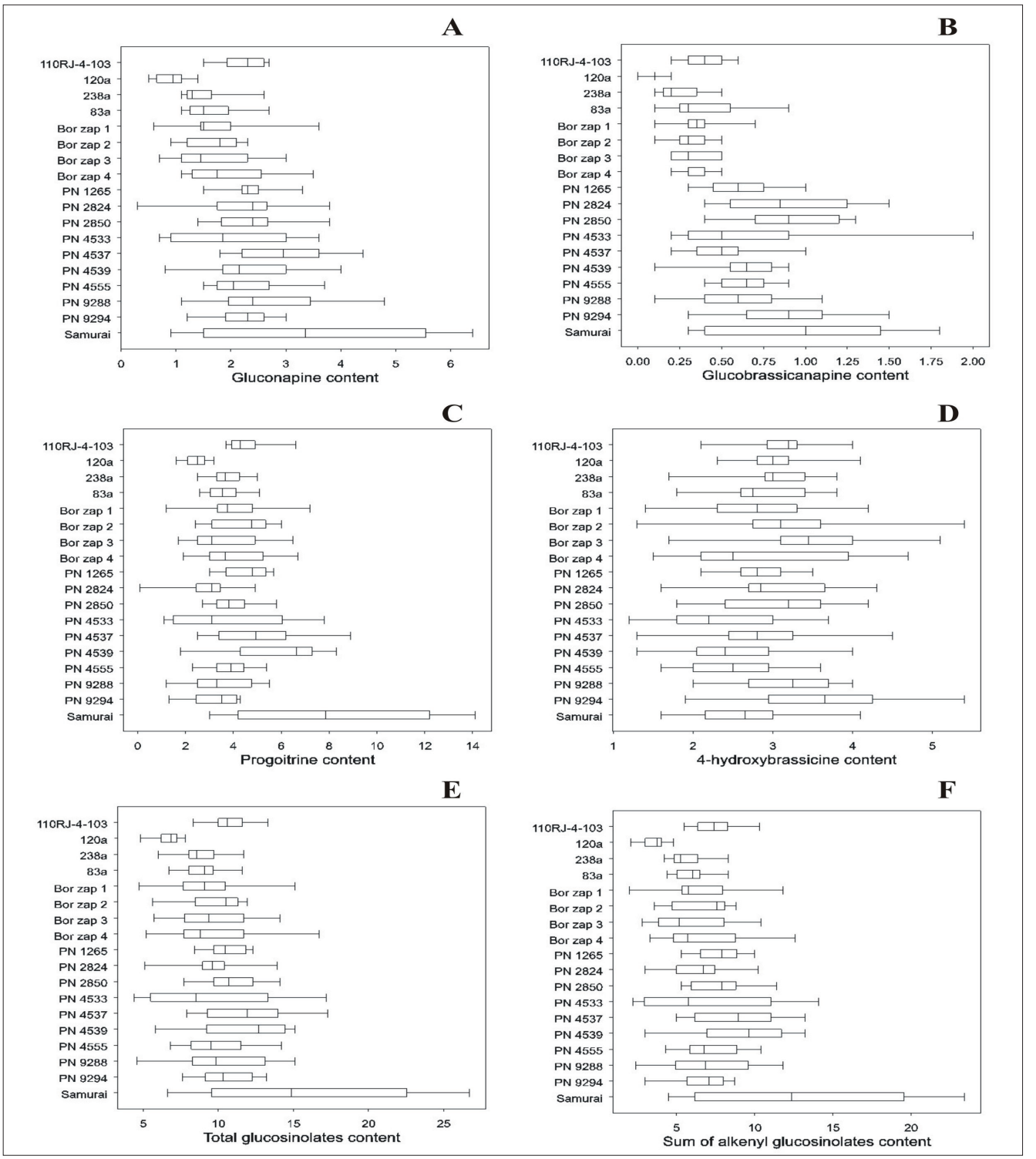

Fig. 1. Glucosinolates content in seed oil of parental lines of F_1 CMS *ogura* hybrids:
A – gluconapine content, B – glucobrassicanapine content, C – progoitrine content, D – 4-hydroxybrassicine content, E – total glucosinolates content, F – sum of alkenyl glucosinolates content

Table 1
Markers associated with gluconapine content in parental lines of CMS *ogura* F_1 hybrids

Marker symbol	Estimates of regression coefficients	P-value	The proportion of total phenotypic variance explained by the marker	Standard error of observations
SA 13~1450 E-AAG : M-CTA; OPA 11~1270	-0.747	0.004	38.2	0.464
OPC 18~750	-0.74	0.005	35.7	0.473
OPW 09~830	0.708	0.006	34.2	0.479
OPY 02~1830	0.720	0.007	33.5	0.482
SA 23~220 E-AGG : M-CTC	-0.936	0.007	33.0	0.483
SA 7~1146 E-ACC : M-CAG; SA 9~450 E-AAC : M-CAT; SA 17~1150 E-ACT : M-CAT; SA 22~1400 E-AGG : M-CTA; SA 22~265 E-AGG : M-CTA; OPP 14~1270; OPN 20~2200; OPA 18~1750; OPA 15~1480; OPA 15~890; OPG 04~1270; OPK 08~1200	1.517	0.008	32.7	0.485
SA 6~1150 E-ACC : M-CAC; SA 6~805 E-ACC : M-CAC; SA 23~1215 E-AGG : M-CTC; SA 23~820 E-AGG : M-CTC; SA 23~145 E-AGG : M-CTC	-1.517	0.008	32.7	0.485
SA 20~270 E-AGG : M-CAG	-1.095	0.009	31.9	0.487
OPA 11~1150	-0.822	0.009	31.4	0.489
SA 11~1600 E-AAC : M-CTA	0.914	0.009	31.1	0.490

Table 2
Markers associated with glucobrassicanapine content in parental lines of CMS *ogura* F_1 hybrids

Marker symbol	Estimates of regression coefficients	P-value	The proportion of total phenotypic variance explained by the marker	Standard error of observations
SA 8~118 E-AGG : M-CAC	-0.447	0.002	41.7	0.195
PGI 02 – 5 phenotyp	0.398	0.002	41.0	0.196
OPW 09~830	0.329	0.003	40.5	0.197
SA 16~330 E-ACA : M-CTT	0.3332	0.003	39.4	0.199
OPY 01~1910	0.336	0.004	37.2	0.202
SA 13~1600 E-AAG : M-CTC	0.3041	0.007	33.7	0.208
OPW 09~890	-0.303	0.008	32.9	0.209
OPL 12~1890	-0.316	0.008	32.1	0.210
SA 22~255 E-AGG : M-CTA	0.298	0.009	31.7	0.211

Table 3
Markers associated with progoitrine content in parental lines of CMS *ogura* F_1 hybrids

Marker symbol	Estimates of regression coefficients	P-value	The proportion of total phenotypic variance explained by the marker	Standard error of observations
SA 22~190 E-AGG : M-CTA	3.263	<0.001	67.0	0.729
SA 7~1146 E-ACC : M-CAG; SA 9~450 E-AAC : M-CAT; SA 17~1150 E-ACT : M-CAT; SA 22~1400 E-AGG : M-CTA; SA 22~265 E-AGG : M-CTA; SA 23~1650 E-AGG : M-CTC; OPP 14~1270; OPN 20~2200; OPA 18~1750; OPA 15~1480; OPA 15~890; OPG 04~1270; OPK 08~1200	4.305	<0.001	61.5	0.788
SA 6~1150 E-ACC : M-CAC; SA 6~805 E-ACC : M-CAC; SA 23~1215 E-AGG : M-CTC; SA 23~820 E-AGG : M-CTC; SA 23~145 E-AGG : M-CTC	-4.305	<0.001	61.5	0.788
SA 22~800 E-AGG : M-CTA	2.234	0.002	42.1	0.967
OPA 16~1900	-2.234	0.002	42.1	0.967
SA 23~260 E-AGG : M-CTC	1.727	0.005	35.4	1.020

Four significant associations of glucobrassicanapine content with nine markers (four RAPD, four AFLP markers and one phenotype) were detected. Two RAPD and one AFLP markers were correlated with the reduction of glucobrassicanapine levels and others with the increase of levels (Table 2). The percentage of phenotypic variance explained by the markers ranged from 31.7 to 41.7%. Also the association between 22 molecular markers (eight RAPD and 14 AFLP) and progoitrine level was established through regression analysis (Table 3). Five AFLP and RAPD markers were linked with low progoitrine content and sixteen with high level of progoitrine in seeds of winter oilseed rape. These molecular markers explained at least 35.4 to 67.0% of the phenotypic variation of the biosynthesis of progoitrine in seeds.

Among the total of AFLP and RAPD markers, for the most important indolyl glucosinolate – 4 hydroxybrassicanapine, it was stated that six RAPD and four AFLP markers were detected (Table 4). Those markers explained from 34.2% to 46.5% of the total phenotypic variance (Table 4).

In this study, 19 markers (RAPD – seven and AFLP – 12) showed a strong association with both the total and alkenyl glucosinolates content in parental lines of F_1 CMS *ogura* hybrids (Tables 5 and 6). Fourteen of them were linked with the highest level of glucosinolate content. The presence of the five AFLP bands represented a decrease in glucosinolate content what makes these markers useful for the quality breeding of winter oilseed rape. The markers explained 48.4 – 57.6 percentage of phenotypic variation in total glucosinolate content and 53.4 to 57.5 percentage of variation in total alkenyl glucosinolate content.

In addition, the obtained results showed a significant linkage between nine AFLP and seven RAPD markers with two alkenyl glucosinalates – gluconapine, progoitrine and also total alkenyl and total glucosinolates content. Five of those were associated with the lowest levels of these anti-nutritional components in seeds of oilseed rape (Tables 1, 3, 5 and 6).

Isozyme markers were the least helpful, because out of five isozyme systems investigated in this study, only PGI

Table 4
Markers associated with 4-hydroxybrassicine content in parental lines of CMS *ogura* F_1 hybrids

Marker symbol	Estimates of regression coefficients	P-value	The proportion of total phenotypic variance explained by the marker	Standard error of observations
SA 21~1650 E-AGG : M-CAT	0.692	0.001	46.5	0.232
SA 5~190 E-ACC : M-CAA; SA 19~1450 E-ACT : M-CTT	-0.577	0.001	45.3	0.235
SA 5~490 E-ACC : M-CAA	0.554	0.002	41.4	0.243
6PGD 5-phenotyp	-0.493	0.003	40.6	0.245
OPP 03~1900	-0.419	0.003	40.4	0.245
OPW 08~1270	-0.409	0.003	40.3	0.245
OPW 08~2120	-0.454	0.003	39.9	0.246
OPN 02~2020	0.403	0.004	36.8	0.252
OPN 02~1830	-0.403	0.004	36.8	0.252
OPG 11~700	0.381	0.006	34.2	0.258

Table 5
Markers associated with total glucosinolate content in parental lines of CMS *ogura* F_1 hybrids

Marker symbol	Estimates of regression coefficients	P-value	The proportion of total phenotypic variance explained by the marker	Standard error of observations
SA 7~1146 E-ACC : M-CAG; SA 9~450 E-AAC : M-CAT; SA 17~1150 E-ACT : M-CAT; SA 22~1400 E-AGG : M-CTA; SA 22~265 E-AGG : M-CTA; SA 23~1650 E-AGG : M-CTC; OPP 14~1270; OPN 20~2200; OPA 18~1750; OPA 15~1480; OPA 15~890; OPG 04~1270; OPK 08~1200	6.080	<0.001	57.6	1.2
SA 6~1150 E-ACC : M-CAC; SA 6~805 E-ACC : M-CAC; SA 23~1215 E-AGG : M-CTC; SA 23~820 E-AGG : M-CTC; SA 23~145 E-AGG : M-CTC	-6.080	<0.001	57.6	1.2
SA 22~190 E-AGG : M-CTA	4.096	<0.001	48.4	1.3

– phosphoglucoisomerase (PGI-2 – 5 phenotyp) (Table 2) was linked with alkenyl glucosinolate – glucobrassicanapine and 6PGD – 6 phosphogluconate dehydrogenase (6PGD – five phenotype) were associated with indolyl glucosinolate – 4-hydroxybrassicanapine (Table 4). The first isozyme represented an increase of the glucobrassicanapine and the second – 6PGD a decrease in 4-hydroxybrassicanapine content.

A significant correlation for all investigated pairs of traits concerning glucosinolates content in seeds of parental lines of F_1 CMS *ogura* hybrids was detected. Gluconapine showed correlation with all alkenyl glucosinolates (glucobrassicanapine r = 0.713 and progoitrine r = 0.726), total glucosinolates (r = 0.916) and total alkenyl glucosinolates (r = 0.906). For 4-hydroxybrassicanapine, main indolyl glucosinolate, the correlation with other glucosinolates was not observed. The most important relationship (r = 0.983) between total glucosinolates content and total alkenyl glucosinolates content was observed. All statistically significant correlations were positive (Table 7, Figure 2). Those correlations showed association of selected interesting markers with loci controlling glucosinolate content in seeds (individual alkenyl glucosinalates, total alkenyl and total glucosinolates content).

The content of seed glucosinolate is controlled by multiple genes and is globally regulated in the cell (Krzymanski, 1970a, 1970b; Uzunova et al., 1995). In another study, many authors pointed to the different number of genes determining the inheritance of total and individual glucosinolate content in seeds of winter oilseed rape (Howell et al., 2003). Magrath et al. (1993) described six unlinked loci which determine the aliphatic glucosinolate profile of *B. napus*. Linkage maps of *Brassica napus* localized QTLs for seed glucosinolate content and individual alkenyl glucosinolates accumulated in seeds of *B. napus* (Uzunowa et al., 1995; Howell et al., 2003; Matuszczak, 2010). Zhao and Meng (2003) suggested the control of different glucosinolates (aliphatic and total) by some common genes. In this study, 19 molecular markers demonstrated an association with the most important individual alkenyl glucosinolates content and total glucosinolates content in seeds of *B. napus*. Rücker and Röbbelen (1994) revealed that in oilseed rape genome two or three genes controlling the inheritance of indolyl glucosinolates, were located in oilseed rape genome. The inheritance of the genes controlling seed indolyl glucosinolates content is independent of loci controlling the levels of seed alkenyl glucosinolates content. Zhao and

Table 6
Markers associated with the sum of all alkenyl glucosinolate contents in parental lines of CMS *ogura* F_1 hybrids

Marker symbol	Estimates of regression coefficients	P-value	The proportion of total phenotypic variance explained by the marker	Standard error of observations
SA 7~1146 E-ACC : M-CAG; SA 9~450 E-AAC : M-CAT; SA 17~1150 E-ACT : M-CAT; SA 22~1400 E-AGG : M-CTA; SA 22~265 E-AGG : M-CTA; SA 23~1650 E-AGG : M-CTC; OPP 14~1270; OPN 20~2200; OPA 18~1750; OPA 15~1480; OPA 15~890; OPG 04~1270; OPK 08~1200	6.370	<0.001	57.0	1.28
SA 6~1150 E-ACC : M-CAC; SA 6~805 E-ACC : M-CAC; SA 23~1215 E-AGG : M-CTC; SA 23~820 E-AGG : M-CTC; SA 23~145 E-AGG : M-CTC	-6.370	<0.001	57.0	1.28
SA 22~190 E-AGG : M-CTA	4.511	<0.001	53.4	1.33

Table 7
Correlation coefficients between analyzed traits

Traits		Content				
		gluconapine	glucobrassicanapine	progoitrine	4-hydroxybrassicine	Total glucosinolates
Content	glucobrassicanapine	0.713**	1			
	progoitrine	0.726***	0.348	1		
	4-hydroxybrassicine	-0.248	-0.091	-0.44	1	
	Total glucosinolates	0.916***	0.634**	0.909***	-0.235	1
	Sum of alkenyl glucosinolates	0.906***	0.607**	0.939***	-0.408	0.983***

* P<0.05; ** P<0.01; *** P<0.001

Meng (2003) showed that the content of indolyl glucosinolate had negative correlations with all glucosinolate components, indicating that an independent locus was responsible for indole methyl synthesis. In the presented study, 10 molecular markers were linked only with more important indolyl glucosinolate, 4-hydroxibrassicine. None of them was linked with seed alkenyl glucosinolates and total glucosinolates content. This is in accordance with different pathways biosynthesis of alkenyl and indolyl glucosinolates. The main GSLs in the seeds of *Brassica napus* are the

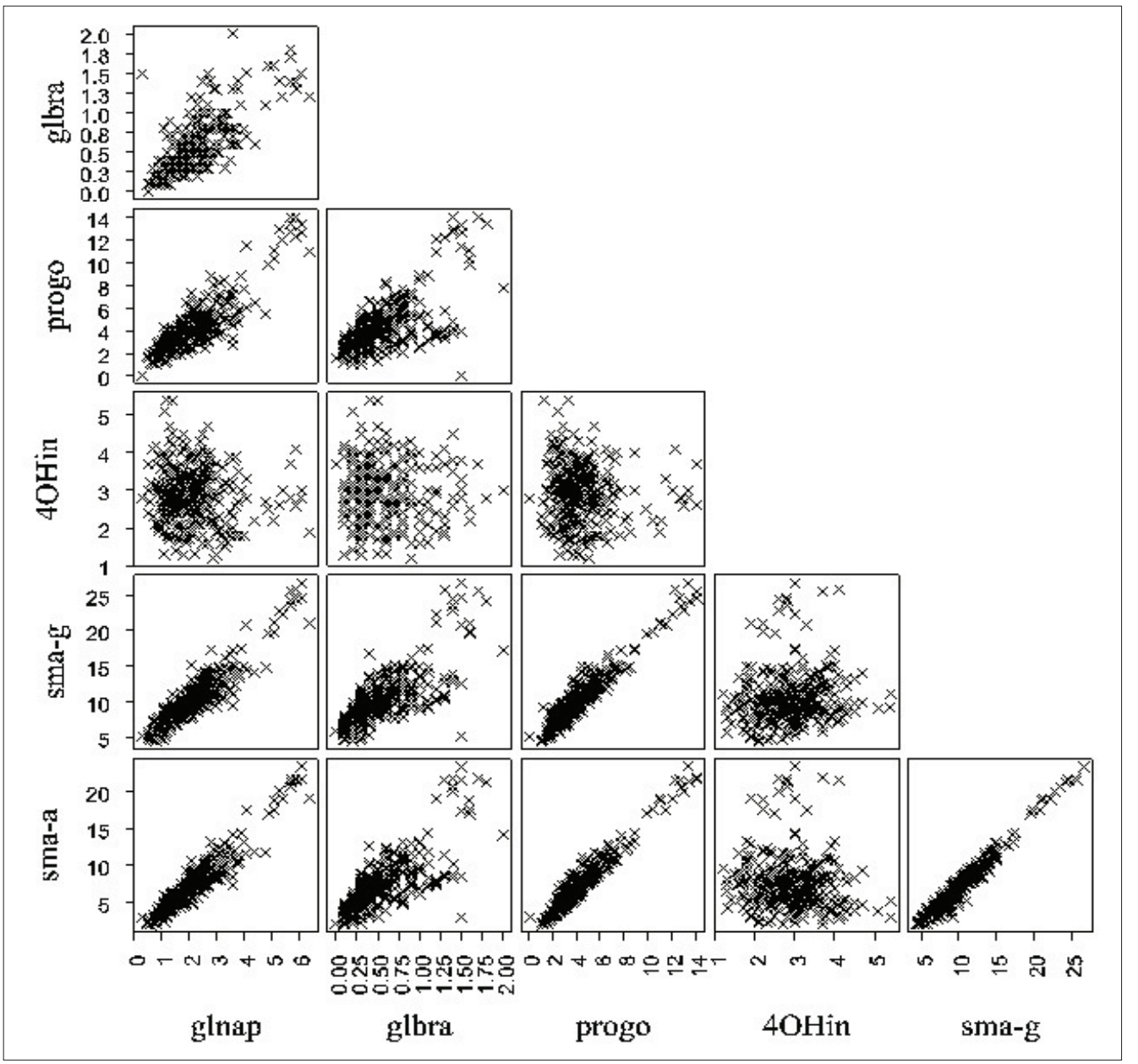

Fig. 2. Relationships between: individual alkenyl, indolyl, sum of alkenyl and sum of glucosinolate contents in seeds of winter oilseed rape
(glnap – gluconapine, glbra – glucobrassicanapine, progo – progoitrine, 4OHin – 4-hydroxybrassicine, sma-g – sum of glucosinolates content, sma-a – sum of alkenyl glucosinolates content)

alkenyl GSLs progoitrine, gluconapine, glucobrassicanapine, which are derived from methionine and the indolyl GSLs- 4-hydroxybrassicine derived from tryptophane.

Prospect for the Future

Selected molecular markers which revealed association with investigated traits must be verified in wide differentiated plant materials which permit the selection of the marker with strong correlation with loci controlling seed glucosinolates content. Those selected RAPD and AFLP markers could be transformed into codominant specific markers, such as sequence characterized amplified regions – SCAR (Bocianowski et al., 2003; Mikolajczyk et al., 2008). New markers may be useful in breeding programmes for developing rapeseed open pollinated and hybrid cultivars with low alkenyl glusosinolates content up to maximal reduction of specific glucosinolate types. The markers reported in this paper may supplement already known markers (Uzunowa et al., 1995; Matuszczak, 2010).

Conclusion

A markers associated with glucosinolates content can be useful in evaluation of some of genetic variation for breeding and selection of genotypes with very low glucosinolates content and the development of low glucosinolate winter oilseed rape cultivars.

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